

Engineering transcriptional gene circuits in probiotics and mini SimCells for bacteria therapy



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Abstract

Numerous bacteria associated with human health (probiotics and pathogens) exhibit inherent properties in terms of targeting, penetrating, proliferating and killing solid tumours through various mechanisms. To rationally redesign these microorganisms into living medicine, there is a lack of genetic software with suitable induction characteristics and compatible cellular chassis with enhanced biosafety, owing to the stringent requirements and controllability during medical practice. Consequently, the development of bacteria therapy lags behind compared to other applications offered by synthetic biology.

The goal of this thesis is to address these two significant challenges to realise the full potential of bacteria therapy. Three different transcriptional control systems were constructed: (1) an aspirin/salicylate responsive regulatory SalR:P_{Sal} system (2) a DNA damage responsive *Vibrio recA* SOS system and (3) a nitric oxide responsive NorR:P_{NorV} system. The system performance was rationally re-designed and optimised based on the detailed molecular mechanism. A universal competitive binding mechanism was found during the tuning process, where the expression level of the regulator played a dominant role in system dynamics and displayed dual activator/repressor features dependent on the state of inducer binding. To enhance the biosafety and usability, these genetic software were characterised and optimised in the cellular context of a probiotic chassis strain, *E. coli* Nissle 1917 and tested in the non-dividing anucleate *E. coli* mini SimCells, which are minicells with intact transcriptional/translational machinery. An optimised purification protocol was developed with a final yield of 5×10^9 mini SimCells/ml and high purity (total elimination of parent cells). Optimised sensor construct loaded in mini SimCells displayed functional responsive dynamics that (1) are robust against environmental fluctuation, (2) displayed faster response time (<30 mins), and (3) maintained medically relevant sensitivity. These findings

validated the programmability of mini SimCells to execute both σ^{54} and σ^{70} based transcriptional circuits and bypass host interference caused by global resource allocation or external fluctuation.

To conclude, this thesis provides a systematic workflow of designing therapy tailored transcriptional software and its functional deployment in mini SimCells. The circuits allow both external controls using established medical methods such as chemo/radiotherapy, aspirin injection or internal triggering via *in-situ* disease biomarkers, pave ways for the future pre-clinical development. Furthermore, this thesis demonstrates the potential of mini SimCells and its design guideline as a minimum version of bacteria-bot systems to achieve robust and sensitive performance, making it a preferred choice in clinical practice.

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Declaration

I declare that the work described within this thesis is my own, except where explicitly stated below.

This thesis is my own composition.

Signed: *xiaoyu chen*

Date: 01/07/2021

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List of Abbreviations

ASA – acetyl salicylate acid (aspirin)

aTc – Anhydrotetracycline

EcN – *E. coli* Nissle 1917

CFU – Colony forming unit

DNA – Deoxyribonucleic acid

DBD – DNA binding domain

DMSO – Dimethyl sulfoxide

DETA/NO – Diethylenetriamine/nitric oxide

D₂O- Deuterium water

EBD – Effector binding domain

FDA – Fluorescein diacetate assay

JCVI – J. Craig Venter Institute

NO – Nitric oxide

OD – Optical density

ODE – Ordinary differential equation

RBS – Ribosome binding site

RNAp – RNA polymerase

siRNA – short interference RNA

shRNA – short hairpin RNA

SCRS – Single-cell Raman spectra/spectrum

SA – salicylate acid

T3SS – Type III secretion system

Chapter 1 Introduction

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1.1 Motivation

Traditional cancer therapy can suffer the drawbacks of (1) adverse side effects in normal cells, (2) being unable to penetrate into the necrotic core and (3) being unable to fully eliminate tumour cells due to the resistance acquired through frequent dosing. These challenges make bacteria therapy and living medicine a promising solution. Extensive advancements have been made in the past two decades in both directions of exploiting more diverse chassis strains and building more complex circuit functions. These advancements and historical hallmarks are summarised and discussed in Chapter 2, section 2.2.

Despite being one of the most promising applications within the field of synthetic biology, in contrast with other applications such as engineering plant microbiota for nitrogen fixation or metabolic engineering for yield optimisation of high-value products, the clinical translation and commercialisation of bacteria therapy has not kept up with the rapid development of its academic progress. To date, attenuated *Mycobacterium bovis*, which was invented 44 years ago, is the only bacterial-based cancer therapy with clinical approval [1]. The complex environments in the human body and stringent clinical requirements pose unique challenges for putting bacteria therapy to use. This can be further decomposed into two subcategories when considering the design of a living

medicine: the challenges of 1) designing and fine-tuning the synthetic transcriptional/ translational genetic control (software) and 2) choosing and building the functioning cell chassis (hardware) to execute the synthetic function.

Challenge 1 (Genetic software)

Numerous transcriptional control and translational control systems have been developed for synthetic biology (more detailed provided in Chapter 2 section 2.4). However, there is a lack of medically applicable control modules with ideal characteristics. An ideal medical application module must satisfy four features: (1) Fast response. This ensures the system can be fully turned on within the half-lives of the given inducer. (2) Minimal basal leakiness. Cargo used in bacteria therapy are often toxic or poses a substantial metabolic burden and thus require tight control and insulation for biosafety. (3) Medically relevant sensitivity. The sensitivity of the control module and induction dynamics need to be fine-tuned to match the medical guidelines of the given inducer so as not to cause any severe side effects in the body. (4) Robustness. The designed circuit in its functioning chassis needs to exhibit and sustained its predicted dynamics when subjected to external fluctuations in the form of oxygen depletion, change in pH and nutrient starvation.

Challenge 2 (Function hardware)

In total, there are seven strains that show early promise for use in bacteria therapy. Each have their own merits and drawbacks (more details provided in Chapter 2 section 2.7). Despite the rapid progress in discovering more human-related microbiomes and efforts in creating a universal chassis, there is a demand for a suitable functioning chassis with enhanced biosafety and reliable performance. Upon entering the human body, the interaction with the human immune system,

resource competition between native microbiomes and constantly changing environments all pose uncertainty and challenges for the designed circuits. Certain pathogenic strains are vulnerable to immune clearance, being cleared by the host before colonisation takes place. Furthermore, genetic instability is another major issue where genetic software can be mutated or silenced via host interference and burden-derived point mutation over time.

Proposed Solutions

In order to expand the current toolbox with more medically usable control units, the first objective of this study was to construct different genetic control units that could be activated via medically relevant triggers with ideal characteristics. As shown in Figure 1.1, three different novel transcriptional controls were developed, which were (1) an aspirin responsive unit (pharmaceutical dosage control unit), (2) a DNA damage response unit (using gamma radiation or UV-induced DNA damage as a spatial control unit) and (3) a nitric oxide responsive unit (*in-situ* disease biomarker control unit). Each circuit was rationally designed according to its molecular mechanism and optimised (Design-Build-Test-Learn) based on the proposed guideline. The systems were further tested and challenged in various growth conditions to test their robustness against external fluctuation.

To address the potential undesired immune response of the host, probiotic *E. coli* Nissle 1917 (EcN) was used as functioning chassis due to its established clinical safety. Recent clinical results demonstrated that EcN is a poor recipient/donor of horizontal transfer DNA, which further strengthens its genetic safety feature [2][3].

To address the genetic stability and host interference of whole-cell chassis, I explored the usage of non-dividing anucleate mini SimCells (Simple Cells) to implement our optimised

transcriptional control construct. The mini SimCells were chromosome-free dwarf cells derived from abnormal cell division with intact transcriptional/translation machinery. In contrast with previous term minicells which have been used as drug delivery vesicles [4][5], I focus on validating its programmability and usage as modular chassis for synthetic biology. For clarity, minicells with preloaded synthetic constructs and displayed intact transcriptional/translational function are termed “mini SimCells”. More details can be found in Chapter 2, Section 2.8. Furthermore, due to the prevalence of similar cell division machinery (MinCDE) in many bacteria species, this method could be adapted to other strains, leading to many different “niche-specific modular chassis” with a wide range of applications.

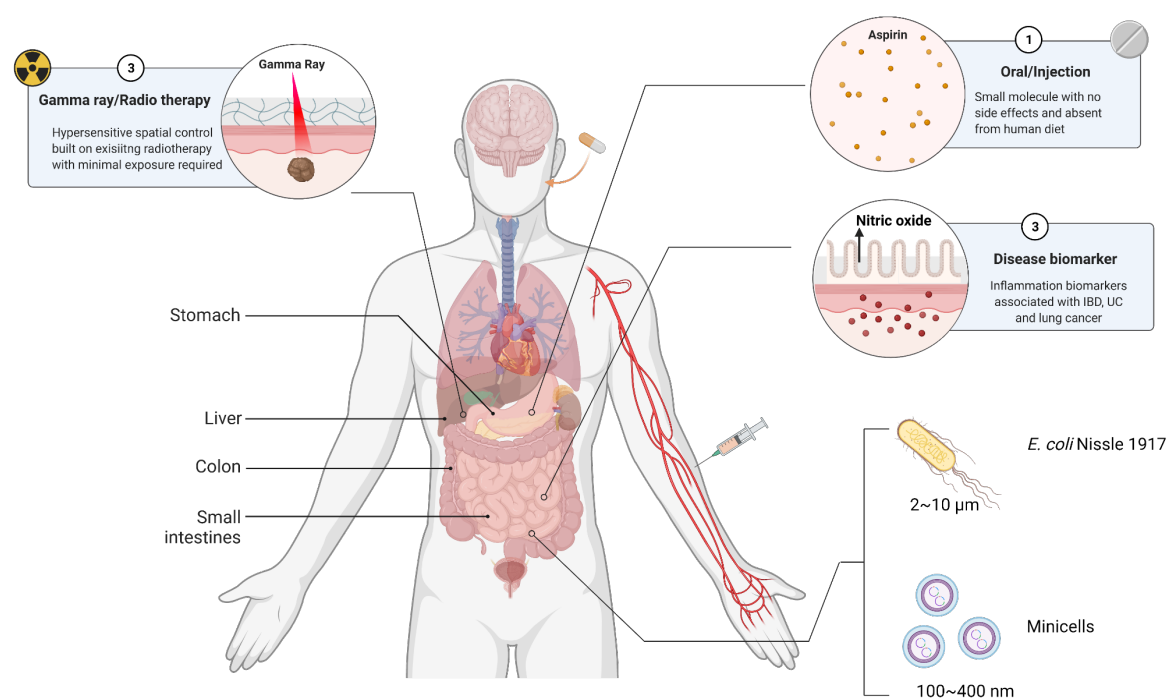


Figure 1.1. Overview of the thesis content.

Three transcriptional control units were developed to expand the toolbox for bacteria therapy. External system control can be done via (1) oral/intravenous uptake of small molecule drug (aspirin) (2) Gamma radiation or UV to trigger DNA damage response in bacteria or triggered via (3) *in-situ* disease biomarker (Nitric oxide). All constructed circuits were constructed using transcriptional bio parts, then assessed and optimised in probiotics EcN and mini SimCells.

1.2 Aims and objectives

In this section, I list the individual milestones for achieving the overall aims of the thesis. The first task is to analyse and isolate suitable sensory circuits from different bacteria species with intrinsic detection properties and then construct different promoter variants based on the existing protein structure and promoter annotation. Following that, the circuit dynamics are fine-tuned through a combinatorial approach: (1) tuning of the intracellular level of the regulator (2) promoter engineering and (3) addition of feedback control. Next, the performance of optimal circuits is validated in EcN under gut-mimic conditions. Finally, the potential of mini SimCells as a modular, safe and programmable chassis used in bacteria therapy is validated using the optimised candidate from each system.

For the first goal of expanding the current genetic software for bacteria therapy, the aim is to construct and fine-tune the proposed three sets of the transcriptional sensory system and tune their expression dynamics in EcN so that each circuit demonstrates:

- 1) A high-sensitivity fit with the medical application guidelines/disease threshold and an operational range relevant to the dosage range used in clinical practice.
- 2) Ideal characteristics (fast response time, low leakiness and high induction fold).
- 3) A robust performance when subjected to external disturbance.

The second goal is to produce and purify minimal cells and test the optimised circuits. There are three key points that need to be validated:

- 1) Characterise the mini SimCells in terms of their motility, size distribution, and potential change in physical/chemical property due to the loss of chromosomes.
- 2) Explore the modularity of the mini SimCells with different family types (σ^{70} and σ^{54}) of transcriptional sensory circuits and validate its performance dynamics.

- 3) Investigate different methods for mini SimCells production and develop a purification method with high purity and yield.

1.3 Key findings and thesis structure

The thesis structure is summarised as follows with key findings highlighted:

In Chapter 2, firstly, I provide the reader with general background knowledge of synthetic biology and bacteria therapy. The basic concepts of synthetic biology and the merits offered by bacteria therapy was firstly introduced in sections 2.1 and 2.2. This is followed by an illustration of the molecular mechanisms of different sensory family members (section 2.3) and existing transcriptional control modules (section 2.4). Next, the tuning methods and toolboxes available for modifying the transcriptional regulation used in this study are outlined (sections 2.5 and 2.6). More in-depth descriptions of the probiotic chassis EcN and other strains used in bacteria therapy are compared and discussed in section 2.7. The production of mini SimCells and the molecular detail of cellular replication machinery MinCDE is outlined in section 2.8 with existing purification and extraction protocols.

Chapter 3 outlines the results of creating an aspirin triggering system (SalR-based) derived from *Acinetobacter baylyi* ADP1. This sensor represents a key family member (LysR) of the LTTR superfamily, which is the largest domain type in the sensory machine of gram-negative strains. Partial results of this section were published in [6]. This key finding of this chapter illustrates:

- a novel competitive binding model of the regulator protein SalR. Based on the model prediction, I obtained a set of highly sensitive aspirin sensors ($\text{LOD}_{\text{aspirin}} = 0.05 \mu\text{M}$) with optimised operational ranges (Inducer F.c = 25) and response time (< 50 mins).

- Furthermore, a quasi XOR logic gate was constructed using an antisense promoter. The results demonstrated tight control over GFP expression near the wild-type level. These circuits could provide tight binary regulation for toxic or burdening protein expression.

Chapter 4 investigates a novel SOS DNA damage report system adopted from *Vibrio natriegens* which is the fastest known dividing organism ~ 10 mins/cycle [7]. This was intended to develop an ultrasensitive spatial triggering system aside from using orally ingestible drugs. The results of this chapter were published in [8]. The final results of this section are a toolbox of highly sensitive and fast-responding (<30 mins) DNA damage sensors that can be triggered via DNA damage compounds (UV or Mitomycin C) with improved robustness and sensitivity. The key finding of this chapter illustrate:

- Rational design of a set of highly sensitive ($LOD_{\text{MitomycinC}} = 16 \text{ nM}$ and $LOD_{302\text{nmUV}} = 1 \text{ sec UV}$) DNA damage sensors through adjusting the base pair composition and numbers of the SOS binding box. The results validated our annotation of P_{VRecA} promoter with an interchangeable region for chimeric promoter design.
- A novel operational HrpRS based amplifier with negative feedback was also constructed that demonstrated a certain degree of disturbance rejection and achieved robust performance when subjected to external noise such as changes in pH, temperature, oxygen and medium richness.
- The optimised circuit can be successfully induced in both planktonic state and biofilm state with similar induction dynamics. The longevity and stability of the system exhibited functionally maintained over 40 hrs of UV exposure via continuous turbidostatic reactors.

Chapter 5 outlines the result for constructing a unique σ^{54} -dependent nitric oxide sensor adopted from *E. coli* Nissle 1917 *norVW* operon. Nitric oxide is a gaseous disease biomarker; an elevated concentration is commonly found in inflammatory bowel disease [9] and lung cancer [10]. The development of a nitric oxide responsive system should allow *in-situ* disease triggering drug release or diagnosis. The circuits were fine-tuned using a similar methodology as shown in SalR. The key finding of this chapter illustrates:

- The regulator NorR also exhibited similar competitive binding mechanisms similar to that of the SalR, despite mediated by different σ families. Therefore, through tuning the regulator level with feedback and rational promoter design, the final optimised tool box displayed a wide operational range (Inducer F.c = 33), high sensitivity ($LOD_{\text{DETA/NO}} = 15 \mu\text{M}$ and $LOD_{\text{NO}} = 26 \text{ nM}$), fast response (<25 mins) and tight control.
- A strong host suppression and system delay occurred upon nitrogen depletion. This host interference can be successfully decoupled upon implementation into mini SimCells.
- Freeze-dried mini SimCell with NO sensor loaded can exhibit a shelf-life over two months stored at room temperature, maintained its medical relevant sensitivity and response time. This provides a more practical in-field application of mini SimCells with preloaded machinery.

In Chapter 6, SimCells are systematically characterised in terms of their motility profile, generation mechanisms, size distribution and Raman spectrum profile. An optimised purification protocol with improved purity (total elimination of parent cells) and yield (5×10^9 mini SimCells/ml) was developed. The optimised candidates from Chapters 3, 4 and 5 were implemented and induced. Both the NorR-based nitric oxide sensor and the SalR-based aspirin sensor exhibited functional dosage response with increased response time and induction fold. Analysis and debugging of the

malfunctioned $P_{V\text{RecA}}$ -based DNA damage sensor were carried out with further insights into signal amplification and certain design constraints for mini SimCells based circuits. The novel finding includes:

- Mini SimCells contain sufficient cellular machinery to execute both σ^{54} and σ^{70} based transcriptional control. It also contains sufficient energy to execute an antisense logic gate under the control of aspirin and tetracycline.
- The majority of mini SimCells (100 ~ 400 nm) preserve intact motility function, which displayed highly efficient movement with a speed of 1~2 body length/sec over a long distance of 20~60 μm .

In Chapter 7, all the key findings of this study are listed and discussed in relation to previous studies in the literature. Future experiments and strategies for troubleshooting the malfunctioned $P_{V\text{RecA}}$ system were listed. Several future aspects of this project are also outlined as natural follow-ups to expand mini SimCells as a platform technology: one is to create a signal integration and decision-making system for disease diagnosis and long-term monitoring, the other is to further investigate the motility feature displayed by mini SimCells and methods in generating sustainable ATP synthesis in mini SimCells. Furthermore, different methods for generating the chromosome-free mini SimCells via dCas9 interference are also proposed and discussed.

1.4 Contribution to literature

The partial work of this thesis is based on the following publications:

J. X. Chen, B. Lim, H. Steel, Y. Song, M. Ji, and W. E. Huang, “Redesign of ultrasensitive and robust RecA gene circuit to sense DNA damage,” *Microb. Biotechnol.*, pp. 1751-7915.13767, Mar. 2021, doi: 10.1111/1751-7915.13767.

J. X. Chen et al., “Development of aspirin inducible biosensors in *Escherichia coli* and SimCells,” *Appl. Environ. Microbiol.*, vol. 85, no. 6, pp. 2959–2977, Mar. 2019, doi: 10.1128/AEM.02959-18. Editor spotlighted in the release.

Y. Song, C. P. N. Rampley, **X. Chen**, F. Du, I. P. Thompson, and W. E. Huang, “Application of Bacterial Whole-Cell Biosensors in Health BT - Handbook of Cell Biosensors,” G. Thouand, Ed. Cham: Springer International Publishing, 2019, pp. 1–17.

Manuscripts under preparation:

J.X.Chen, B.J Wang, I. P. Thompson, W.E.Huang, “Rational redesign and characterisation of nitric oxide biosensors in *E. coli* Nissle 1917 and mini SimCells”. Journal article

J.X.Chen, W.E.Huang, “Characterisation of mini SimCells as a modular and motile chassis for programmable living medicine.” Journal article

J.X.Chen, W.E.Huang, “Living medicine: a practical guide in designing therapy tailored genetic circuits and choosing the right functioning chassis.” Review article

Chapter 2 Background

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This chapter introduces the background of synthetic biology, its downstream application in bacteria therapy and the basic design components for constructing a living bacteria bot. This is further concentrated to introduce the architecture of the transcriptional control and the molecular mechanism involved for different types of transcription factor (TFs) mediated

activation/repression. Next, the tools and characterisation methods used for tuning and improving its performance used in this thesis are illustrated and discussed. Subsequently, a literature summary with most of transcriptional systems and chassis strains used in bacteria therapy is briefed and compared. Finally, the detailed procedures for minicell generation/purification and the associated molecular mechanism are provided and illustrated.

2.1 Synthetic biology

Synthetic biology is a cross-disciplinary field that rationally designs and recreates biological organisms using a high-throughput Design-Build-Test-Learn (DBTL) cycle [11]. Early studies focused on using standardised and characterised genetic elements to construct simple logic gates. Since the construction of the first synthetic toggle switch in 2000 [12], the field has shown significant progress in developing circuit modules such as fixed bandwidth filters [13], genetic amplifiers [14], edge detectors [15] and numerical operators [16]. More recent developments shift towards building more complex processing systems such as oscillators [17] and memory recorders [18]. Synthetic biology distances itself from earlier 'cut and paste' molecular cloning and moves towards a more systematic structure using control-theory-guided design. This is aided by rapid developments in computational biology, automation, DNA synthesis and sequencing technology. The current state of the art allows users to design *ad hoc* DNA and simulate gene circuit function based on *in silico* thermodynamics [19] or machine learning algorithms [20]. Thus far, the most complicated synthetic sensing system that has been constructed in a single cell contains a maximum of 12 orthogonal sensing pathways [21]. At a larger scale, the construction of an entire recoded *E. coli* genome was achieved in 2019, resulting in a strain that uses synthetic codons to translate artificial proteins and obtain total immunity against bacteriophages [22]. Furthermore,

the logic-gate building is not limited to using only DNA or RNA anymore, as *de novo* protein-based logic gates have also been made possible due to progress in structural biology and advanced protein folding model prediction [23].

In contrast to the previous digital revolution era, synthetic biology aims to impact most industries such as agriculture and healthcare. Its downstream applications include, but are not limited to, (1) engineering microbes with nitrogen-fixing capacity for non-legume plants [24], (2) a single-cell factory for producing high-value compounds using wastewater or hazardous material [25][26], and (3) living medicine in which engineered microbes can act as pathogen seekers [27], disease detectors or drug delivery vehicles [28]. The development of synthetic biology shares many similarities with electronic engineering. As illustrated in Figure 2.1, a typical workflow for constructing a synthetic bacterium can be divided into two main parts: the design and encoding of the 'genetic software' and the loading to its 'functioning cellular hardware'. The objective of genetic software design is to characterise functional DNA fragments and reconstruct them into logic circuitry with computational capability. The raw material is often transcriptional regulatory units or molecular machinery that were initially discovered through metagenomics mining using bioinformatics [29] and DNA sequencing [30]. These raw materials are then annotated and characterised into interchangeable bio-parts. These bio-parts consist of genetic modifying tools (DNA), transcriptional control (mRNA) and translational control modules (Protein), each offer different merits and drawbacks. Figure 2.2 shows a condensed timeline for modules and circuit development in recent years.

One of synthetic biology's central aims is to reprogramme biosystems and design artificial organisms for different applications. However, the vast difference in and diversity of microbial translational and transcriptional profiles pose challenges in the reusability and function prediction

of the designed circuits. In the effort to unify the 'programming language' of the cell chassis, one solution is to rebuild an entire artificial cell with simplified and standardised function modules, even though there is still an ongoing debate about whether a 'universal' chassis exists. An 'all-known' synthetic chassis that can cover a specific range of scenarios has been attempted, namely from three angles: 1) Minimal cell chassis through top-down genome deletion; 2) Bottom-up assembly of cell-like lipid vesicle contained with simplified molecular machinery and 3) Chromosome-free chassis through chromosome degradation or abnormal division.

The top-down approach represents efforts in finding and deleting 'non-essential' genes and reduces biological redundancy to a bare minimum, only maintaining essential biological functions such as transcription, translation, and replication. A most recent example of the top-down approach is the JCVI-Syn3.0 project (2016) [31], a 50% reduction of synthetic *Mycoplasma mycoides* genome based on the JCVI-Syn1.0 cell first constructed in 2010, marked as the first self-dividing synthetic organisms. In contrast, the bottom-up approach has a more extensive history and was initially developed to answer questions about how cells and life evolved on earth. Typically, this involves generating multifunctional liposome vesicles using microfluidic devices, together with other bioactive molecules to synthesise and mimic functions such as cellular adhesion, chemotaxis and redox reaction. A third approach in generating chromosome-free cells is using I-CeuI endonucleases to degrade host chromosomes [32][33] or mini SimCells by manipulating cellular division mechanisms to produce bioactive dwarf cells with no chromosomes [34]. A condensed timeline for chassis development is illustrated in Figure 2.3.

The ultimate goal for genetic software and functional hardware is to produce a unified operating chassis system that is compatible with different types of genetic software, thereby enabling standardised genetic programs to be reused in different applications. This emphasis on

programmability and modularity differs significantly from the traditional approaches to obtain genetically modified organisms. (Figure 2.1)

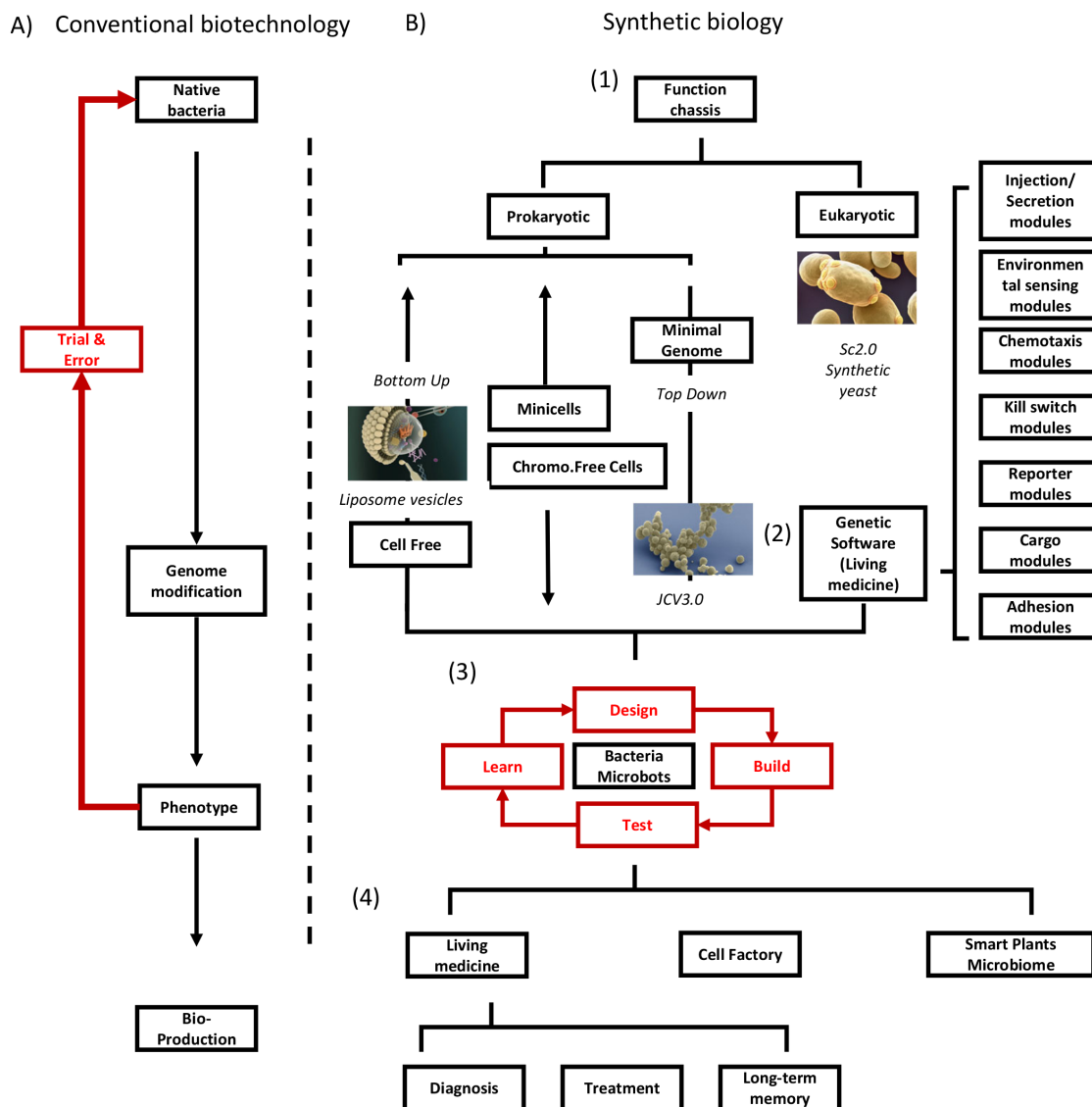


Figure 2.1 Comparison of workflow between conventional biotechnology and synthetic biology

A) A conventional workflow using genetically modified bacteria often involves several rounds of trial and error based on tinkering and expert knowledge. In contrast, B) synthetic biology adopts a more systematic approach that involves (1) constructing a niche-specific function chassis and (2) design-and-synthesis-compatible genetic software. The function validation and optimisation iterate through a (3) DBTL cycle adopted similarly in other engineering disciplines. Due to the extended modularity of each genetic part and the programmability of the synthetic chassis, (4) downstream applications of such an approach can cover a much broader range of scenarios and scale more quickly than the conventional methods.

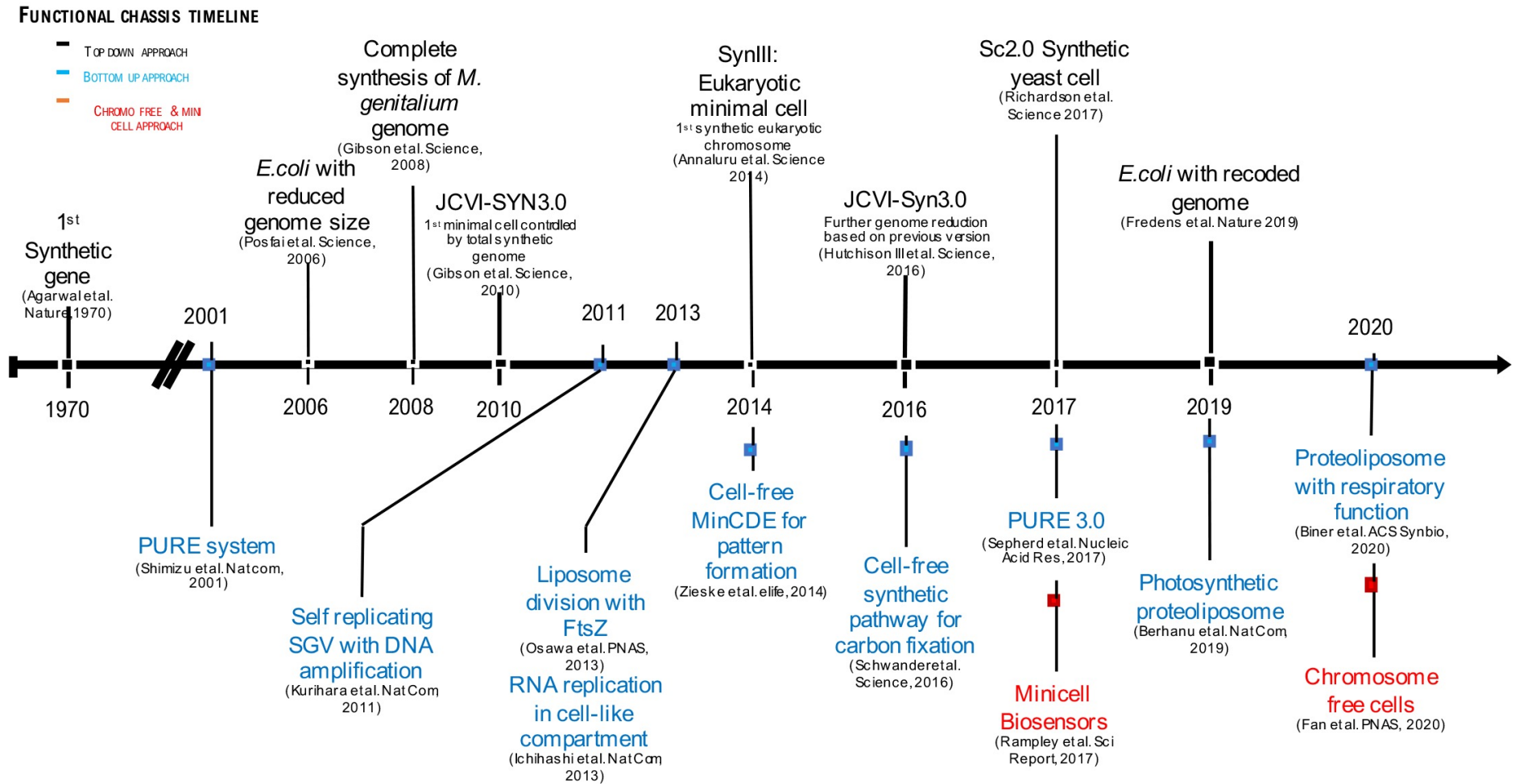


Figure 2.3 Condensed timeline of universal cell chassis development.

The top-down approach (black) represents efforts in deleting non-essential genes, which are raised as a product of natural evolution and genetic drift. The bottom-up and cell-free approach (blue) represents efforts in synthesising cell-like lipid vesicles with simplified functions such as DNA replication, photosynthesis, cell division and respiration. In Minicell or Chromosome-free (red) approaches, cells contain no host chromosome while simultaneously maintaining their transcriptional and translational function for a certain period of time.

2.2 Bacteria therapy and living medicine

2.2.1 Bacteria therapy and its promises

Bacteria therapy is one of the most promising applications in synthetic biology. Engineering microbes that can function as smart-living therapeutics for *in-situ* diagnosis, drug delivery and disease recording *in vivo* holds enormous potential [36]. This section will introduce the general background of bacteria therapy and discuss several advantages over existing chemotherapy or radiotherapy, followed by a break-down list of existing modules for constructing a bacteria therapy microbe.

The idea of using bacteria for medical treatment is not new, especially in cancer therapy. In the 1890s, Willian Coley administrated killed *S. pyogenes* and *S. marcescens* cells to cure patients with cancers [37]. However, this idea was quickly abandoned due to the lack of practicability (impurity in deactivated live bacteria) and the rapid development of radiotherapy and chemotherapy. Due to the recent progress of synthetic biology and immunology, the concept of living medicine has reattracted people's attention as a solution for many issues encountered by traditional approaches such as drug resistance in tumours, inadequate tumour penetration and nonspecific tissue damage [38][39]. Genetically engineered bacteria are ideal vectors for delivering the various types of cargo in a controllable manner.

Bacteria offer several unique advantages over traditional drug delivery systems. Both gram(-) and gram(+) strains have demonstrated selective colonisation in tumour tissue [36]. For instance, *S. typhimurium*, uropathogenic *E. coli* CFT073 and *E. coli* Top10 have been shown to colonise tumour cells at densities of more than 1×10^8 cfu/gram [40]. Specifically, probiotic *E. coli* Nissle 1917 has demonstrated preferential colonisation of tumour tissue over normal liver tissue with a ratio of 1000:1 [41]. Mice models treated with antigen-decorated bacteria exhibited significant repression of tumour growth [42]. Secondly, it has been reported that the bacterium can

reside in the gut from over one week to nearly a month. This extensive time length provides flexibility and sufficient duration for any *in vivo* treatment. Time taken for bacteria colonisation start to appear in the tumour was shown to be between 48–72 hrs upon oral transfection [41][43]. Omar et al. [44] created an oscillatory drug release system that was tested over 40 days. The results showed a 30% reduction in tumour activity over time. However, in some cases, long residency time can pose potential problems such as burden-derived random mutation [45] or horizontal gene transfer [46] between the host gut microbiome and synthetic microbes. Methods such as genetic kill switches and auxotrophic knockout can be used to limit and control the residency time [47].

Thirdly, there is a diverse range of payload type bacteria that can be loaded with therapeutic agents. Bacterial surfaces enriched with amide bonds that are prevalent on the cell surface can be encoded with different materials including RNA aptamers and nanoparticles through covalent linking [48]. Bacteria can be also be preloaded with a chemotherapeutic drug in their cytoplasm or have it translated upon induction. Depending on the application, the cargo can be cytokines, cytotoxic agents, pro-drug converting enzymes or siRNA [49]. The high specificity and loading capacity is especially advantageous for anti-cancer agents with severe systemic toxicity such as cytokine IL-2 [50] and IL-8 [51]. Furthermore, in contrast to the passive chemotherapeutic drugs which rely on site-specific accumulation and free diffusion through the tumour, evidence showed that *Clostridium novyi* and other strains could penetrate tumour areas that chemotherapy and radiation were unable to reach due to large and poorly vascularised areas in the tumour core [52]. At the same time, this higher penetrability into anaerobic and necrotic regions makes bacteria therapy more effective in treating quiescent cells, which do not respond to chemotherapeutic agents that target actively dividing cells [38]. (Figure 2.4) Bacteria can acquire energy through their surrounding environment and exhibit chemotaxis towards specific tumour metabolites, [53]

which leads to a higher drug concentration in the distal area and less overall long-term systematic toxicity. The timing for cargo release can be precisely controlled through tuning and induction via external signals.

With regard to how bacteria gain entry into the tumour in the first place, the precise mechanism underlying bacteria entry and tumour colonisation are still unclear. Some have speculated that this might be due to both passive and active mechanisms. Active migration into the necrotic core may occur via bacteria sensing and attraction by tumour-related secondary metabolites such as serine, aspartate and ribose. This is supported by Zhao et al., who employed auxotrophic strains (*Salmonella*) to demonstrate increased specificity towards tumour nutrients such as leucine and arginine [54]. Furthermore, the immunosuppressive and anaerobic condition provided by the tumour microenvironment is ideal for bacteria proliferation and for bypassing host clearance. Passive mechanisms involve bacteria-derived overproduction of TNF- α and other pro-inflammatory cytokines, which cause an influx of blood into tumours and passively flush bacteria into the tissue [55]. Bacteria can colonise robustly once they have entered the tumour due to the immunosuppressive characteristics and anaerobic conditions inside the core.

The bacteria's interaction with the host and the tumour is extensively discussed in a review [49]. Although different strains display distinctive tumour suppression features, the killing mechanisms of non-genetically modified strains can be roughly categorised into three methods: (1) resource competition [56]; (2) indirect recruitment of immune cells such as CD8⁺ T-cells [57]; (3) direct killing mediated via bacterial toxin or TLR5 signalling which suppresses tumour proliferation [58].

In summary, living bacteria can offer four unique advantages: high specificity, high penetrability, programmability, and lower systematic toxicity. This field has developed rapidly

with the aid of higher complexity genetic circuits such as conditional triggering *in-situ* drug delivery and recording disease state to function as a memory unit. The use of bacteria therapy in hyperammonaemia [59], inflammatory bowel diseases [60], phenylketonuria [61] and various cancers have demonstrated early pre-clinical success. Further cross-disciplinary studies using microelectronics and synthetic bacteria to create a point-of-care device have also been implemented in the swine model for real-time remote monitoring of internal bleeding [62].

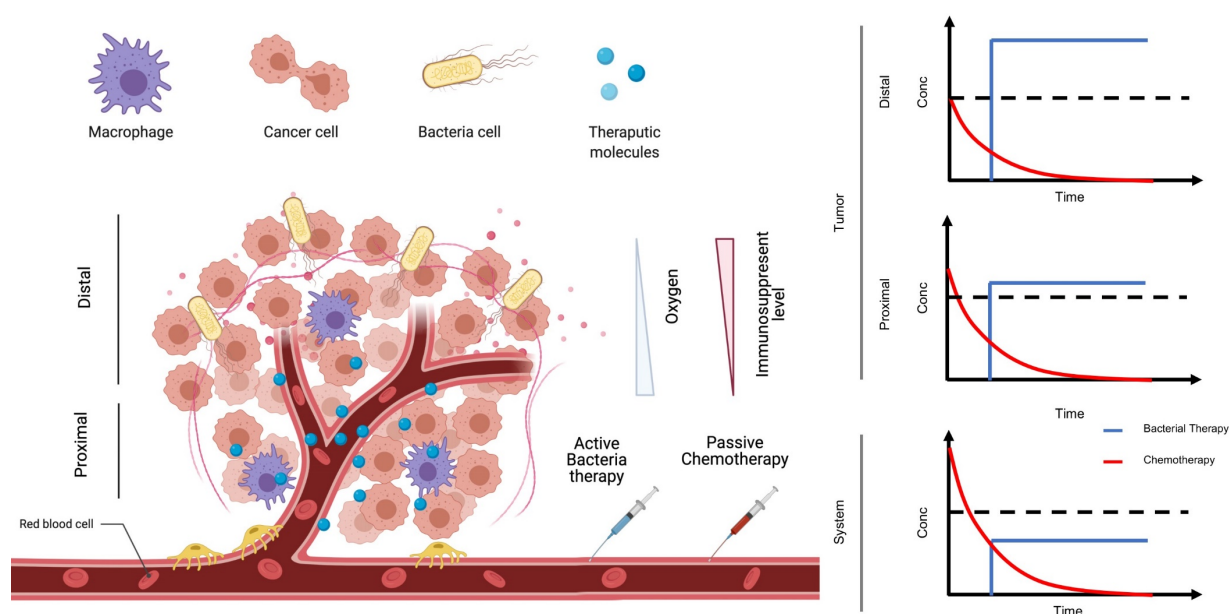


Figure 2.4 Schematic of bacteria therapy and its pharmacokinetic profile.

Figures were redrawn from [36]. The blue line indicates bacteria therapy that colonises both proximal and distal regions of the tumour upon entry. Due to the high penetrability into the necrotic anaerobic cores, the concentration of drugs produced via bacteria is higher in the distal area and remains over a long period. In contrast, traditional chemotherapy (red line) requires a high initial dose to counter the decrease with time due to host metabolism, resulting in high systemic toxicity. In the distal area of the tumour, the oxygen-depleted and immunosuppressed environment favours bacterial colonisation. Together, bacteria therapy offers lower systemic toxicity with higher penetrability and efficiency over a long period of time.

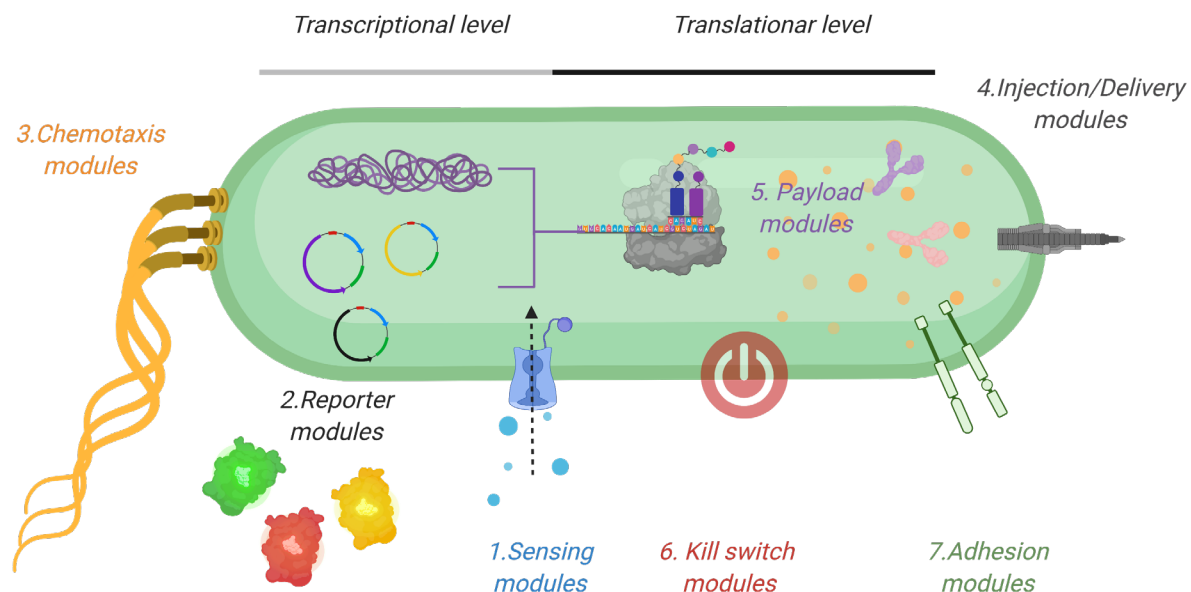


Figure 2.5 Modular components of genetic software used in bacteria therapy.

Typical genetic software involved in bacteria therapy can be primarily categorised into seven types. The genetic code determines different functions that can be either encoded on host chromosomes or plasmids. Engineered bacteria can be controlled via (1) sensing modules (blue) at both transcriptional (DNA, mRNA) and translational (protein) levels. Alternatively, the sensing module can be wired to produce a signal via (2) reporter modules (red, green, yellow fluorescent protein) or to control bacterial migration (yellow) via (3) chemotaxis modules. Other functional modules such as (4) injection modules (grey) allow intracellular injection of short-lived (5) cargo (purple) such as RNA or protein. (6) Kill-switch modules (red) ensure the contingency and biosafety of bacteria therapy. Specificity can be improved using (7) cell-specific adhesion modules (green) such as nanobodies or adhesin.

2.2.2 Constructing a bacteria doctor

Recent progress in developing different functional modules for medical applications can be classified into seven types of genetic parts. As shown in Figure 2.5, each module is designed to improve either (1) specificity of tumour targeting (i.e., adhesion modules), (2) penetration ability (i.e., chemotaxis modules) or (3) biosafety (i.e., kill switch). Other modules, such as injection modules, depend greatly on the payload type, which is commonly coupled with agents (DNA and RNA) that are easily degraded in the extracellular environment. This broad range of different modules can be used to form a signal-processing and decision-making bacteria-bot to perform

many intricate tasks. However, a synthetic microbe with all designed modules in place has yet to be developed, which is potentially due to (1) incompatibility or interference between different modules, (2) cellular resource competition and (3) crosstalk upon co-expression [63]. Within these modules, a suitable sensor/input module is the most critical unit to achieve coordination and robust control of the synthetic microbes, a well-designed sensor unit ensured the timing of drug release, expression of surface-displayed nanobodies, adhesion to tumour cells and self-destruction could be coordinated and adjusted in different medical usages. For sensing modules alone, much effort has been made to build the 'standardised', 'decoupled' and 'abstracted' control units to meet different applications [64]. However, few control modules exist that demonstrate ideal characteristics for medical usage; commonly used logic gates rely heavily on well-established modules such as P_{LacI} , P_{TetO} and P_{Bad} , which are non-ideal for long term usage or achieving tight regulation. More details are discussed in section 2.4.

The choice of hardware chassis is another critical factor to be considered. There are currently seven different types of strain chassis that have been used to treat different forms of tumours and other gut-related diseases. Strains such as *Salmonella* [65], *Clostridium* [66] and *Listeria* [67] have demonstrated strong anti-tumour behaviour and immune-activating features (the recruitment of CD8⁺ T cells and overproduction of interferon- γ). However, usage of these pathogenic strains poses safety concerns in medical regulation. Although attenuated immunogenic mutant strains with altered lipid composition exist for *Salmonella* [68][69], the strains still suffer from early host immune clearance. Probiotics candidates are a safer chassis choice and can avoid early immune clearance by the host before colonisation can take place. Among various probiotics strains, *Lactobacillus* [70], *Bacteroides* [71] and *Bifidobacterium* [72] have all demonstrated some degree of success. However, limitations for engineering these strains are made by a lack of accurate

genetic manipulation tools for precise knock-in/out and their obligated anaerobic growth condition. These obligate anaerobes also have limited colonisation capacity in (well-oxygenated) peripheral regions of the tumour. In this project, EcN (*E. coli* Nissle 1917) was selected as the preferred candidate with these restrictions in mind due to 1) its proven clinical safety and recent evidence for its genetic safety, 2) ease of genetic modification, and 3) its facultative aerobic profile. More details of EcN and other strains will be introduced and discussed in section 2.7.

2.3 Bacterial transcriptional sensory architecture

To reconstruct a synthetic biosensor, we must understand its native form. Bacteria contain different transcription factors (TFs) that respond to various chemicals and environmental stimuli. These transcription-factor-based sensory systems can be largely categorised into a) single component families and b) two-component families. In a typical two-component system, as shown in Figure 2.6, upon signal activation via the binding of inducer molecules, a membrane-bound histidine kinases sensory domain will become auto-phosphorylated and transfer its phosphoryl group to a coupling intracellular sensory regulator. Subsequently, this will trigger expression for the downstream gene.

In contrast, in a one-component system, a single sensing regulator controls the downstream gene expression. Such a dual functional regulator often contains an effector-binding domain (EBD) and a DNA-binding domain (DBD). EBD is responsible for binding with the inducer molecule and conferring structural changes (oligomerisation) to transfer this signal to the DBD, where the DBD is responsible for later binding and regulating the operator DNA and initiating the transcription of mRNA. The one-component system displays a greater domain variety than the two-component system. Therefore, they are often correlated with the central metabolism of the bacteria. Although it is difficult to create chimeric EBDs and DBDs, thus making the one-component system less

versatile than the two-component system, they represent the dominant family in signal transduction [73] of prokaryotes and have been extensively studied for usage in detecting aromatic compounds, heavy metals and antibiotics [74]. Furthermore, the two-component system relies on using a phosphorylation module for signal passage, and undesired crosstalk could happen between synthetic constructs and the native system [75]. The balance between the membrane sensory domain and the intracellular regulator needs to be carefully calibrated to ensure steady performance. For these reasons, I will be focusing on the one-component system and refereeing TFs for the regulatory system in this work.

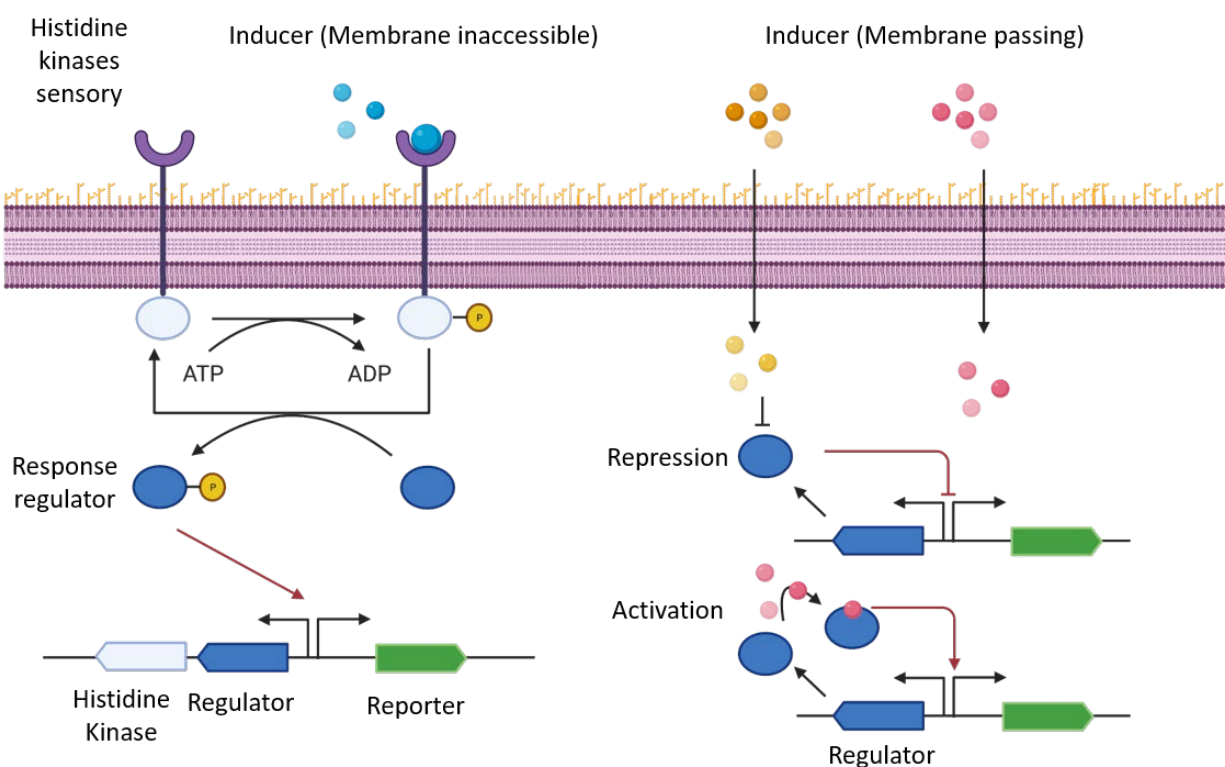


Figure 2.6 Bacterial sensing through one-component and two-component systems.

Bacteria use two different mechanisms to sense their surroundings. For detecting molecules that are unable to pass the membrane, a two-component system is used. Upon binding with the surface-attached sensory (histidine kinase), this triggered a series of downstream signal transfers in the forms of phosphorylation at the cost of ATP. In contrast, a one-component system involves

transcription factors that can bind both DNA and the inducer chemicals. Based on different sub-family types, this can lead to either activation or repression for the downstream gene.

For TF-mediated transcription initiation, the regulation mechanisms can be primarily categorised into three different types based on the regulatory features. As shown in Figure 2.2, the regulator can block the operator site and prevent the RNAP complex from binding in a repressor model. Upon activation by the inducer, the regulator can be released from the DNA and initiate the transcription. A variant of such a model is the aporepressor model, the regulator of which functions as a repressor in its activated form. Promoter activation is more complicated than repression, which often involves the interaction of the regulator with α subunit of the RNAP complex or DNA bending.

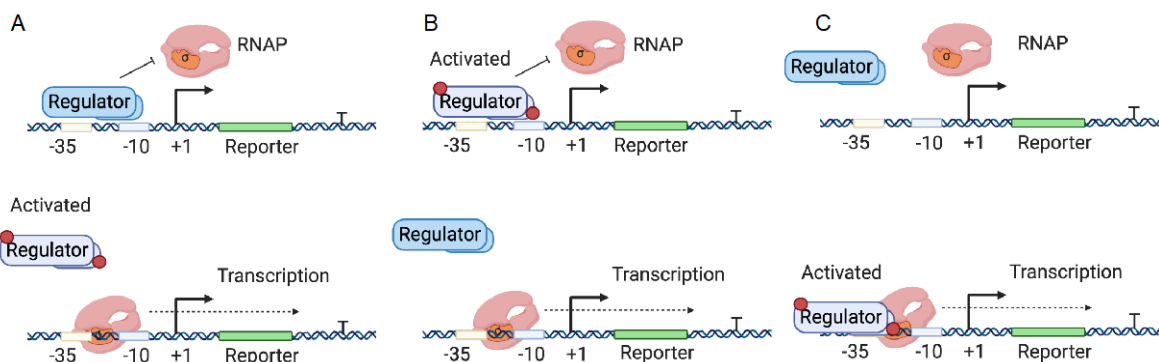


Figure 2.7 Schematic diagram for transcriptional control in the one-component system via TFs, A) repressor, B) aporepressor and C) activator.

Binding of the regulator to its operator site (-35 & -10) can affect the transcriptional initiation through three different mechanisms. Upon binding with the inducer (red dot) and induced conformation change, the regulator can either A) alleviate and release the promoter binding site B) repress or C) recruit or participate in the transcription initiation with RNAP complex.

2.4 Existing transcriptional controls used in bacteria therapy

Of the well-characterised circuits, only a handful remained good candidates for use in bacterial therapy. A summary of regulatory circuits is outlined in Table 2.1. For triggering units induced by

small molecules, the inducer target needs to 1) be well tolerated at high doses, 2) not pose any side effects or toxicity over long-term usage, and 3) be absent in a normal diet to avoid false induction. By this guideline, both L-arabinose and aspirin are good inducer candidates. The P_{Bad} promoter and its regulator AraC are well characterised in multiple chassis systems and have exhibited tight control in most cases [76][77]. In the mouse model, probiotic EcN tested with the P_{Bad} system have shown functional dose-dependent gene expression in colonised tumours upon L-arabinose injection [40]. For sensing aromatic compounds such as aspirin, the LysR transcriptional regulators (LTTRs) represent the largest transcriptional regulators family found in bacteria [78]. The regulator protein is usually 300 amino acids long, composed of an N-terminal DBD binding and an iconic C-terminal HTH (helix-turn-helix) EBD binding site. Several LysR candidates can respond to a variety of aromatic compounds such as benzoate, pentachlorophenol and muconate. The aspirin/salicylate responsive regulator, NahR has been adopted to construct an aspirin-inducible [79] drug expression system and has been tested in the mouse model. The construct was tested in *Salmonella* and resulted in a significant tumour reduction over a five-day period [79].

Another promising option for external control is to use UV light/radiation to spatially regulate the region of expression. The existing optogenetics module (400 ~ 705 nm) cannot sufficiently penetrate the human body (0.5 ~ 4.5 mm being the penetration depth of skin) [80]. Therefore, gamma radiation and SOS DNA damage sensing systems have been used as spatial triggers for drug expression. Native *Salmonella* and *Clostridia* SOS systems have been implemented to make gamma-inducible systems that respond to 2 Gy of gamma rays [81][43]. Compared with small-molecule switches, gamma-ray induction based on site-specific DNA damage can overcome diffusion limitations, such as non-homogeneous mixing, and offer accurate spatial control.

A third promising method is to use disease-related metabolite biomarkers as inducers. For instance, nitric oxide is an important target that participates in many important aspects of host-microbiome interaction, angiogenesis, cell cycle and cancer development [82]. A high level of serum nitric oxide is often a sign of gut inflammation [9] and is associated with lung cancer [83]. The disease state cut-off for nitric oxide in inflammation gut disease is $>13.65 \mu\text{M}$ [9]. Archer et al. employed a nitric oxide sensor for recording disease state that includes an *E. coli*-derived NorR regulatory module combined with a *fimE* recombinase to control the bidirectional expression of RFP and GFP [84]. Results demonstrated successful tracking and recording in inflamed mouse ileum explants within six hours of detection. Other inflammation-related biomarkers such as thiosulfate and tetrathionate responsive units have also been characterised in the mouse model as a potential diagnosis platform for colon colitis [85]. However, this approach employed indirect sensing of the secondary metabolites from the primary oxidative biomarkers H_2S .

Finally, regulating circuits that respond to the tumour micro-environments have been developed based on the hypoxia-sensing fumarate and nitrate reduction (FNR) regulator [86]. In the absence of oxygen, iron-sulphide clusters trigger a conformation change of FNR to induce the promoter firing. However, considering the heterogeneity and complex tumour structure, the well-oxygenated peripheral region might pose challenges towards this type of sensory circuit. Other self-regulating quorum-sensing circuits have been constructed to induce a self-suicide cell lysis cycle upon reaching a critical population [44]. This results in an oscillatory protein expression and drug release every 60 mins. This system is useful once stable colonisation can form within the tumour core. A number of other potentially valuable small-molecule-sensing circuits are also listed in Table 2.1 for future investigation. In this study, I focus on three of the most promising transcriptional control systems.

2.5 Characterisation of transcriptional control units

To characterise the transcriptional units, their translational output needs to be quantified and converted into a detectable signal. Different fluorescent proteins are often used in this case, among other options such as bioluminescence, colour or proenzyme. Figure 2.8 demonstrates a typical dosage response for biosensor output (Y) as a function of inducer concentration (X). The output function (Y) can be described as:

$$Y = \text{Baseline} + \frac{X^{\text{Hill slope}} * (S_{\text{max}} - S_{\text{min}})}{X^{\text{Hill slope}} + EC50^{\text{Hill slope}}}$$

Operational range: This also refers to the working range of a biosensor which defines the inducer range between the highest sensitivity and saturated concentration.

Dynamic range: The signal output range between the maximum (S_{max}) and minimal signal (S_{min} or baseline).

Minimal sensitivity: describes the highest inducer level, where a higher concentration beyond this point would only give rise to a saturated response at the steady-state rather than dosage-dependent expression.

Maximum sensitivity (Limit of detection): defines the minimal inducer level that can trigger significant protein expression to be distinguished from the basal line in a given response time.

Hill slope: describes the steepness of the dosage response curve. A high Hill slope often indicates a more 'digital switch' response and often implemented for decision-making circuits. In contrast, a sensor with a low Hill slope but a wide operational range is often implemented as a biosensor to monitor the concentration of inducer chemicals over a wide range.

EC50: Half maximal effective concentration defines as the chemicals concentration which induces a half-point response between the baseline (S_{min}) and saturated response (S_{max}) after a specified time.

Leakiness/Baseline: this defines the signal protein expression in the absence of an inducer (S_{min}).

Due to random collision of the RNAP complex (intrinsic cellular) noise or non-tight regulation, protein can leak from poorly designed promoters. This is especially important for toxic or cell-burdening protein expression.

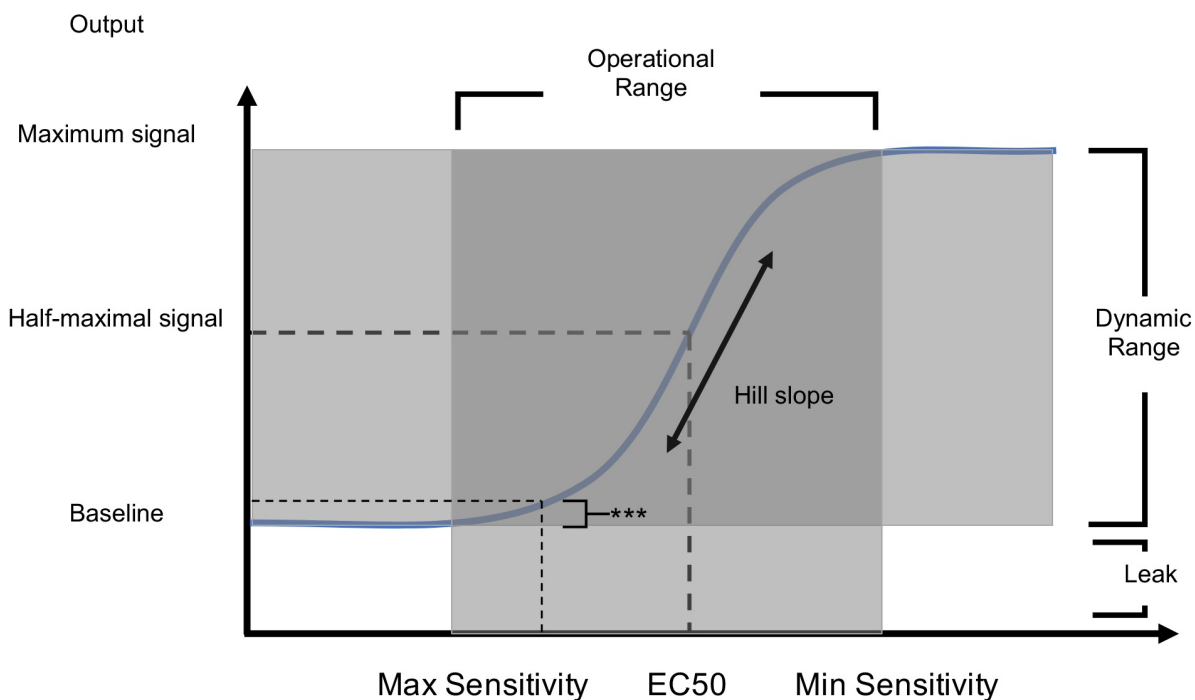


Figure 2.8 Terms for defining expression kinetics of a typical biosensors.

Dosage responsive dynamics of a typical biosensor and term for describing the performance. The X-axis represents the concentration of the input signal/inducer concentration. In contrast, Y-axis represents the protein expression of the signal protein (Bioluminescence/Fluorescent protein).

Table 2.1. List of small molecule inducible transcriptional systems.

Inducible regulators	Inducer	Original/functional strain	Comment	Ref
AcuR	Acrylate	<i>Rhodobacter sphaeroides</i>	Possible false induction by the esters in the foods	[87]
CdaR /day	Diacids: glucarate, galactarate and glycerate	<i>E. coli</i> MC4100	Possible false induction by the organic acids in the foods	[88]
PrpR	Propionate	<i>E. coli</i> DH10B		[89]
CymR	P-isopropyl benzoate (cumate)/ Cuminic acid	<i>E. coli</i> DH5 α , S-17/ λ pir, K-12, Top10, BL21(DE3); <i>Pseudomonas putida</i> F1, <i>Staphylococcus aureus</i> ,	Toxic inducer to human	[90][21]
XylS	Benzoate or m-toluate	Gram-negative bacteria, including <i>Pseudomonas putida</i> ; <i>E. coli</i> K-12 LE392; <i>Azotobacter vinelandii</i>		[91]
LuxR	N-acyl-L-homoserine lactone (AHL), N-(3-oxohexanoyl) homoserine lactone	<i>Vibrio fischeri</i>	Widely used quorum sensing promoter. However, it could have potential false induction by esters in food	[92][93]
TtgR	Flavonoids such as naringenin, phloretin and genistein	<i>Pseudomonas putida</i>	Flavonoids has potential therapeutic effects. However, due to its prevalence in fruits and vegetables, there is potential false induction by diet.	[94]
PI	CI repressor/ Heat inducible	<i>E. coli</i>	Leaky expression with difficulties in fine-tuning threshold	[95]
GBP	GBA (γ -aminobutyric acid)	<i>Pseudomonas fluorescens</i>	Human neuron transmitter	[96]
Mvfr	Dynorphin	<i>Pseudomonas aeruginosa</i>	Non-specific quorum sensing signalling using human neuron transmitter as inducer. However, a high dosage of such drug can be addictive.	[97]
OprF	INF-g	<i>Pseudomonas aeruginosa</i>	Human interferon sensing system.	[98]
SSRT2	Somatostatin	<i>Helicobacter pylori</i>	Growth hormone inhibitor, the sensor units demonstrate high sensitivity.	[99]
QseE/QseF	Epinephrine/adrenaline	<i>Enterohemorrhagic E. coli</i>	Human stress hormones epinephrine (Epi) and norepinephrine are sensed through QseC HK, which initiates a complex signalling cascade to regulate virulence gene expression in enterohemorrhagic <i>Escherichia coli</i> (EHEC).	[100]
QseC	Norepinephrine / Epinephrine			[101]
Caf1A	IL-1 β	<i>Yersinia pestis</i>	Pro-inflammatory cytokine is crucial for immune response.	[102]
ZntR	Zinc	<i>E. coli</i>	A dynamic range of 0- 1.1 mM can be detected. Potential false induction through a normal diet such as meat and dairy product.	[103]
AraC	Arabinose	<i>E. coli</i> KS272, LMG194, DHB4, LMG145, LMG89, DH5 α		[104]

LacI	Isopropyl β-d-1-Thiogalactopyranoside (IPTG)	<i>E. Coli</i>	Sugar analogue/ Polysaccharides that are commonly found in fruit, vegetables. Choline is an essential nutrient, and there is potential false induction through a normal diet.	[105]
rhaBad	L-Rhamnose; L-Mannose	<i>E. coli</i> MG1655; <i>E. Coli</i> JM109, W3110, BW3110, BL21		[106] [107]
FruR	Fructose	<i>E. coli</i> JS006 cells		[108]
TreR	Trehalose			
BetI	Choline chloride	<i>E. coli</i> BL21		[109]
VanR	Vanillic acid	<i>E. coli</i> BL21	Non-specific biosensor for detecting aromatic aldehydes that can be potentially therapeutic. However, potential false induction by structural similar chemicals that are present in food.	[110]
NahR	Sodium salicylate	<i>Salmonella</i> spp.	First examples of <i>in vivo</i> regulation of synthetic bacteria using aspirin/salicylate. An ideal drug candidate due to its safety and high clinical tolerance.	[79]
MphR	Erythromycin	<i>E. coli</i> Top10	The inducer is an antibiotic that might disrupt the host native microbiome and is non-ideal for long term usage.	[111] [112]
TetR	Tetracycline	<i>E. coli</i> DH5αZ1/EcN		[113][114]
NorR	Nitric oxide	<i>E. coli</i> Top10	Secondary derive of the gut inflammation biomarkers H ₂ S, which also share many other significant roles in cellular development, immune signalling and cell fate.	[84]
ThsSR	Thiosulfate	<i>E. coli</i> Nissle 1917		[85]
TtrSR	Tetrathionate			[85]
HucR	Uric Acid	<i>E. coli</i> DH5α and DH10B	Potential biomarkers for gout.	[115]
SoxR and OxyR	H ₂ O ₂	<i>E. coli</i>	Detection of oxidative stress caused by free radical molecules. A potential method for the indication of gut inflammation. However, it cannot distinguish specific oxidative compounds.	[116]
FNR	Oxygen	<i>Salmonella</i> spp.	FNR system is an oxygen-responsive transcription factor naturally present in <i>Salmonella</i> and <i>E. coli</i> . It can be used in combination as conditional control upon bacteria colonisation in the anaerobic core. Limited expression will occur in the well-oxygenated area near the blood vessel.	[117]
ChuA	Heme	<i>Bordetella pertusiss</i> <i>E. coli</i>	Useful transcriptional regulatory unit for detecting internal bleeding.	[118]

2.6 Toolboxes for dynamical tuning of transcriptional genetic circuits

Tuning of the genetic circuits typically focuses on generating a library using different combinations of the genetic components to adjust the operational and dynamical ranges. To fine-tune a synthetic circuit, there are global, transcriptional, translational and post-translational approaches. As shown in Figure 2.9, a simple plasmid-encoded expression system contains six modules that can be interchanged to fine-tune the output dynamics. The overall aim using different toolboxes is to alter the overall DNA $\rightarrow$ mRNA $\rightarrow$ Protein flux. These methods include are but not limited to: engineering the upstream binding site of the promoter [119], swapping the ribosome binding site [120], modifying RNA stability [121], changing the strength or direction of the terminator [122], additional tagging to alter protein stability [123] and other protein engineering methods such as rational design of chimeric protein [124] or translational regulation (riboswitch) [125]. Comparative analysis of the merits and drawbacks for different methods are well summarised in [126] and [127].

In this work, I will focus only on transcriptional tuning methods and the usage of feedback motifs. Other translation-mediated control methods have been well summarised in review [128] and [129].

2.6.1 Fine tuning through using simple sequence repeats

Egbert et al. [120] introduced a tuning mechanism that used simple sequence repeats at the RBS after the Shine-Dalgarno region (AGAGGAGAAA) to attenuate the transcriptional strength. Using different lengths of these repetitive spacers allows them to expand the targeted gene expression over a thousand-fold range. Four different combinations were examined (AT, A, AC and T), where the AT spacer showed the best attenuation gradient. Upon every AT insertion (a

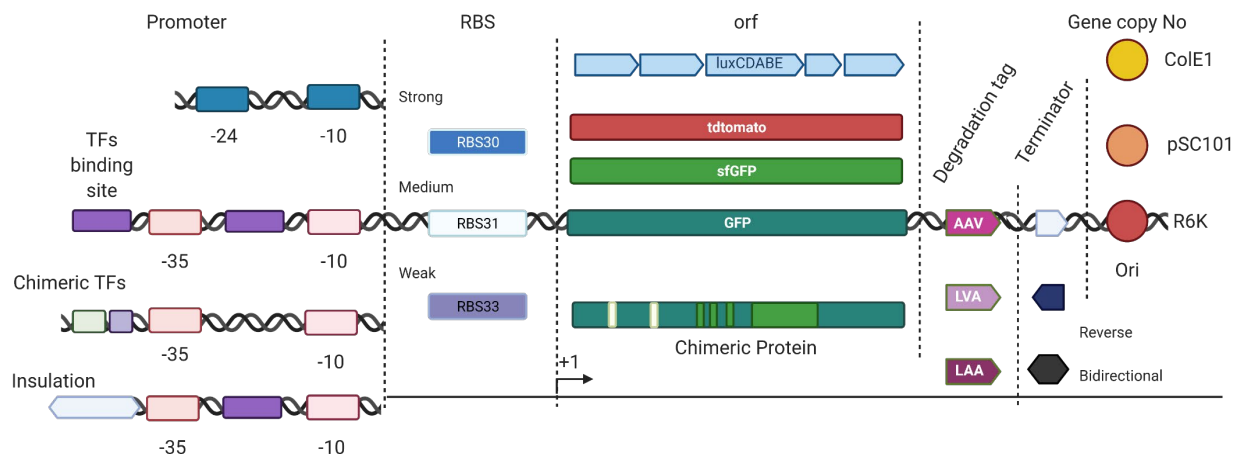


Figure 2.9. Toolbox of tuning transcriptional constructs.

The typical transcriptional biosensor design can be split into six parts: promoter design, ribosome binding site attenuation, tuning of the reporter module and terminator. The plasmid copy number can be altered using different replications of origin to control average number of plasmids/cells. Protein degradation tags can be added to adjust the protein half-life and commonly used to remove the signal masking for non-degradable proteins.

total spacer length of AT₆ and AT₁₂ was tested), a linear inverse correlation between the fluorescence and the spacing length was observed. This provided a convenient way to attenuate the expression amount of the target gene. The longer the spacer, the weaker the expression. However, such method can be affected by the cellular context of the host, thus the actual correlation coefficient could vary in different hosts. The linear correlation between expression level and spacer length is maintained for over 200 generations in *E. coli*.

2.6.2 Feedback motifs with different traits

The transcriptional network with autoregulation occurs more frequently than nonregulated networks in the *E. coli* regulatory system [130]. These patterns include self-regulated negative feedback or positive feedback and more complex network motifs based on these (multi-input feedforward loop, two-node positive feedback etc). This inspired further questions to understand

what evolutionary advantage these motifs could equip for biological systems and how they were involved in multiple cellular function, such as perfect adaptation, noise rejection and cellular memory [131][132].

Uri Alon et al. [133] have constructed a mathematical model to capture these dynamical behaviours and have outlined potential traits for each motif offered. For a negative autoregulated motif, the transcriptional rate of the target gene can be written as an input function regards to the inducer concentration S_x :

$$f(S_x) = \frac{\beta}{1 + \frac{X^*}{k_d}} = \frac{\beta}{1 + \frac{X_T}{k_d} \frac{1}{1 + (\frac{S_x}{k_x})^n}} \quad \text{Equation(1)}$$

where each symbol is denoted as:

X^* – activated repressor in the presence of inducer,

X_T – total repressor amount,

S_x – inducer concentration,

k_d – rate constant of promoter and repressor protein,

k_x – rate constant of protein inducer and protein

n – Hill coefficient of the inducer protein binding.

For the positive autoregulation, this can be written as follows:

$$f(S_x) = \frac{\beta X^*}{(X^* + k_d)} \quad \text{Equation(2)}$$

Both design motifs have been adopted in transcriptional architecture to execute different functions. In particular, positive feedback regulation is a foundational building block for circuits to exhibit cellular memory and persist signal oscillation. The switch-like behaviour allows the external transient signal to be converted into a sustained response. This plays a vital role in energy

metabolism and host survival. Such a design motif has been adopted to amplify genetic signals [134], synthetic bistable switches [135] and synchronised population oscillators [44].

In contrast, the negative autoregulation motif allows circuits to speed up the response time [133]; these types of motif are often found in cellular sensory responses to toxin or predator metabolites. Furthermore, negative autoregulation confers increased robustness of steady-state expression levels against the fluctuation of environmental noise [136].

2.6.3 RNAP collision-based transcriptional tuning

Antisense transcriptional regulation is another prevalent motif adopted across all organisms. About 30% of transcriptional start sites in *E. coli* were found to be located in the antisense transcript direction or within the opening reading frame [137]. Similarly, transcriptomic studies have revealed that about half of the genes were regulated and coupled with antisense regulation in the human pathogen *H. pylori* [138]. Such methods frequently occurred for genes that require tight regulation, such as toxic genes or virulence factors [139][140]. Antisense regulation as a universal tuning strategy was systematically reviewed Brophy et al [141]. A large library of 5,668 combinations between constitutive antisense promoters and unidirectional terminators was investigated. The library aimed to fine-tune three regulatory circuits in a NOT logic fashion. The results demonstrated an over 30-fold change in output and the strength of antisense promoter follows a linear relationship with the output threshold. As shown in Figure 2.10C, this offers a convenient method for tuning the response threshold without modifying the input promoter and related RBS. Several mechanisms have been proposed for the downregulation caused by the antisense promoter firing. For the promoters that are >200 bp apart, the modelling results suggested that the sense promoter and antisense promoter interfere with each other mainly via the collision of the actively transcribing RNAPs [142]. However, other mechanisms are not mutually exclusive;

the transcribed antisense mRNA could bind and subsequently abolish the translation of sense RNA. [141]

2.6.4 Transcriptional signal amplification

Numerous studies outline the potential usage of whole cell bacterial biosensors for *in vivo* disease diagnosis [75]. However, the complex and heterogeneous environment found in the human gut has proven to be challenging, leading to an unpredictable performance and low signal-to-noise ratio. Furthermore, serum and urine samples exhibited inhibition towards normal cell physiology. thus pre-dilution of clinical samples is often required before the *in vitro* usage of biosensors [143]. This makes signal amplification modules a pivotally important tool. An ideal transcriptional amplifier should reliably scale-up the inducer signal and exhibit a tuneable analogue transistor dynamic. Several existing methods are shown case below.

Recombinase/Integrase-based amplification method. Site-specific recombinase (serine and tyrosine recombinase classes) is class of enzyme that catalyses the inversion or deletion of a gene depending on the recognition site orientation. For a DNA sequence marked with opposing recognition site, the recombinase catalyses unidirectional inversion. Conversely, for a gene marked between aligned sites, deletion of the flanked DNA occurred. This unique property has been used to build memory circuits [144], counters [141] and switches [145] upon receiving external stimulus.

On the other hand, if the transcriptional activity of the sensor promoter is lower than that of the recombinase modules, the circuits can be used as a transcriptional signal amplifier. Bonnet et al. have constructed six different types of the one-layer logic gate with amplification function based on two types of recombinases [146]. All constructed gates exhibited an amplification gain ranging from 1.8 ~ 129 F.c. The constructed circuits were then used to test against human clinical samples (serum and urine) for glucose and nitrogen oxides [147]. This type of system often has

minimal leakiness in its OFF state and is tightly regulated throughout passages. However, it also suffers from heterogeneous gene expression, which leads to a mixed population of ON/OFF cells. Creating a closed negative feedback loop with excisionase has been shown to increase system robustness by generating a more uniform population [148].

HrpRS based cascading amplification method. Using the *hrp* (hypersensitive response and pathogenicity) regulatory gene found in *Pseudomonas syringae*, Wang et al constructed a tuneable transcriptional amplifier with a gain range over 8.1 ~ 20.3 F.c [14], as shown in Figure 2.10 D. Upon receiving a sensor input, the HrpRS will be expressed and form a heterohexameric complex for subsequent activation of the P_{HrpL} , which in turn amplifies the small transcriptional input from the sensor into a larger GFP output. Using the repressor module HrpV, which suppresses the formation of the heterohexameric complex, the circuits can be tuned with different amplification gains.

The hrpRS amplifier has been proven to be highly modular and further developed into a three-layer cascading amplifier [149]. The initial arsenic sensor input was connected with three transcriptional amplification modules (ultrasensitive phage activator RinA and extracytoplasmic function sigma factor ECF11) in sequence. The results showed a much higher amplification gain range from 66 ~ 110 F.c and a much higher sensitivity (750-fold ~ 5000-fold increase) than the HrpRS amplifier alone.

T7 RNAP based amplification method. The T7 RNA polymerase system was initially derived from the bacteriophage system and has been shown to reallocate more than half of the total cellular resource upon activation [150]. This system was developed to become IPTG responsive and is widely used for recombination protein expression. Such high transcriptional flux has also been engineered to become a heavy-metal sensor. Kim et al. have constructed CadC-controlled T7

RNA transcription systems to improve the cadmium/lead sensor's sensitivity and output dynamics [151]. Such a system achieved a 9.2-fold boost in sensitivity compared to the original CadC regulatory system.

Despite the well-established and commercialised pET system, the highly fragmented T7-derived expression system has been constructed by Shapiro et al. for creating orthogonal genetic switches [152] and a 'resource allocator' [153]. This offers better flexibility in constructing a multiple-signal integration system. More importantly, the resource allocator can allow temporal control of different genes to avoid retroactivity, which is a common issue in the large circuits when downstream gene expression is in competition with the upstream gene, given only a limited RNAP and ribosome are available.

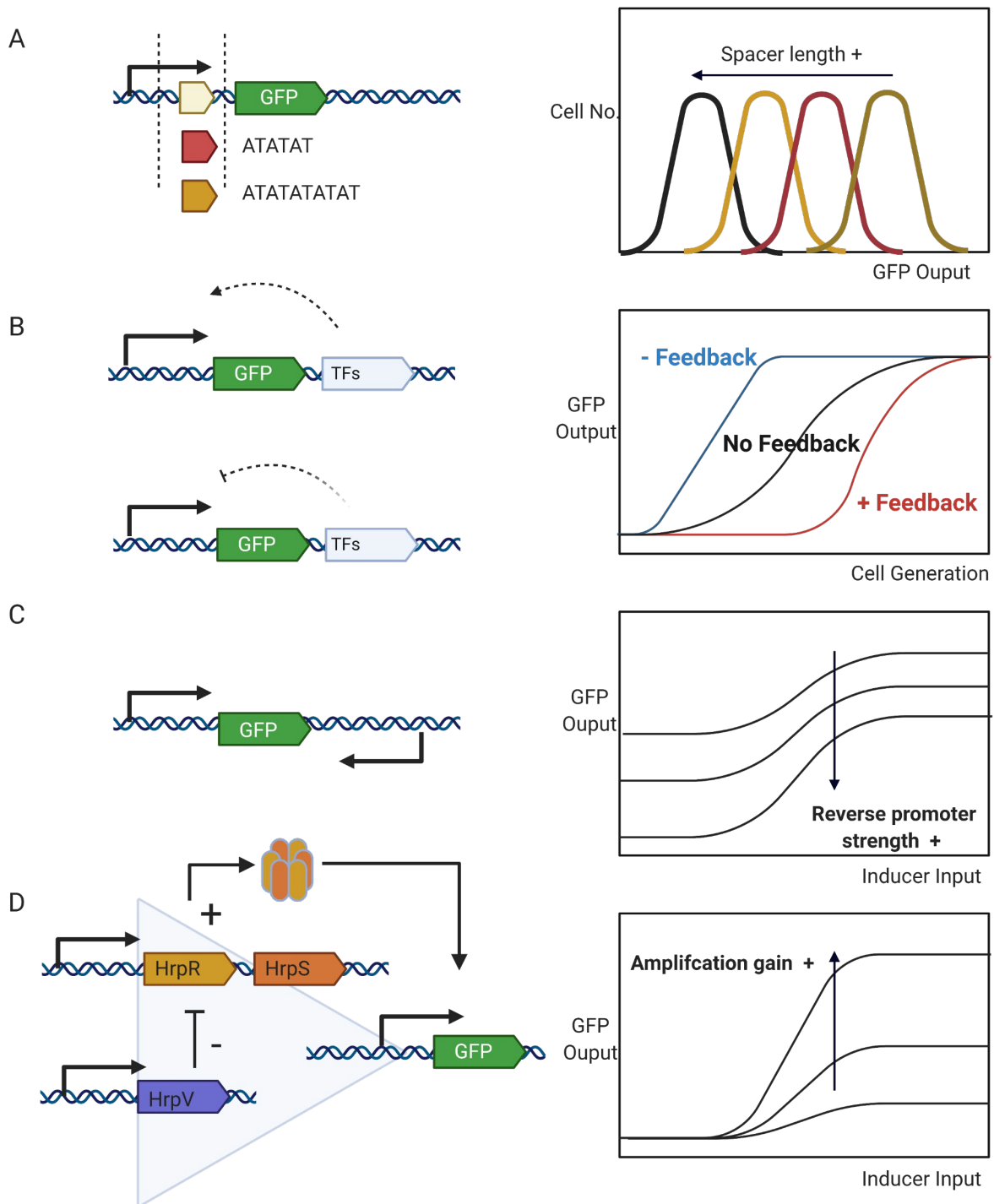


Figure 2.10 Different methods for tuning the dosage response curve of a typical biosensor.

A) RBS-strength attenuation using repetitive AT repeats. The longer the AT repeats, the weaker the RBS strength. This can be reflected as a linear correlation between the GFP output and the AT spacer length. B) Different feedback motifs and their associated characteristics. Autoregulated

negative feedback can speed up response time under the cost of the same cellular energy, while positive feedback allows switch-like dynamics and system memory. C) RNAP collision constructs, as the increased strength of the reverse promoter, the dosage response curve of the forward promoter can be shifted downward. D) HrpRS-based amplification construct. The initial sensor signal can be wired to control the formation of heterohexamer HrpRS, this subsequently controls the ultrasensitive response of P_{HrpL} with a higher flux of GFP production. The HrpV module can be controlled separately to tune the amplification gain through dissociating the formation of heterohexamer.

2.7 In vivo and clinical usage of engineered bacteria

The vast quantity ($10^{11} \sim 10^{12}$ microbes/ml) and species diversity of microbes (>1000 kinds) that have co-existed with humans provide a plethora of chassis choices for bacteria therapy [154]. Among the existing cases, seven different chassis strains have been studied extensively with clinical results demonstrating early promise for cargo delivery or *in-situ* diagnosis/treatment. A non-exhaustive list of existing cases has been summarised in Table 2.5. Below I discuss the distinctive features exhibited by different strains and give general guidance for choosing the suitable strain based on different niches. Ionic features of each strain are also illustrated in text boxes shown in Figure 2.11. The average residency time exhibited by each strain is summarised in Table 2.3.

Bacteroides. *Bacteroides* spp is a class of gram-negative, facultative anaerobic symbionts. *Bacteroides thetaiotaomicron* is the most prevalent and abundant species in the human gut [155]. It constitutes 40–45% of the total gut microbiome population. Long-term stability analysis of the human microbiome has shown that *Bacteroids* spp can achieve robust colonisation within a five-year study across different diets, gender and age [156]. Indeed, *Bacteroides* exhibits a diverse metabolic profile that can break down most of the diet-derived carbohydrates and lipids [157]. These traits allow *Bacteroides* to be a suitable chassis for treating a chronic disease or for building memory circuits embedded for long-term monitoring. Given the late start compared to other

commensal strains, not many well-characterised genetic tools exist for *Bacteroides*. However, progress has recently been made in using *Bacteroids* spp for treating metabolic disease. For instance, *Bacteroides ovatus* has been engineered to break down porphyrin, a rare marine polysaccharide found in the normal diet [157]. Furthermore, CRISPR interference and recombinase-based memory systems have both been implemented and tested functionally using *Bacteroides thetaiotaomicron* [158].

Salmonella spp. *Salmonella* spp is a class of gram-negative facultative anaerobic bacterium. *Salmonella typhimurium* is the most characterised strain for tumour treatment and has been shown to have a high degree of tumour-specific colonisation in both *in vivo* studies and clinical trials [159][53]. As a human pathogen, several modified *Salmonella* strains (strains VNP20009 [69], A1-R [54] and CRC2631 [160]) have been made for attenuated virulence, auxotrophic growth or truncated cell invasion to increase its biosafety[161]. Due to its pathogenic origin, its secretion system and cell invasion mechanism have also been explored to transfer DNA or siRNA to a mammalian cell [162][163]. Another interesting feature exhibited by *Salmonella typhimurium* is that it displayed 14.5 times faster average swimming speed than *E. coli*, and colonised tumour tissues with a hundredth of the dosage [164]. The importance of its motility is further demonstrated by overexpression of the regulator *flhDC* in chemotaxis, yielding five times more accumulated mass in tumour colonisation in comparison to the wild type [165]. Several ongoing clinical trials using *Salmonella* have been summarised in Table 2.5.

Listeria monocytogenes. *Listeria monocytogenes* (LM) is a gram-positive facultative anaerobic bacterium commonly associated with listeriosis. LM is highly immunogenic and can elicit both adaptative and innate immune responses. Upon invading a host cell, LM produces a pore-forming protein, listeriolysin O (LLO), which allows evasion and entry into the cellular

cytoplasm of the cell. Degraded peptide produced by LM can then be passed onto class I histocompatibility complex (MHC) and activate CD8⁺ T cells. At the same time, engulfed bacteria trigger an antigen presentation of CD4⁺ T cells and a MHC response [166]. Based on this unique immune-triggering property, attenuated LM strains have been assessed as ideal tumour vaccine candidates. The first live-attenuated LM-based HPV vaccine was conducted in 2001, where 15 patients with metastatic cell carcinoma received treatment and showed an increase in median survival rate [167]. Numerous clinical trials are ongoing, with different fusion protein markers based on LM targeting lung, prostate and HPV associated cancers [168].

Clostridium. *Clostridium* spp is a class of gram-positive, spore-forming, strictly anaerobic bacterium. The first usage of *Clostridium* spore for cancer treatment can be traced back to 1978 [169], as a well-recognised safe chassis, the spore-forming feature and strict anaerobic feature ensures *Clostridium* only germinates and proliferates within the hypoxic/necrotic cores of solid tumours or the rectal region. *Clostridium novyi* has been demonstrated to show selective growth in the hypoxic region of solid tumours and cause tumour regression [170][171]. Direct injection of *Clostridium* spores has also achieved a similar degree of success [66]. The administration of using the spores directly bypasses several challenges associated with oral delivery, such as cell viability drop after exposure to bile salts, stomach acids and digestive enzymes.

Lactobacillus/Lactococcus. Both *Lactobacillus* spp and *Lactococcus* spp are gram-positive, non-sporulating, strict anaerobic probiotics with a long history used in food and agriculture. The safety of use and protection against pathogen strains attracted much attention in altering them to become therapeutic carriers or vaccines. However, the low G-C content in their genomes and a lack of effective recombination tools make them difficult to engineer. Nevertheless, recent advances in creating and characterising genetic toolboxes of *Lactobacillus* spp has become

available [172]. The gram-positive membrane is advantageous for protein secretion, and *Lactobacillus* spp naturally exhibits several immune-modulating effects. These properties have been exploited to develop mucosal vaccines by expressing surface/extracellular antigens such as HPV E7 [173], HIV [174] and influenza virus [175]. Similar to EcN, *Lactobacillus* does not exhibit long-term colonisation of the human intestine. However, early clinical trials suggest most *Lactobacillus* strains can colonise in the human gut within 5–12 days [176][177], which is long enough for most *in vivo* treatment.

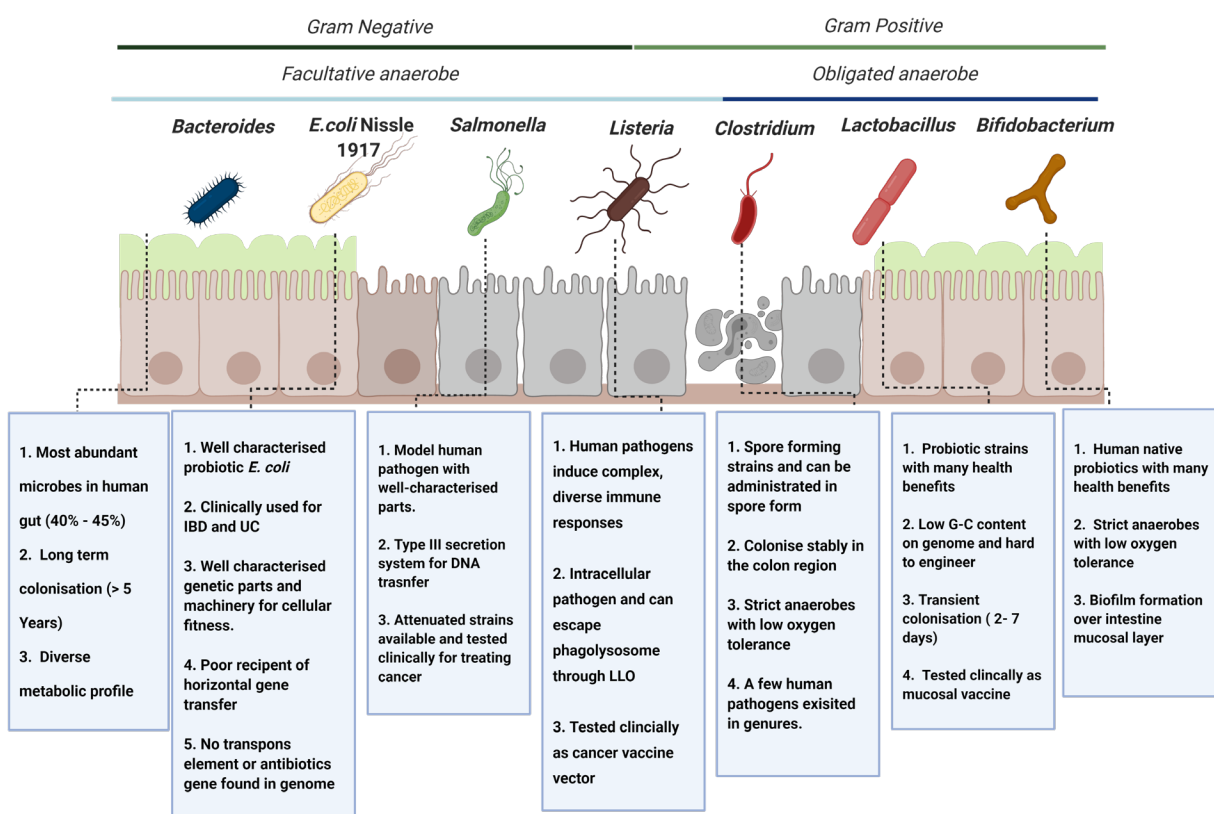


Figure 2.11 Seven most common strains used for bacteria therapy.

Seven commonly used strains in bacteria therapy. The metabolic and gram profile is shown at the top. Genus of human pathogens is shown in the region with grey intestinal marks. The iconic features of each strain are described in the boxes at the bottom.

Bifidobacterium. *Bifidobacterium* spp is a class of gram-positive, obligated anaerobic probiotic bacterium. Similar to *Lactobacillus* and EcN, *Bifidobacterium* exhibits several health-promoting effects such as the prevention of diarrhoea, modulation of the host immune system and the improvement of lactose intolerance [178]. As a stable probiotic community (2–14%) that is resident in the human gut, native bifidobacterial microbiota remains stable during an individual's life span with little variation across different age groups [179]. Numerous studies have exploited this feature to investigate the long-term host-microbiome interactions to gain insight into the gastrointestinal transit time [180] or the impact of physiological state under different environmental conditions [181]. Further examples using *Bifidobacterium* as a carrier include delivery of human growth factors [182], antibodies [183] interleukins [184] and anti-tumour compounds [185].

Choosing the right strains. To choose an ideal chassis system for bacteria therapy, multiple factors need to be considered depending on the application niche. For instance, the large intestine poses an anaerobic or semi-anaerobic condition (with oxygen dissolved in water) [186], thus genetic circuits that are not affected by oxygen are preferred here. The strict anaerobes listed above are a better choice in this regard. However, this would complicate the downstream characterisation step and manufacture for later medical translation.

Furthermore, it is critical to assess whether designed strains can successfully colonise in the designated region. Previous *in vivo* studies indicated that *E. coli* displayed a much higher growth rate in the intestine mucosa layer than in the lumen [168]. Such a variation in growth conditions could have a non-ideal impact on the dynamics of the circuit.

Residence time and interaction between the host immune system and synthetic bacteria is the most critical factor. Non-ideal immune clearance, especially for those strains with immune

activation, can often occur before the colonisation can establish. This was observed in phase I clinical trial of administered *Salmonella*. The reticuloendothelial host system clears the majority of the strains within 24 hrs [187]. Increasing dosage is not a viable solution since severe dose-dependent side effects were reported using an attenuated strain *Salmonella* VNP20009 [188].

An indirect solution is to decorate the pathogenic cellular membrane with probiotics (EcN) outer shell as a camouflage [189], or simply to use probiotics strains such as *Lactobacillus*, *Bacteroides*, *Bifidobacterium* and *E. coli* Nissle 1917 as starting chassis. In contrast to the non-model and hard-to-engineer probiotics (*Lactobacillus*, *Bacteroides*, *Bifidobacterium*), *E. coli* Nissle 1917 poses many intrinsic advantages. As illustrated in Figure 2.12, EcN has been used initially to treat IBD (inflammatory bowel disease) and other gastrointestinal conditions [190]. The facultative anaerobic feature allows it to tolerate well-oxygenated areas in the peripheral tumour region [41]. Evidence demonstrated that EcN produces two different microcins [171] and possesses seven different kinds of iron-scavenging siderophores [172], thus allowing it to out-compete pathogens such as *Salmonella* spp and *Listeria* spp. EcN also interacts with the host's intestinal barrier by forming a protective biofilm layer [191]. This has been clinically demonstrated to maintain the integrity of the intestinal barrier and stimulate anti-inflammatory activities [2]. Importantly, in contrast to *Bifidobacterium* or *Bacteroides*, EcN does not possess long-term colonisation capabilities, thus making it a safer candidate to test with genetic modification. The genome of EcN is 0.4 Mb larger than *E. coli* K12 with no known virulence factor detected [192]. A bioinformatics study of EcN also reviewed no transposon elements or antibiotics resistance found in its genome [192]. Further *in vivo* studies also indicated that EcN acts as a poor recipient of horizontally transferred DNA [2]. This ensured a relatively safe starting point for its genetic containment.

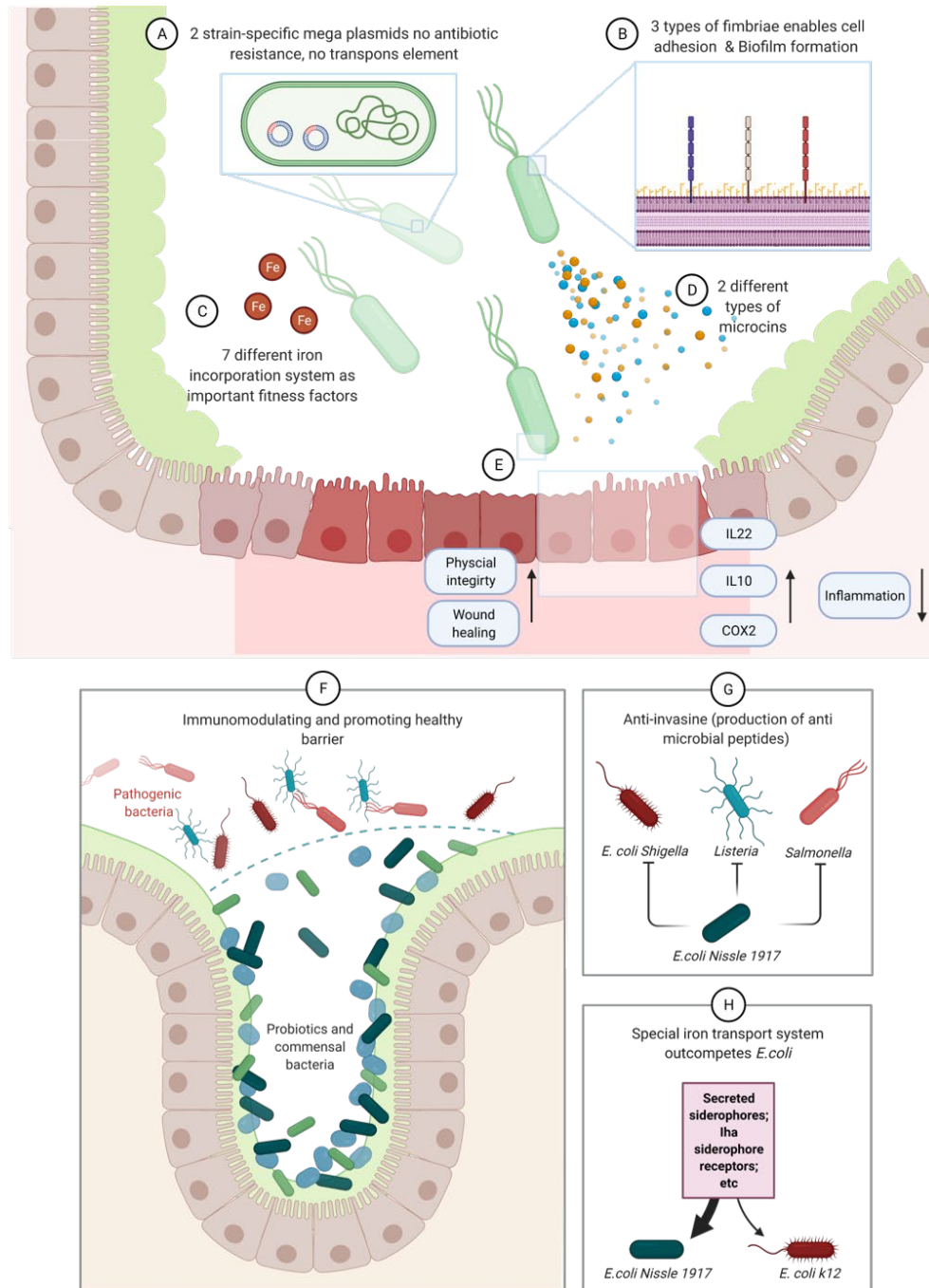


Figure 2.12 Characteristics and health benefits of *E. coli* Nissle 1917

A. Genomic material of EcN composed of genome size of 5.1 Mb and two cryptic plasmids B) three different types of fimbriae enable cell adhesion & biofilm formation over the intestine mucosal layer. C) seven different iron incorporation systems and D) two different microcins allow enhanced fitness and competitive advantages towards pathogens. E) The protective layer of EcN biofilm has several immune-modulating effects and maintains the health barrier function. F) Growth outcompete with *E. coli* k12 and killing pathogens like *Shigella*, *Listeria* and *Salmonella* have all been demonstrated clinically.

An additional advantage of using EcN as a chassis for bacteria therapy is the well-established tools and characterised parts in *E. coli*. Although minor genome differences existed between the lab *E. coli* strains and EcN, the transcriptomics is highly conserved between EcN and its ancestor *E. coli* k12 in both biofilm and planktonic states [193], therefore making most of the characterised genetic part easily transferable into EcN [193]. In conclusion, this strain was considered to be the optimal choice for use as a chassis strain in this study

2.8 Minicells and their application

2.8.1 Min system and bacterial division

Minicells are chromosome-free, nano-sized (100~400nm), non-dividing, dwarf cells generated as a product of abnormal bacterial cell division. For clarity purposes, only minicells with intact transcription/translational machinery and preloaded constructs will be termed as mini SimCells (simple cells). Chromosome-less minicell production was initially found in *E. coli* and a *B. subtilis* mutant back in 1967 [194]. Later, minicell-producing strains have been found in both gram-positive strains (*Lactobacillus*) [195] and gram-negative strains (*Salmonella*) [196]. The Min system plays a vital role in regulating the spatial and temporal location of the FtsZ rings. The precise molecular mechanism involved in minicell production is shown in Figure 2.13. The *E. coli* Min system is composed of three essential Min proteins, MinC, MinD and MinE, forming a gradient oscillation between the dipolar ends. MinC acts as an inhibitor of FtsZ (Figure 2.13C) and binds with the MinD dimer. Meanwhile, MinD anchors itself to the inner membrane and forms a cluster at the polar end in a density-dependent manner. Upon MinE binding, ATP is hydrolysed and releases the MinD from its membrane-binding state to reinitiate another cycle of oscillation. This repeated oscillation creates a time-average void area of MinC in the middle of cells to guide the formation of FtsZ ring complex and perform equal size division (Figure 2.13D).

Previous reports indicated that alteration of any Min component could affect the overall oscillatory dynamics, leading to defective division and minicell phenotype [197]. Methods in the generation of minicells include combinatory deletion of all *minCDE* orf or frame-shift mutation in a single *minCD* orf [198]; an alternative method using overexpression of FtsZ to overpower the MinCD [199] can also lead to minicell formation. Existing studies of the structural mechanism of MinCDE interaction and its oscillatory behaviour have been well summarised in review [197] and [200].

2.8.2 The minicell in medical usage

The minicell was initially used as a model chassis for studying the phage invasion and the molecular structure of the bacteria secretion system under Cryo-EM [201]. It was only recently that minicell was designed as a carrier module to apply in cancer therapy, in contrast to the conventional liposome-based carrier. Minicells can be encapsulated with a wide variety of chemotherapeutic with different charges, hydrophobicity and solubility [5]. Doxorubicin, a chemotherapeutic drug used to treat brain tumours, was loaded into minicells with a loading capacity of 10^6 /minicell [202]; in comparison, same-size liposomes can only be loaded with 10,000 drug molecules. Through the surface attachment of a bispecific antibody, the minicell can anchor to specific tumour types and gain entry via endocytosis. The increase in specificity significantly reduces the systematic toxicity of Doxorubicin administration [183]. Furthermore, cargo delivery of siRNA/shRNA has also been successfully implemented to compromise drug-resistant tumours via knockdown multidrug resistance genes. The loading capacity of RNA into minicells was reported as ($\sim 12,000$) siRNA and shRNA (~ 100 copies) [44]. Another key advantage of using minicells is that the intact bacteria membrane allows several key functions to be retained. For instance, the *Salmonella* minicell has been exploited as a nano-injection machine for antigen

delivery [203]. The results indicate that minicells are able to maintain the proton gradient and assemble functional T3SS injection machinery for eliciting CD-8+ response upon antigen delivery. More *in vivo* and clinical studies have been summarised in Table 2.4. These results outline a great potential for minicells competing with the conventional nanomedicine as a programmable chassis with higher load and specificity. However, the programmability of minicells was not pursued and investigated in detail.

In contrast with using whole-cell chassis, the non-dividing minicells could allow genetic circuits to be faithfully executed without potential mutation or growth-burden-derived function silencing. The small size could also facilitate faster diffusion and penetration through the tumour surface. Lastly, due to the prevalence of Min systems in bacterial replication, minicells with different strains of origin can be generated to ensure flexibility upon different application niches.

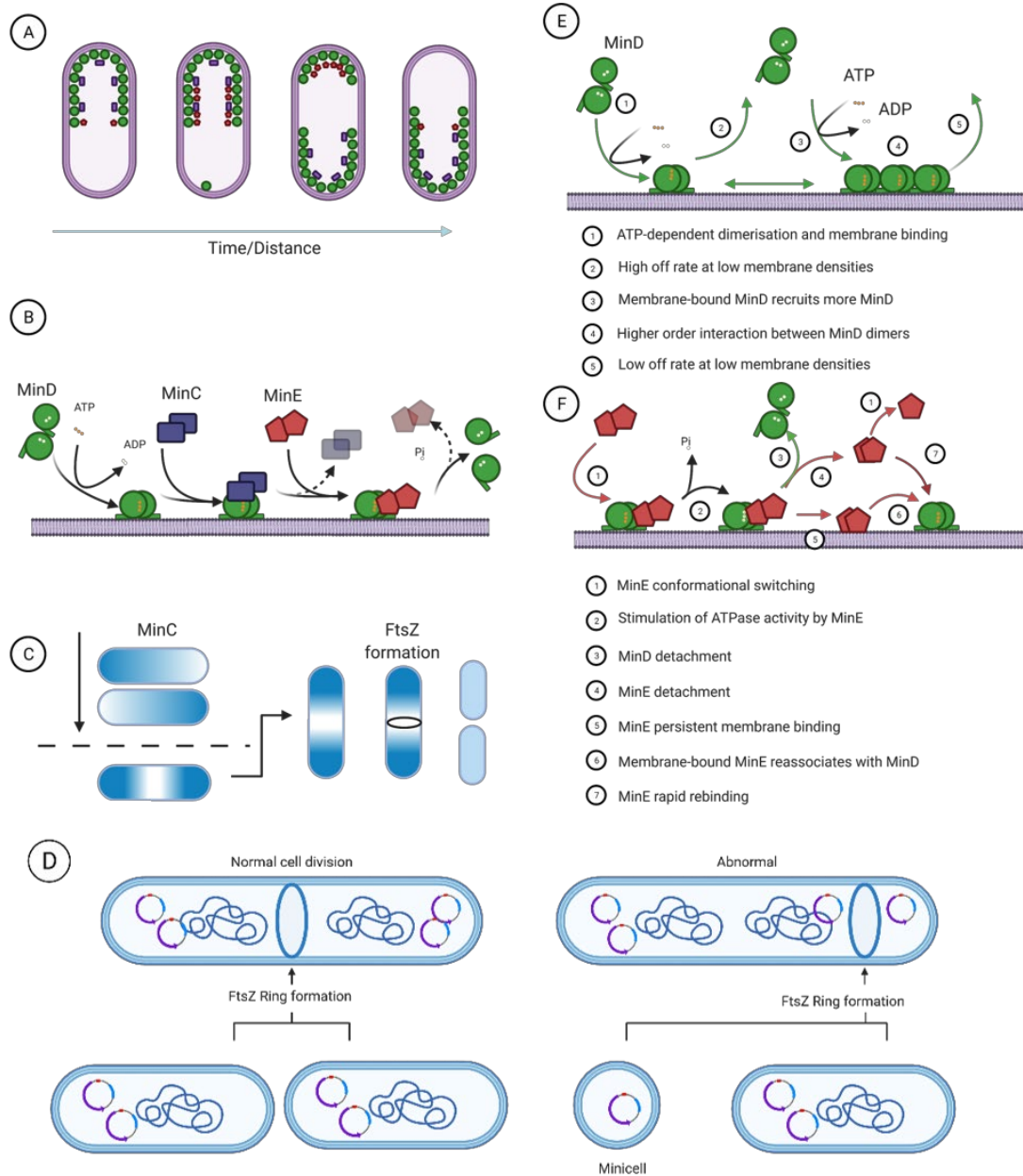


Figure 2.13 *minCDE* machinery for controlling binary division.

Schematics diagram for A) MinCDE oscillation within the cells. MinD attaches to the cellular membrane and oscillates between the dipole ends of the cell. Key components are colour-coded in green (MinD), purple (MinC) and red (MinE) B) Detailed interaction between each element. C) Spatial average of the MinC concentration position the precise location of FtsZ ring in the middle of the cell for binary fission. D) disruption of the spatial gradient formation ($\Delta minCD$, $\Delta minC$ or $\Delta minD$) can lead to FtsZ ring formation at either of the dipole ends, subsequently forming chromosome-free minicells and elongated diploid daughter cells. E) Membrane-bound MinD dimer displayed density-dependent cooperativity to ensure it concentrated at the dipole region and F) MinE dissociated the membrane-bound MinD dimer via ATP hydrolysis thus releasing it for the oscillatory cycle. Figures partially adopted from [204].

2.8.3 Purification and extraction

To date, there is no purification protocol available for large-scale minicell production. Several different lab-scale methods have been developed to purify and isolate the minicells from their parent cells. These different methods can be split into 1) Physical methods, 2) chemical methods, 3) genetic methods, and 4) combinatorial methods. The relative merits and drawbacks of each approach will be discussed. The yield and purity achieved by each method are summarised in Table 2.2. Despite numerous efforts to obtain pure minicell culture, there is no systematic approach in analysing the critical determinant step in the overall purification process. Often, one or more methods are combined to yield the best results.

Physical methods. Physical separation mainly relies on the difference in size/density between the parent cells and minicells. Differential centrifugation followed by centrifugal enrichment in a sucrose gradient has been adopted in [205]. Low-speed centrifugation was initially carried out to remove the large cell pellets. The remaining supernatant was then resuspended and further separated in a 5%~20% sucrose gradient. This process was repeated until the minicell was concentrated and enriched in one layer. The reporter yield (minicells/parent cells) of the final product lies in the range of $1:10^4 \sim 1:10^6$ [205]. However, the overall process is energy-intensive and sensitive to human error, thus requires additional chemical treatment. Furthermore, the usage in the sucrose gradient is toxic to *E. coli* cells and may affect the biosynthesis activity of minicells [206].

Chemical methods. Chemical methods generally involve using cell-wall lysing antibiotics or cell-wall inhibiting chemicals. An inhibitor of cell wall biosyntheses such as D-cycloserine [207], Vancomycin [208] and β -Lactam-derived antibiotics (Ceftriaxone, penicillin) [206] were all tested to kill the parent cells while leaving the non-dividing minicell unaffected. Notably, such

methods generally require the parent bacterium to be at its exponential growth phase. Therefore, one or two hours of incubation at its optimal condition is usually required before the addition of drugs. The drawback of such a method is that different bacteria often possess different levels of intrinsic tolerance for antibiotics, thus limiting this protocol's transferability when a new type of parent cell is used. Antibiotic usage can fit well with small-lab scale production; however, they are much more difficult for the later translation and scale-up in large-scale fermentation.

Genetic method. Genetic modification has been applied on the host chromosome to encode either a 1) conditional expression of a toxin gene or 2) contain essential gene deletion to make auxotrophic parent cells. A thermoinducible suicide system was deployed to eliminate parent cells, in this case, a restriction endonuclease (I-CeuI) was placed under a heat-sensitive promoter $P_{\lambda_{R-L}}$. Upon temperature shift to 42°C, I-CeuI will create a double-stranded break on the host chromosome at multiple sites, therefore killing any potentially contaminated parent cells. Minicell-producing strains with *dapA* and *proAB* deletion [33] have shown auxotrophic growth dependence on diaminopimelic acid (DAP), and externally supplemented proline. Therefore, to increase the purity of minicells, the parent cells can be killed via removal of the supplemented nutrients and incubated overnight. A further example is the development of an autolysin (Holin/lysin) system [209] under the control of a P_{Bad} promoter to trigger cell lysis only for actively dividing populations, thus leaving the minicells unaffected.

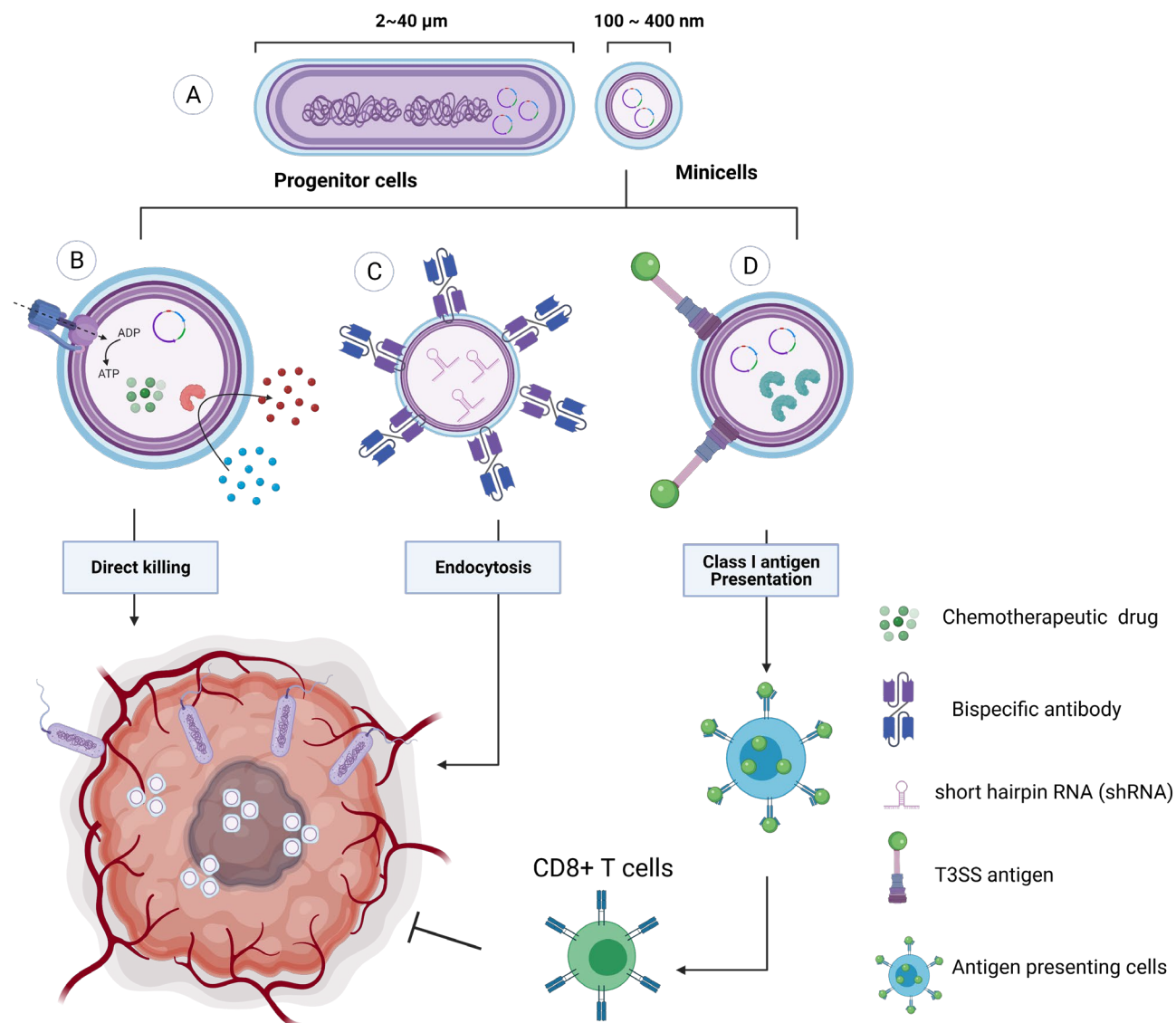


Figure 2.14 Minicells and their application in bacteria therapy.

A) Minicells are produced via abnormal cell division with an average size of 100~400 nm in diameter. Due to the intact membrane structure, it offers unique advantages when compared to a cell-free system, existing usage of minicells in medical application such as B) loading of chemotherapeutic compounds or proenzyme C) DNA or shRNA transfer via bispecific antibody derived endocytosis. D) immune activation (CD8 +) via inducible expression of antigen on minicell membrane. Furthermore, due to its high surface/volume ratio and small size, it could offer better diffusion and penetration through the porous structure displayed on the tumour surface.

Table 2.2. Existing purification methods for minicells.

Parental strains	Purification type	Yield	Contamination	Ref
<i>Salmonella typhimurium</i>	Low speed centrifugation combined with Opti-prep (Iodixanol) gradient	$1.67 \pm 0.7 \times 10^{12}$ per preparation (500 ml starting culture)	0.0001% of original	[203]
<i>Escherichia coli</i> x925	Rate-zonal centrifugation and sucrose gradient	6×10^8 per preparation	0.00001% of original	[205]
<i>Escherichia coli</i> PB14	Antibiotics (Ceftriaxone) treatment with filtration	3.81×10^{10} per preparation (200 ml starting culture)	n.a	[206]
<i>Escherichia coli</i> x925	Antibiotics (Penicillin) with gradient centrifugation	1.8×10^8 per preparation (200 ml starting culture)	0.0002% of original	[210]
<i>Escherichia coli</i>	Antibiotics (D-cycloserine)	n.a	n.a	[211]
<i>Escherichia coli</i>	Holin/lysin system	2.05×10^{10} /ml from 1 L starting culture	0.0000025%	[209]
<i>Escherichia coli</i> k12	Auxotrophic mutation (proA ⁻ B ⁻ dapA ⁻) and thermal inducible of endonucleases I-CeuI	1.5×10^6 /ml	0.00006% of original	[33]

Table 2.3 Resident time and seeding density for different tumour colonised bacteria.

Gram	Bacteria type	Seeding dosage in tumour (CFU)	The native population in gut %	Resident time	Ref
Gram negative	<i>Bacteroides</i>	n.a	40~45	>5 years	[156]
	<i>E. coli</i> Nissle 1917	10^8 - 10^9	n.a	>32 days	[212]
	<i>Salmonella</i>	2.5×10^8	n.a	4~7 days	[165]
	<i>Listeria</i>	1.5×10^7 ~ 2.5×10^7	n.a	1~6 weeks	[213]
Gram positive	<i>Clostridium</i>	1×10^4 ~ 3×10^6 spores	n.a	n.a	[214]
	<i>Bifidobacterium</i>	10^6	2~14	>6 years	[215]
	<i>Lactobacillus</i>	6×10^9	n.a	2~7 days	[176]

Table 2.4. Existing cases for minicells and their applications. (Encapsulating chemotherapeutic drug, protein, DNA and RNA)

Delivery Cargo	Strains	Genotype	Comments	Ref
Cytolysis protein toxin. Perfringolysin O	<i>E. coli VAX12B4</i>	<i>MG1655 (Δlac-pro, ΔlpxM, ΔdapA, attBλ::proBAΩpTac::ftsZΩc1857-PR-PL::I-CeuI)</i>	Cytolysin toxic protein delivery via minicells with membrane decorated integrin to target bladder tumour.	[33]
Nucleoprotein of choriomeningites virus (LCMV) and DNA vaccine.	<i>E. coli MPX1B9</i>	<i>MG1655(F⁻, λ⁻, ilvG, rfb-50, rph-1, zac::aph, lacIq, Ptac ftsZ20, ΔphoA)</i>	Co-delivering viral nucleoprotein and DNA vaccine for immunisation.	[216]
Y.pseudotuberculosis invasion protein and plasmid DNA	<i>E. coli MPX1B9</i>	<i>MG1655(F⁻, λ⁻, ilvG, rfb-50, rph-1, zac::aph, lacIq, Ptac ftsZ20, ΔphoA)</i>	Characterising the internalisation of minicells upon invasion binding with mammalian cell (Cos-7) surface.	[217]
Doxorubicin and pH insertion peptide (pHLIP)	<i>E. coli Nissle 1917</i>	EcN (<i>ΔminCD:cm</i> ; minE over-expression)	Chemotherapeutic drug delivery via minicells and microenvironments triggered payload activation.	[218]
Doxorubicin	<i>S. typhimurium</i>	<i>Δ minCDE- strains</i>	Chemotherapeutic drug delivery via EGFR receptor targeting minicells with surface bispecific antibody for brain tumours.	[202]
	<i>S. typhimurium</i>	<i>Δ minCDE- strains</i>	Similar to above, however, minicells were loaded with bispecific antibodies for targeting neuroblastoma cells.	[219]
	<i>S. typhimurium</i>	<i>Δ minCDE- strains</i>	Similar to above, minicells were loaded with mesothelin antibody for treating mesothelioma.	[220]
Doxorubicin Fluoropaclitaxel Vinblastine Cisplatin	<i>L. monocytogenes</i>	<i>minCDE knock out</i>	A characterisation study of chemotherapeutic drug delivery via different minicells chassis (payload loading, residency time and efficacy)	[5]
	<i>P. aeruginosa</i>			
	<i>S. flexneri</i>			
	<i>S. enterica</i>			
siRNA	<i>S. typhimurium</i>	<i>Δ minCDE- strains</i>	Short hairpin RNA delivery via minicells targeting tumour cell surface. Internalised shRNA targets and knocks down expression of protein conferred multidrug resistance.	[221]
n.a	<i>E. coli JM109</i>	<i>E. coli JM109 (Δ minCD)</i>	An increase in yield over 80% was achieved via overexpression of FtsQ, ftsL, ftsW, frsN and ftsZ at the same time, <i>minD</i> and <i>minC</i> deletion.	[222]
First 104 aa of SopE protein with antigenic peptide (SopE-OVA)	<i>S. typhimurium</i>	<i>mind:cat</i> chromosome insertion	Protein delivery using minicell as a vaccine with Type III secretion system for injection of antigens, thus achieving immunisation upon membrane contact.	[203]
n.a	<i>C. glutamicum</i>	<i>C.glutamicum (ΔNCgl1366, ΔparA)</i>	Production of minicells via endotoxin free strains.	[223]

Table 2.5. Existing studies explored different strains for bacteria therapy.

Metabolic profile	Strain name	Objectives /Function	Genetic circuits design/Strains modifications	Administrative route	Organisms	Ref
Gram negative	<i>E. coli Nissle 1917</i>	Activation of multiple chemotherapeutic prodrugs	n.a	Injection	Mouse	[224]
		Using genetic engineered probiotics in the mouse model to detect liver metastases through urine samples	LacZ reporter system that are induced by IPTG, convert LuGal and produce Luciferin as output	Oral	Mouse	[41]
		Expression and secretion of human defensins	LacO+T7 expression system with IPTG inducer	Oral	In vitro	[225]
		Expression of NAPEs and suppress obesity	Constitutive expression	Oral	Mouse	[226]
		Sense and kill pathogen <i>Pseudomonas aeruginosa</i>	Quorum sensing induced by pathogen molecule AHL	Oral	Mouse	[227]
		Treating Hyperammonaemia	L-arg biosynthesis regulator ($\Delta argR, \Delta thyA$) knocked out for up-regulating the ammonia converting L-arg synthesis pathway	Oral	Human	[59]
		Treating Phenylketonuria	n.a	Oral	Human (Phase I/IIa)	[228]
	<i>E. coli Top10</i>	Induction of CD4+ and CD8+ response by the colonisation of <i>E. coli</i> TOP10 on tumour	Constitutive expression	Injection	Mouse	[229]
	<i>Escherichia coli K12 MG1655</i>	Deliver gene FLAG-tagged protein (a kinase-negative mutant of IKK2) to tumour-associated phagocytic cells	Constitutive expression	Injection	Mouse	[230]
	<i>Salmonella. Typhimurium</i>	Drug-loaded liposomes were linked using biotin onto the bacteria outer membrane surface	n.a	Injection	In vitro	[231]
		Engineered bacteria with site-specific expression systems for payload delivery.	FF+20*-HlyE (hypoxia-inducible) promoter with cytotoxic protein HlyE as anti-tumour payload	Injection	Mouse	[117]
		Bacteria were engineered to survive only in anaerobic conditions.	Essential gene (asd) was placed under the control of the hypoxia-conditioned promoter	Injection	Mouse	[232]
		Construction of hypoxic triggering promoters	Optimised FNR-Box based promoter GFP with a 15-fold increase upon induction.	Injection	Mouse	[86]
		Attenuated <i>S. typhimurium</i> expressing chemokine CCL21 controlled by ompC promoter;(Osmolarity trigger))	Attenuated <i>S. typhimurium</i> expressing chemokine CCL21 controlled by ompC promoter; The production of CCL21 in vitro in bacterial cultures is >50 pg/ug.	Injection	Mouse	[51] [233]

		Attenuated Δ DppGpp <i>S. typhimurium</i> for the targeted delivery of the <i>Vibrio vulnificus</i> derived flagellin (FlaB)	Expression induced by L-Arabinose; Strain defective in guanosine 5'-diphosphate-3'-diphosphate synthesis (Δ ppGpp <i>S. typhimurium</i>), resulting in an avirulent strain in BALB/c mice.	Injection	Mouse	[234] [235]
		Explore genetically engineered <i>Salmonella</i> as a potential treatment for metastatic osteosarcoma.	Attenuated <i>S. typhimurium</i> χ 4550 produce IL-2 factor	Oral	Mouse	[236]
		Bacterium to lyse synchronously at a threshold population density and to release genetically encoded cargo	Quorum regulator (<i>luxI</i>) was attached with both cell lysis (<i>phi</i> X174 gene) and therapeutic haemolysin HlyE protein, and thus when cell reaching critical density, bacteria will lyse and drug release	Oral	Mouse	[44]
	<i>Salmonella. enterica</i>	Tumour suppression induced by <i>S. enterica</i> carrying inducible 5-fluorocytosine-converting enzyme (cytosine deaminase)	XylS2-depndent Pm promoter which can be induced by acetyl salicylic acid (ASA) both <i>in vivo</i> and <i>in vivo</i> .	Injection	Mouse	[79]
		Drug delivery using bacteria loaded with nanoparticles contain chemotherapeutic doxorubicin	n.a	Injection	In vitro	[237]
	<i>Salmonella QS</i>	Quorum sensing controlled <i>Salmonella</i> produce GFP around tumours that only initiates drug expression in the tightly packed colonies present within tumours.	GFP expression via <i>lux</i> quorum sensing system which inserted into <i>Salmonella</i> chromosome.	Injection	Mouse	[238]
	<i>Salmonella typhimurium</i> VNP20009 and VNP200010	Expression amplification of the targeted molecules upon cancer colonisation.	Integrated the P _{Bad} promoter into the quorum-sensing machinery from <i>Vibrio fischer</i> in <i>Salmonella</i> to enable signal amplification upon reaching critical density.	n.a	In-vitro	[239]
	<i>Salmonella CRC2631</i>	<i>Salmonella</i> membrane loads with nanoparticles to improve penetration efficacy and specificity for tumour entry	Surface loaded with streptavidin-conjugated fluorophores and sucrose-conjugated gold nanoparticles. Surface display of customised surface peptide for biotin binding.	n.a	In-vitro	[240]
	<i>Vibrio cholera</i>	The light-emitting protein encoded on bacteria	n.a	Injection	Mouse	[241]

Gram positive	<i>Bacteroides spp</i>	General expression of GFP and tuning by inducing reagent	TetR system with synthetic aTc as inducer	Oral	Mouse	[242]
	<i>Bacteroides thetaiotaomicron</i>	A synthetic tool sets for engineering commensal strain <i>Bacteroides</i>	CRISPRi system with IPTG, L-rhamnose, chondroitin sulphate as inducer	Oral	Mouse	[158]
	<i>Listeria monocytogenes</i>	Express HPB E7 antigen as cervical cancer vaccine	n.a	Injection	Mouse	[243]
		Express HPV16 E7 protein as cervical cancer vaccine	A genetically modified attenuated recombinant LM4 Δ hly: E7 strain was constructed to express and secrete the LLO-E7 fusion protein.	Injection	Mouse	[244]
		Vaccine vector for delivering membrane protein of <i>Aeromonas hydrophila</i>	Attenuated strains with <i>actA</i> , <i>inlB</i> , <i>dal</i> and <i>dat</i> gene deleted.	Injection	Mouse	[245]
		Immune activation and induce the proliferation of antigen-specific T lymphocytes.	Replication-deficient strain (Δ dal Δ dat) to express and secrete human CD24 protein	Injection	Mouse	[213]
	<i>Bifidobacterium Longum</i>	Investigate the tumour colonisation specificity of <i>Bifidobacterium</i>	n.a	Injection	Mouse	[246]
	<i>Bifidobacterium adolescenti</i>	Transport endostatin gene to solid tumours. Transformed <i>B. adolescentis</i> germinated in the tumour tissue. Strong repression of angiogenesis was observed.	Constitutive expression	Injection	Mouse	[247]
	<i>Bifidobacterium pseudocatenulatum</i>	Use of <i>Bifidobacterium</i> to restore the obesity-induced altered vascular function	n.a	Oral	Mouse	[248]
	<i>Lactobacillus jensenii</i>	Prevent transmission of SHIV virus	Constitutive expression	Vaginal	Rhesus macaque	[249]
	<i>Lactococcus lactis</i>	LBD/ Expression of anti-inflammatory cytokine (IL-10)	Constitutive expression	Intragastric	Mouse	[250]
	<i>Lactobacillus gasseri</i>	Delivery of GLP-1/ Proliferation factor for converting epithelial cells into insulin-producing cells	Constitutive extracellular secretion of GLP-1	Oral	Mouse	[251]
	<i>Clostridium acetobutylicum</i>	Rat Interleukin-2 (IL2) secreted by <i>Clostridium acetobutylicum</i> DSM792 to a level of 800 μ g/l	Constitutive expression	n.a	In vitro	[50]
	<i>Clostridium difficile</i>	Expression of anti-inflammatory metabolite, β -hydroxybutyrate.	Constitutive expression and administration using <i>Clostridium</i> spores.	Colon	Mouse	[252]

2.9 Outlook

This chapter provides the reader with a basic background concept of synthetic biology and bacterial therapy, emphasising the existing cases of transcriptional genetic software and functioning hardware cells. The following chapter will introduce the construction of three different transcriptional control systems that are (1) aspirin drug dosage control (Chapter 3), (2) DNA damage control (Chapter 4), (3) NO disease biomarker control (Chapter 5), and finally, the overall assembly and characterisation of the optimised circuit into mini SimCells. (Chapter 6).

Chapter 3 Pharmaceutical dosage control: SalR:P_{Sal} based aspirin sensors

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In the previous two chapters, I outlined the advantage of bacteria therapy and the current shortage of therapy tailored control units. In this chapter, I characterise and design aspirin responsive circuits based on the P_{Sal} promoter and its regulator SalR originating from *Acinetobacter baylyi* ADP1. A novel competitive binding model has been constructed and validated for SalR, where it acts as a dual activator/repressor. Through fine-tuning the regulator level of SalR, the circuits can be fine-tuned to exhibit (1) minimal system leakiness to near background level or (2) amplified system output. Partial results of this chapter have been published in [34].

3.1 Introduction

An ideal control module used in bacteria therapy should exhibit the following traits. At the system level, the chassis strains should be safe and non-immunogenic for human usage. The inducer should have no side effects on human health and be absent in a normal diet. At the circuit design level, the regulatory circuits should have minimal cross-talk and only be triggered by specific inducer candidates. Circuits used in bacteria therapy emphasise safety, thus require minimal basal leakiness and the tight control for the release of potential cytotoxic drugs used to treat cancer.

Aspirin is a commonly used anti-inflammatory and pain-relief drug. Its clinical safety has been established since 1897 [253]. Recent animal studies also suggest its efficacy in tumour suppression [254] and immune modulation [255]. For a normal adult, the clinically safe dosage of aspirin is 75 to 300 mg per day, and therapeutic blood concentration for adults is between 111 and 555 μM , whereas the toxic concentration is between 832 and 1,665 μM [256]. The biological half-life of aspirin is 2 to 3 hrs for low dosages and 15 to 30 hrs for large dosages [257]. These features make aspirin an ideal inducer for external drug control.

The salicylate (SA) and aspirin (ASA) responsive module *nahR/P_{Sal}::xylS2* has been previously reported, which is a *Pseudomonas* species-derived gene circuit that has been applied in the regulated expression of *Salmonella*-derived toxin genes at the millimolar level [79]. In this study, a simple and sensitive aspirin/salicylate-sensing SalR/P_{Sal} system was isolated from *Acinetobacter baylyi* ADP1 and its function was tested in various *Escherichia coli* strains. As a member of the LysR-type transcriptional regulator (LTTR) superfamily, SalR controls the degradation of the salicylate pathway and regulates its own promoter, P_{Sal}. [258], a well characterised ‘sliding dimer’ mechanism of the LTTR promoter activation is shown in Figure 3.1. It was hypothesised that the effector-free form, SalR_r, can also compete with the binding of SalR_a,

its counter effector-binding form. Therefore, the dynamical performance of the sensor can be conveniently fine-tuned by controlling the expression quantity of SalR. Two basic sensory constructs and two optimised versions were designed based on the SalR regulatory system. The basic designs include one with positive autoregulation (PAR) and one under the control of a strong constitutive promoter (simple regulator form: SRS). The results demonstrate that through using a weak expression of SalR in SRS format, the induction strength can be significantly improved, thus validating the SalRr / SalRa competitive binding model. Furthermore, the PAR construct was connected with an antisense promoter (PAR_Rev_RNAP), and through turning on the reverse flux of RNAP transcription and truncating of the forward *salR* transcripts, tight system control and suppression of basal leakiness to a near background level can be achieved. This highly sensitive and tightly controlled system is functional in both probiotic strains and mini SimCells.

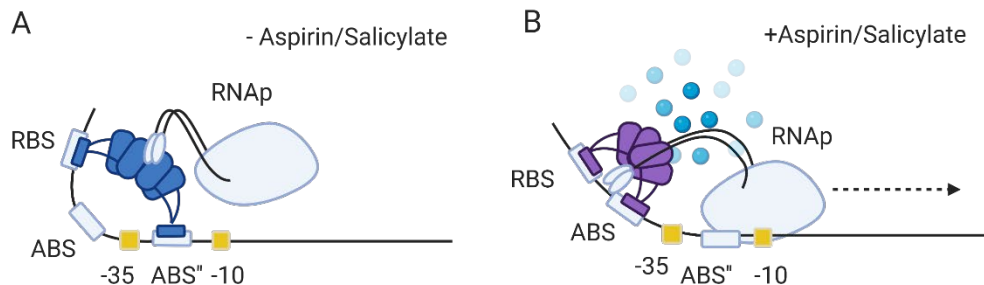


Figure 3.1. The sliding dimer mechanism involved in LysR transcriptional activation.

A) In the absence of an inducer (salicylate or aspirin), the LysR regulator forms a homotetramer with binding sites at the regulator binding site (RBS) and the activating binding site (ABS''). This causes DNA to bend and prevent the access to α -CTD of the RNAP complex to the promoter. B) In the presence of the inducer, the LysR carries a series of conformation changes and the homotetramer slides from ABS'' to ABS, exposing the -35 and -10 sites for RNAP binding. This movement also directs and recruits the α -CTD to promote the transcription initiation.

3.2 Material and methods

Chemicals and reagents

All reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise noted. Antibiotics (Kanamycin and Ampicillin) were obtained from Fisher Scientific (UK). Sterile 1x phosphate-buffered saline (PBS) solution was made by diluting 10x PBS solution stock purchased from Fisher Scientific (UK) with deionised water. For antibiotic usage, Table 3.1 lists the final working concentrations used for growing, selecting and induction experiments of *E. coli* DH5 α and *E. coli* BL21DE3. For *E. coli* Nissle 1917, 60 μ g/ml of Kanamycin was used as the final working concentration.

Table 3.1 Antibiotics stock and working concentration.

Antibiotic	Final working concentration (μ g/mL)	Ref
Kanamycin (Kan ^R)	50	[259]
Ampicillin (Amp ^R)	100	-
Tetracycline (Tet ^R)	12.5	-
Chloramphenicol (Cm ^R)	25	-
Carbenicillin (Carb ^R)	100	-

- Denotes "Same as above".

Strains and plasmids

The strains and plasmids used in this study are listed in Table 3.2 and Table 3.3. The original plasmids used in this study with backbones (pMK or pMA) were synthesised by GeneArt (UK) and transformed into *E. coli* DH5 α cells, which were made chemically competent beforehand. For making standard chemically competent cells, the protocol from the NEB (UK) vendor site was used without alteration. For making electrocompetent cells, the protocol adopted from Barrick lab was used [260]. *E. coli* Nissle 1917 (EcN) was acquired from Ardeypharm GmbH (Herdecke,

Germany). *E. coli* DH5 α and *E. coli* S17 (for plasmids with R6K γ ori) were used as storage strains, 35% (v/v) glycerol was supplemented for long-term storage in a -80 °C freezer.

Table 3.2 Strains used in Chapter 3

Strain Name	Genotype	Ref
<i>E. coli</i> DH5 α	<i>F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZAM15 Δ(lacZYA-argF) U169, hsdR17(<i>r_K⁻m_K⁺</i>), λ^-</i>	-
<i>E. coli</i> BL21:DE3	<i>F⁻ ompT gal dcm lon hsdS_B(<i>r_B⁻m_B⁻</i>) λ (DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>]) [<i>malB⁺</i>]_{K-12}(λ^S)</i>	-
<i>E. coli</i> MC1000	<i>F⁻ λ^- Δ(ara-leu)7697 [<i>araD 139</i>] B/r Δ(codB-lacI)3 galK16 galE15 e14- <i>mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r-m+)</i></i>	-
<i>E. coli</i> JM109	<i>endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14- [F' traD36 proAB+ lacIq lacZAM15] hsdR17(<i>r_K-m_K⁺</i>)</i>	-
<i>E. coli</i> S17	<i>endA1 hsdR17 glnV44 (= supE44) thi-1 recA1 gyrA96 relA1 ϕ80dlacΔ(lacZ)M15 Δ(lacZYA-argF) U169 zdg-232: Tn10 uidA::pir+</i>	-
<i>E. coli</i> Nissle 1917	Ardeypharm GmbH wild type	Herdecke, Germany [198]
<i>E. coli</i> MC1000: Δ minD	<i>F⁻ λ^- Δ(ara-leu)7697 [<i>araD139</i>] B/r Δ(codB-lacI)3 galK16 galE15 e14- <i>mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r-m+) ΔMinD KanR</i></i>	
<i>E. coli</i> Top10	<i>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZAM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ^-</i>	Invitrogen
ADPWH_C Δ salR	Mutant of ADPWH_ <i>lux</i> , partial DBD with kanamycin insertion	
ADPWH_N Δ salR	Mutant of ADPWH_ <i>lux</i> , absence of four bases (ATAA) encoded for residue 195 Ile of <i>salR</i>	
ADPWH_ <i>lux</i>	Closed loop autoregulated system; <i>luxCDABE</i> is inserted between <i>salA</i> and <i>salR</i> under control of P _{Sal}	

Table 3.3 Plasmids constructed in Chapter 3

Short Abbreviation	Plasmids	Description	Ori	Ref
PAR	pMK_P _{Sal} _GFP_SalR	Optimised SalR sensor with + feedback	ColE1	-
PsalAR_Full	pMA_P _{Sal} _GFP_SalA_SalR	Non-optimised <i>salAR</i> operon cloned with an insertion of gfp	ColE1	-
PAR_Amp ^R	pMK_P _{Sal} _GFP_SalR_Am p ^{R*}	pMK_P _{Sal} _GFP_SalR sensor with Amp ^R inserted; Kan ^R deleted.	ColE1	-
SRS	pMK_P _{roD} _SalR_P _{Sal} _GFP	SalR sensor with no feedback. SalR expression under P _{roD} promoter.	ColE1	-
SRS*	pMK_P _{J109} _SalR_P _{Sal} _GFP	SalR sensor with no feedback. SalR expression under P _{J109} promoter	ColE1	-
AraC_Rev_RN Ap	pMK_P _{Sal} _GFP_SalR_RN Ap_P _{Bad} _AraC	Antisense <i>Pbad</i> promoter attached at the end of <i>salR</i> orf (sensor with feedback)	ColE1	-
-	pBW201_P _{J109} _GFP	Constitutive GFP expression with P _{J109}	p15A	[14]
-	pBW206_P _{J106} _GFP	Constitutive GFP expression with P _{J106}	p15A	[14]
-	pBW204_P _{J115} _GFP	Constitutive GFP expression with P _{J115}	p15A	[14]
-	pBW201_P _{roD} _GFP	Constitutive GFP expression with P _{roD}	p15A	[14]
AraC_Rev_RN Ap_AT3	pMK_P _{Sal} _GFP_SalR_RN Ap_P _{Bad} _AraC_AT3	Antisense <i>Pbad</i> promoter with attenuated RBS binding site (3 AT repeats inserted)	ColE1	-
AraC_Rev_RN Ap_AT5	pMK_P _{Sal} _GFP_SalR_RN Ap_P _{Bad} _AraC_AT5	Antisense <i>Pbad</i> promoter with attenuated RBS binding site (5 AT repeats inserted)	ColE1	-
TetR_Rev_RN Ap	pMK_P _{Sal} _GFP_SalR_RN Ap_P _{tetO} _TetR	Antisense P _{tetO} promoter was attached at the end of <i>salR</i> orf in replacement of P _{Bad} promoter.	ColE1	-

- Denotes plasmid constructed "in this study".

Table 3.4 Primers used in Chapter 3

Primer Name	Sequence 5' → 3'	Target Constructs
Fragment PAR_Amp ^R REV	CACGCCAGACGGCCCGCGGAACCCCTATTTGT TTATTTTCT	pMK_P _{Sal} _GFP_SalR_Amp ^{R*}
Fragment PAR_Amp ^R FOR	TTATTGATTGCGGCCTTACCAATGCTTAATCAG TGAGGCACC	
<i>ProD</i> _Fragemen t_For	TTAATCTCTAGTACTCTAGTAAAAGTTAAACAA AATTATTTGTAGAGGGAAACC	pBW201_P _{roD} -GFP
<i>ProD</i> _Fragemen t_Rev	GCCGCTTCTAGAGTTCTAGAGCACAGCTAACAC C	
<i>ProD</i> _Vector_F or	ACTTTTACTAGAGTACTAGAGATTAAAGAGGAG AAATACTAGATGCGTAAAGGAGAAG	
<i>ProD</i> _Vector_R ev	CTGTGCTCTAGAACTCTAGAAGCGGCCGC	
J109_Promoter_ For	ACTGAGCTAGCTGTAACTTCAAAATTTAAATC TAAATATCAATGTTTTAAGATCATAATGATGG	pMK_P _{J109} _SalR_P _{Sal} _GFP
J109_Promoter_ Rev	CCTAGGGACTGTGCTAGCTCACACAGGAAAGTA CTAGATGGACCTGTC	
QC_SRS_For	CAATTAATTACAGAACAGAGATAACTTTT	pMK_P _{roD} _SalR_P _{Sal} _GFP
QC_SRS_Rev	GTCTCCCTGAATATATTATACGAG	
QC_PAR_For	CCGCATCTTCAAAGATCTAATTTA	pMK_P _{Sal} _GFP_SalR
QC_PAR_Rev	CTTTTGAAAGATCCCAACGAA	
SalR-fwd-Anti	CCCAGTTAATTGTTTAGATCTTTACAGAACAGA GATAAC	pMK_P _{Sal} _GFP_SalR_RNAP_ P _{Bad} _AraC
SalR-rev-Anti	CCTCATGGGCCTTCCTTTTCAC	
Pbad-fwd-Anti	GGAAGGCCCATGAGGTTATGACAACCTTGACGGC TACATCATTTCACTT	
Pbad-rev-Anti	AAACAATTAACCTGGGCCTCCTGTTAGCCCCAAA AACG	
Q5 sdm AT-fwd	ATATTTTCCTCCTGTTAGCCCCAAA	pMK_P _{Sal} _GFP_SalR_RNAP_ P _{Bad} _AraC_AT3
Q5 sdm AT rev	ATATATATTTACAGAACAGAGATAACTTTTTTG GATCTAAACAATTAACCTGGGCATAGATCCTTTC TCCTCTTTAGATC	
PtetO_fwd	TGAAAGGAAGGCCCATGAGGCGCCAGATATCGA CGTCTTAAG	pMK_P _{Sal} _GFP_SalR_RNAP_ P _{tetO} _TetR
PtetO-rev	CCCAGTTAATTGTTTAGATCTTTACAG	
salR_fwd	CCCAGTTAATTGTTTAGATCTTTACAG	
salR_rev	CCTCATGGGCCTTCCTTTTC	

Molecular cloning and primer design

For all molecular cloning operations carried out in this study, standard protocol from the NEB vendor site was used. This included miniprep, PCR clean-up, HiFi (Gibson) Assembly, endonuclease digestion, dephosphorylation and PCR. Primers were designed using NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and visualised using SnapGene®. To design Gibson cloning primers, NEBuilder (<https://nebuilder.neb.com/>) was used. Detailed components of protocols used are listed below. Codon optimisation was carried using Jcat (<http://www.jcat.de/>) online codon optimisation tool based on the codon usage in *Escherichia coli* K12. DNA quantity and quality were assessed using Nanodrop function imbedded with TeCan Spark® plate reader. For general guidelines, DNA purity requires both $260/280 > 1.8$ and $230/260 > 2$ to be matched for subsequent cloning or plasmids transformation.

Double Restriction digestion

Component	Total volume 25 µL
CutSmart buffer	5 µL
DNA template (500ng)	-
Restriction enzyme No.1	1 µL
Restriction enzyme No.2	1 µL
Nuclease-free water	-

Single Restriction digestion

Component	Total volume 25 µL
CutSmart buffer	5 µL
DNA template (1 µg)	-
Restriction enzyme	1 µL
Nuclease-free water	-

T4 ligation

Component	Total volume 20 μ L
Vector DNA	100 ng
Insert DNA (Molar Ratio Vec:Ins = 1: 3)	-
2x T4 ligase buffer	10 μ L
T4 DNA ligase	1 μ L
Nuclease-free water	-

Q5 High fidelity PCR

Component	Total volume 50 μ L
Q5 Polymerase	0.5 μ L
10 μ M Primers fwd	2.5 μ L
10 μ M Primers rev	2.5 μ L
Q5 5x reaction buffer	10 μ L
10 μ M dNTPs	1 μ L
DNA template (10ng)	-
Nuclease-free water	-
DMSO*(Optional)	1.5 μ L

*DMSO is added for High-GC content template to improve yields and specificities of PCR priming reaction.

Colony PCR

Name	Total volume 50 μ L
5x DreamTaq Master Mix	25 μ L
10 μ M Primers fwd	2 μ L
10 μ M Primers rev	2 μ L
Nuclease-free water	-
Dissolved colony pick*	1 μ L

*Bacterial colony was picked and dissolved into 5 μ L of nuclease-free water to ensure homogeneity upon liquid transfer.

Q5-Site directed Mutagenesis

Component	Total volume 25 μ L
Q5 HotStart High Fidelity 2x Master Mix	12.5 μ L
10 μ M Primers fwd	2.5 μ L
10 μ M Primers rev	2.5 μ L
DNA template (25ng)	-
Nuclease-free water	-

Bacteria transformation

100 ng of plasmid was added to 100 μ L of chemically competent cells and incubated on ice for 30 mins, being pipetted up and down to ensure the mixture was fully homogenised. This was then heat shocked at 42 °C for 45 secs. Eppendorf tubes containing the cells and plasmids was put back on the ice to rest for 5 mins. Subsequently, 950 μ L of prewarmed (37 °C) SOC medium was added and incubated at 37 °C, 150 rpm for 1 hr for the cells to recover. The recovered cells were then plated onto agar plates with appropriate antibiotics for overnight selection. For EcN, to increase the positive colony number, a concentration step was added after the 1 hr recovery, and cells were centrifuged at 2500 g for 5 mins. 800 μ L of supernatant was then removed and the remaining 200 μ L was re-dissolved by carefully pipetting and plating the concentrated cells onto agar plates. For the preparation of chemically competent cells and electrocompetent cells, protocols adopted from Cold Spring Harbor (<http://cshprotocols.cshlp.org/>) were used.

Flow cytometry

Transformed *E. coli* DH5 α cells were induced with a gradient of aspirin concentration (final concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50 μ M) in kanamycin (50 μ g/mL) LB for 16 hrs in a 200-rpm shaker at 37 °C. The induced culture was then analysed using FACSCalibur™ (BD Bioscience), with 100,000 events being captured for each sample (condition

gating: 30 secs or 100,000 events). Background blank medium and sonicated debris control were both subtracted. Data were then exported to FlowJo® for subsequent analysis and visualisation.

Circuit induction and GFP kinetics measurements

The stock solution of L-Arabinose, glycerol and aspirin was made in deionised water and sterilised using a 0.22 µm syringe filter for inducing cells containing SRS and PAR, respectively. Through diluting the overnight cell culture, the initial OD was set to be 0.1 for all induction experiments. A total volume of 200 µl (cultured bacteria cells and inducing aspirin) was loaded in a single 96-well black-sided, clear-bottomed plate (Nunc, UK) in quadruplicate. The plate was then placed into a BioTek Synergy HT microplate reader (BioTek Corporation, UK), maintained at 37 °C with reads of fluorescence (excitation: 480 nm, emission: 520 nm) and optical density (OD) at 600 nm. Readings were recorded every 15 mins for 16 hrs. Shaking was applied for 10 secs prior to each reading.

Medium recipe

For experimental conditions in PBS (phosphate buffer saline) and M9, prior to the induction of the transformed bacteria, cells were centrifuged and washed with PBS and M9 respectively, three times at 4 °C, to remove any residue of LB broth. To achieve micro-aerobic condition, 50 µl of mineral oil was added on top of 200 µl reaction volume in a 96-well plate and sealed with a gas-impermeable membrane.

Visualisation of bacterial cells

Fluorescence of induced bacterial cells or SimCells was visualised using a Motica BA210 digital microscope with Moticam 580INT display output after 16 hrs of induction. 5 μ l of each sample was taken from the well and placed on a glass slide with a coverslip. Images were taken at 400 $\times$ magnification. Light micrographs were subsequently analysed using ImageJ version 1.50b, with fluorescent maxima automatically counted after consistent thresholding.

FeCl₃ assay and FDA assay for aspirin salicylate quantification

FeCl₃ assay protocol was adopted from [261]. To study the aspirin conversion by the bacteria, wild type *E. coli* Nissle 1917 was cultured with 5 mM aspirin and incubated at 37 °C, 200 rpm. Samples were collected at time points 0, 3 and 16 hrs. Cell pellets were removed by centrifuge at 4000 g for 10 mins. The supernatant was then mixed with freshly prepared FeCl₃ solution. Absorbance reads were taken using a Tecan Spark® Multimode microplate reader. To quantify the esterase activity, fluorescein diacetate (FDA) hydrolysis assay was used with protocol available at the vendor site (Thermo Fischer®). To prepare the cells in different growth phases, each *E. coli* subtype was grown to OD₆₀₀ ~ 0.3 (Log) and OD₆₀₀ ~ 1.2 (Stationary) in LB; this was then calibrated to the same OD ~ 0.1 with the addition of LB medium. 10 μ l of 24 μ l FDA stock was added for each sample. Fluorescence reads were taken every 15 mins and normalised against OD growth.

3.3 Results

3.3.1 Original sensor performance isolated from *Acinetobacter baylyi* ADP1

The sensory circuits were initially characterised and constructed in their native context, P_{Sal} promoter regulated open reading frames *salA* and *salR* were cloned with insertion of a *gfp* orf into a pMK backbone (ColE1 Ori) (Figure 3.2 B). However, upon transformation into *E. coli* DH5 α , such circuits failed to respond to 100 μ M aspirin at either 30 °C or 37 °C (Figure 3.2 A). A slight induction expression of GFP was observed only when supplied with 2 mM aspirin at 30 °C. It is speculated that such insensitivity might be due to the *salA* orf, which can convert salicylate/aspirin into catechol (Figure 3.2 C). To solve this, the SalR regulator was codon-optimised according to *E. coli* codon usage and the *salA* orf was removed.

3.3.2 Heterogeneity expression of PAR and SRS circuits in *E. coli* DH5 α

Two different designs with the removal of *salA* orf and codon-optimised *salR* orf were constructed, a simple regulation system (SRS) and a positive autoregulated circuit (PAR). As shown in Figure 3.3, in the SRS design, the expression of *salR* is under a strong constitutive promoter (P_{roD}), which is chosen for its strong and context-independent expression of targeted genes [262]. In the PAR circuits, *salR* is regulated by its own promoter, P_{Sal}, to form a positive feedback loop. All other genetic modules remained the same (RBS_B0030 and plasmids backbone). Both circuits were transformed in *E. coli* DH5 α and induced with an aspirin gradient (0, 2.5 and 50 μ M were shown). Green fluorescent protein (GFP) expression was measured at the single-cell level using flow cytometry and a fluorescence microscope (Figure 3.3, Appendix Figure A-1). GFP expression in both SRS and PAR can be significantly induced by 2.5 μ M aspirin (Figure 3.3 A).

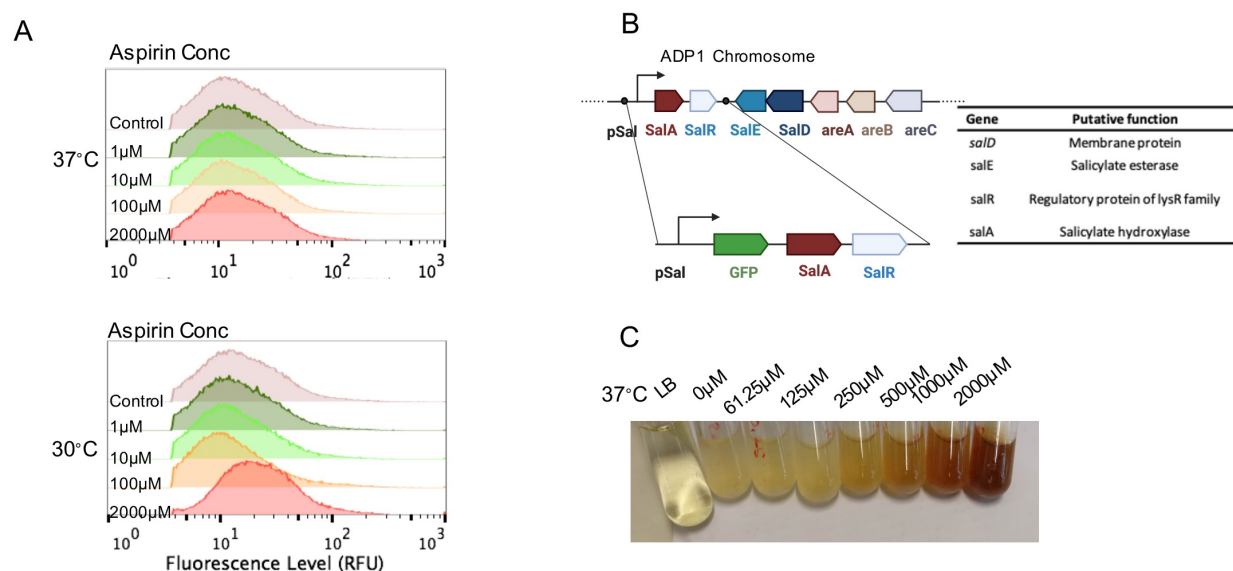


Figure 3.2. Original performance of the native circuits.

A) Induction kinetics of the original circuits ($P_{Sal_gfp_salA_salR}$) using a gradient of aspirin (0, 1, 10, 100, 2000 μ M) at 37 °C and 30 °C, respectively. FACS population = 50,000. Background removed using sonicated cells and LB blank medium. B) Schematic diagram of the *salAR* catabolic cluster on the *Acinetobacter baylyi* ADP1 and the making of $P_{Sal_gfp_salA_salR}$. C) Catechol production via SalA with increasing aspirin gradient over 16 hrs, incubation at 37 °C.

Density plots of the front scatter (FSC) reads against the GFP channel showed a single uniform population of GFP-expressing cells in SRS construct throughout the induction gradient, ranging from 3.67 ± 0.12 to 32.11 ± 1.45 GFP relative fluorescent units (RFU). In comparison, PAR constructs exhibited a higher fluorescence intensity output at a steady-state than SRS, and a higher fold of change. Conversely, the basal leakiness was significantly lower in the case of SRS than that of PAR. From the flow cytometry and fluorescent microscopy results, the PAR circuits demonstrated a slight bistable population, ranging from 9.69 ± 0.76 RFU (low) to 165.16 ± 5.52 RFU (high), while induced SRS circuits demonstrated a stable uniform population. The leaky and bistable expression of GFP observed in PAR confirmed that both circuits behaved as their original design intended.

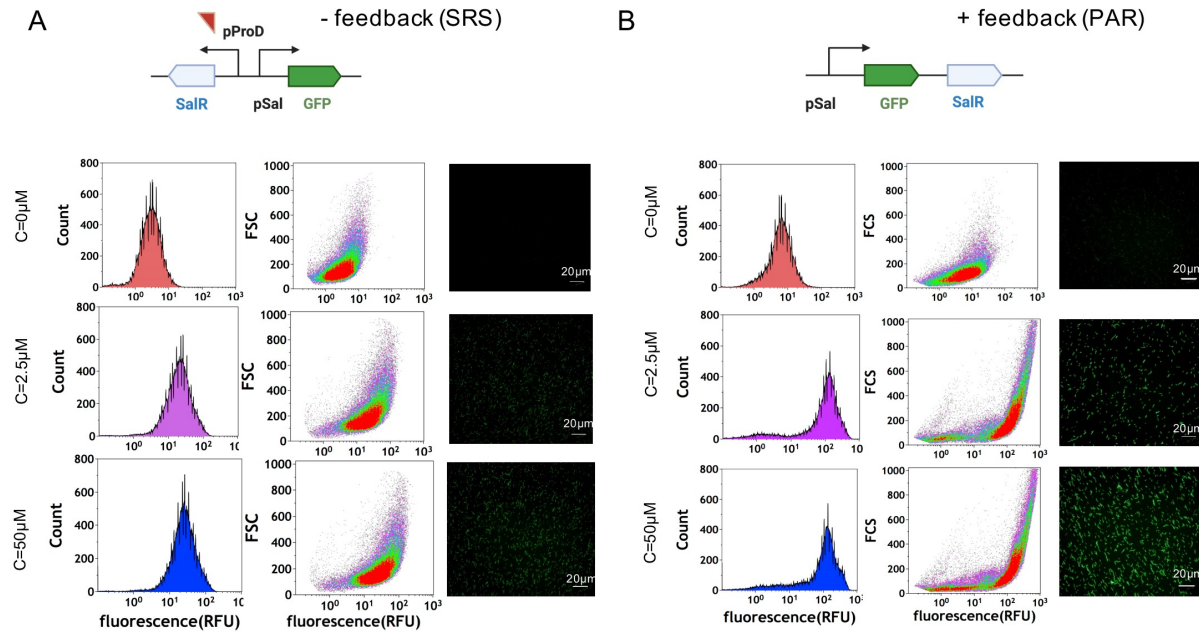


Figure 3.3. System performance after codon optimisation.

Single-cell (FACS) induction profile of *E. coli* DH5 α transformed with the SRS (-Feedback) and PAR (+Feedback) circuits in response to aspirin. A population of 100,000 cells from uninduced (red), 2.5 μ M aspirin (magenta), and 50 μ M aspirin (blue) treatments were observed for each SRS and PAR circuit. The circuit configurations are illustrated at the top. Histograms of fluorescence are plotted on a logarithmic scale to render the wide range of biosensor activation. Background removed using sonicated cells debris and blank LB medium. Scale bar = 20 μ m.

The aspirin-inducible regulatory system is controlled by the SalR regulatory protein, originally from *Acinetobacter baylyi* ADP1 [263]. SalR belongs to the LTTR superfamily, the largest family of regulators in prokaryotes [264], which contains an N-end DNA binding domain (DBD) and a C-end chemical recognition domain (CRD) [265]. Based on previous reports of characteristic features in the LTTR family [266] and the ‘sliding dimer’ mechanism illustrated in Figure 3.1, it was hypothesised that the effector-free form, SalR_r, is able to bind the P_{Sal} promoter to repress transcription. This SalR/P_{Sal} regulation was validated using a salicylate biosensor, ADPWH_{lux}, that was previously constructed by inserting a promoterless luxCDABE cassette between *sala* and *salR* of the *sal* operon in *A. baylyi* ADP1 [263][266]. To examine and confirm the molecular mechanism, two SalR mutants, ADPWH_N Δ *salR* and ADPWH_C Δ *salR*, were

constructed with the N terminus and C terminus disrupted from ADPWH_*lux* respectively, (Appendix Figure A-2, Table 3.2). In the mutant ADPWH_C Δ *salR*, four bases (ATAA) in the CRD domain of the *salR* gene were deleted using the *sacB-km* counterselection method [267]. It was hypothesised that this deletion did not prevent the mutated SalR from binding the P_{Sal} promoter, because the N terminus was unaltered. In the case of ADPWH_N Δ *salR*, the DBD of the *salR* gene in ADPWH_*lux* was knocked out by inserting a kanamycin resistance gene [263] into the ClaI cutting site. Hence, this ADPWH_N Δ *salR* mutant had completely disrupted the DNA binding motif of SalR and was unable to bind the P_{Sal} promoter.

Although both SalR mutants ADPWH_N Δ *salR* and ADPWH_C Δ *salR* were unable to respond to salicylate acid, the background levels of bioluminescence expression were different (Figure A-2): the average background bioluminescence expression of ADPWH_N Δ *salR* was $5,968 \pm 48$, which is significantly higher than that of both ADPWH_C Δ *salR* ($3,222 \pm 66$; $P < 0.001$) and ADPWH_*lux* ($3,857 \pm 99$; $P < 0.001$). ADPWH_C Δ *salR* with a mutated C terminus chemical recognition domain (but unchanged N terminus DNA binding domain) should bind and repress the P_{Sal} promoter, reducing the background expression of bioluminescence, similar to ADPWH_*lux*, which has an intact *salR*. In contrast, disruption at the N terminus in ADPWH_N Δ *salR* completely alters the SalR structure, making it unable to bind the P_{Sal} promoter and leading to a higher background expression of bioluminescence (Appendix Figure A-2). Collectively, the results suggest that uninduced SalR or the effector-free form, SalRr, should bind and repress the P_{Sal} promoter.

3.3.3 SalRr/SalRa competitive binding model construction and validation

According to the *salR* gene mutagenesis results and induction kinetics described above, it was hypothesised that both SalRr and SalRa can competitively bind the P_{Sal} promoter. To assess

the hypothesised mechanism and investigate how variation of different aspects of the SRS and PAR circuits affect the aspirin-regulated performance, a mathematical model was developed to simulate and predict the performance of the three gene circuits. The interaction considered in our model for the SRS and PAR systems is outlined in Figure 3.4 A and is implemented using deterministic differential equations and binding models which describe gene regulation and expression. This process treats the SalR regulatory mechanism as a biased competition between binding of SalRa, which activates the P_{Sal} promoter, and SalRr, which occupies the binding site and represses the P_{Sal} promoter. For the SRS circuit, the competition between overexpressed SalRr and aspirin-activated SalRa is expected to produce low GFP expression levels. This competition has little effect on the PAR circuit, as its SalR level is tuned by a self-regulated feedback loop.

An ODEs-based model was created to describe the activator/repressor behaviour, in which the concentration of SalR is governed by:

$$\frac{d[SalR]}{dt} = S([\alpha], [salR])\alpha_1 - \delta_1[SalR] \quad - \quad \text{Equation(1)}$$

for the PAR circuit,

$$\frac{d[SalR]}{dt} = \alpha_2 - \delta_1[SalR] \quad \text{Equation(2)}$$

for the SRS circuit, and the output of the circuit (GFP) concentration is governed by (both circuits)

$$\frac{d[GFP]}{dt} = S([\alpha], [salR])\alpha_3 - \delta_2[gfp] \quad \text{Equation(3)}$$

where the regulating function that describes the interaction between SalR and inducer concentrations and the P_{Sal} promoter is given by

$$S([\alpha], [salR]) = \frac{K_R}{K_R + [salR]} \left(L + (1 - L) \frac{[salR]}{[salR] + K_A} \frac{[\alpha]^n}{[\alpha]^n + K_\alpha^n} \right) \quad \text{Equation(4)}$$

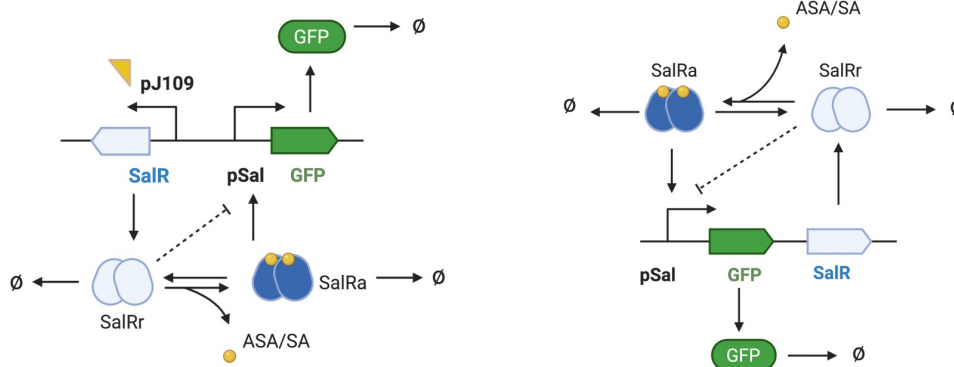
in which α is inducer concentration, L is the leakage rate from the P_{Sal} promoter, K_R defines the saturation point for repressive binding with P_{Sal} , K_A defines the saturation point for activating SalR

binding with P_{Sal} , and K_a defines the saturation point for inducer interaction with the circuit. Therefore, equation (4) reflects the balance expected between repressive SalRr binding and inducer-dependent activation of SalRa binding. The structure of the model is built on the well-established Michaelis–Menten equation and Hill function [130]. Strategy for parameters fitting and value used for simulation are provided in the appendix (Appendix Table A-1) and [34].

To validate the proposed model, plate reader experiments were carried out to analyse the induction kinetics of SRS and PAR circuits over a range of inducer concentrations and these data were compared to the simulated results from the mathematical model. The expression profile for both the SRS and PAR circuits in LB media is shown in Figure 3.4 B. The fluorescence and optical density (OD) were monitored for 16 hrs over a range of aspirin concentrations between 0.05 and 50 μ M. GFP expression in both SRS and PAR circuits was detectable within 40 mins, which is close to the theoretical minimum time required for GFP maturation [268]. No significant difference in growth rate was observed between the SRS and PAR circuits (Appendix Figure A-3). Experimental results from the expression of PAR and SRS circuits are in good agreement with the mathematical model simulation (Figure 3.4 B). In the SRS circuit, the abundant SalRr competitively occupied and repressed the P_{Sal} promoter, reducing the possibility of SalRa binding, which repressed overall GFP expression in the presence of aspirin and lowered the baseline level. The threshold concentration of aspirin required to activate GFP expression in the SRS circuit was 0.05 μ M, with a low basal leakiness (Figure 3.4 B). The PAR circuit tuned the strength of SalR expression through a positive feedback loop and reduced the competitive repression, resulting in a much stronger GFP expression with the same activation threshold concentration of 0.05 μ M (Figure 3.4 A). However, a significant increase in basal expression was also observed. Further

testing in a different growth medium confirmed that the difference between the induction profile of SRS and PAR is independent of carbon source and growth state. (Appendix Figure A-4).

A



B

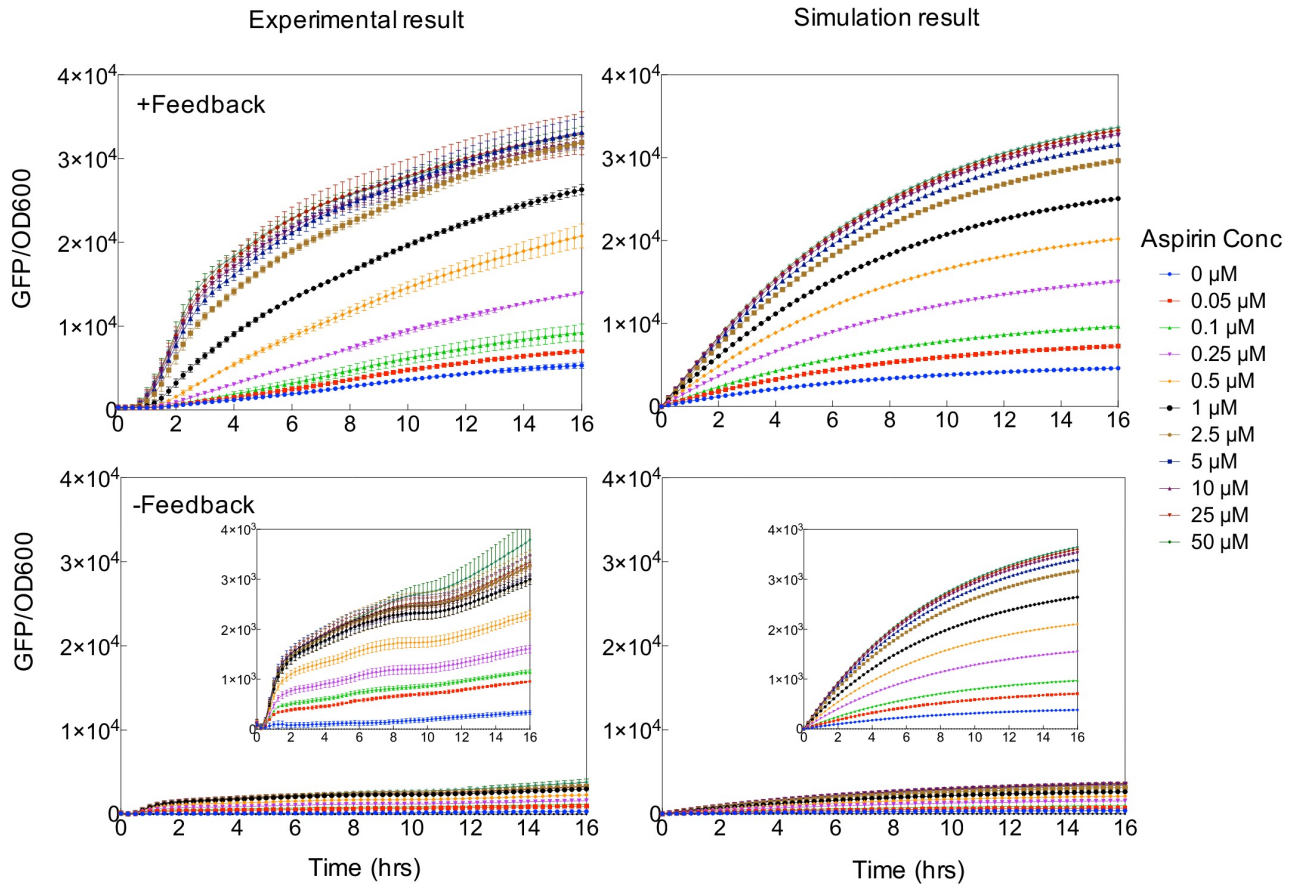


Figure 3.4 Simulation and experimental results of induction profile.

A) Model architecture for the competitive binding between inactivated state SalRr and activated state SalRa in both SRS and PAR circuits. B) Dosage response curve between simulated results

and experimental results of SRS (P_{roD} -salR- P_{Sal} -gfp) and PAR (P_{Sal} -gfp-salR). Aspirin gradient used is (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 μ M). N=3. The standard deviation and mean value of three biological replicates were plotted in each graph.

To redesign the SRS circuit, the expression of *salR* under the control of the constitutive promoter with different strengths was examined. Promoter P_{roD} along with three weaker promoters, P_{J109} , P_{J115} , and P_{J106} , were fused with the *gfp* gene separately, and the strength was evaluated by detecting GFP expression levels. Figure 3.5 C shows that P_{roD} was the strongest promoter and P_{J109} was the weakest promoter among the four promoters P_{roD} , P_{J109} , P_{J115} , and P_{J106} , which is consistent with previous reports [262][14]. Subsequently, the P_{J109} promoter (five times weaker than P_{roD}) was characterised and replaced P_{roD} promoter in the construction of SRS* (Figure 3.5 A) (P_{J109} -salR- P_{roD} -gfp). Interestingly, the GFP expression level in the SRS* circuit was 3.5-fold higher than that in the original SRS circuit, and the new SRS* showed an intermediate leakiness of GFP expression compared to the SRS circuit (Figure 3.4 B and Figure 3.5 C). The limit of detection for the SRS* circuit remained 0.05 μ M ($P < 0.05$). This is in good agreement with the competitive binding hypothesis, suggesting that the SRS* circuit with the weak promoter P_{J109} should produce less SalR than the SRS circuit, ease the SalRr/SalRa competitive binding on the P_{Sal} promoter, and increase the GFP expression level (Figure 3.5 B). By applying the same parameters, the mathematical model fit the experimental results well and predicted that the transcriptional rate from P_{J109} is $\sim 15\%$ of that from P_{roD} , similar to the output ratios observed in Figure 3.5 D. Collectively, the *salR* mutagenesis experiment, the behaviour of the SRS*, SRS and PAR circuits, and mathematical model fitting all suggest that the hypothesis of SalRr/SalRa competitive binding is reasonable.

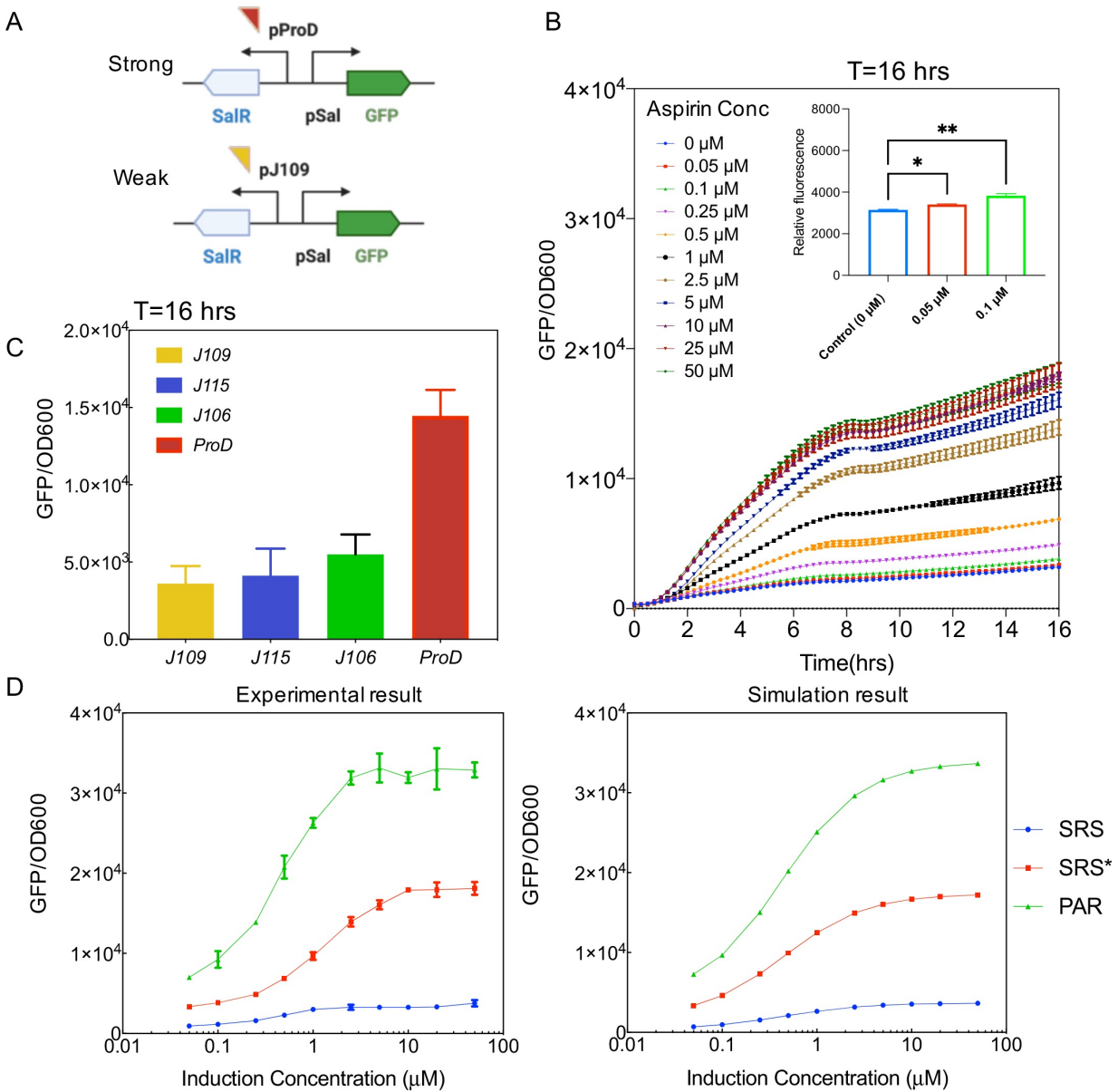


Figure 3.5. SalR attenuation through a weaker promoter

A) Replacement of the strong constitutive promoter (P_{roD}) into the weak constitutive promoter (P_{J109}), the new constructs termed SRS* (P_{J109} -salR- P_{Sal} -gfp). B) Aspirin induction kinetics for the circuits over 16 hrs. The aspirin gradient used is (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50 μ M). N=3. A detection limit of 0.05 μ M was recorded as shown in an enlarged bar chart. Paired t tests were carried out to determine the significance. * $P < 0.05$, ** $P < 0.005$. C) Characterisation of a set of constitutive promoters in *E. coli*. N=3. The standard deviation and mean value of three biological replicates were plotted in each graph.

3.3.4 System performance tested in *E. coli* Nissle 1917

To verify whether our proposed working mechanism holds true in probiotic strains, the SRS, SRS*, and PAR circuits were then cloned into a probiotic strain, *E. coli* Nissle 1917. Considering that the gut environment is semi aerobic, *E. coli* Nissle 1917 with SRS, SRS*, and PAR circuits were characterised under both aerobic and oil-sealed conditions. To simulate the potential growth competition with a host gut microbiome [269], a nutrient-limited environment was also tested. A small amount of glucose (0.4% w/v) was supplied as a sole carbon source, as it is a common carbohydrate source in the human diet. *E. coli* Nissle 1917 with SRS, SRS*, and PAR circuits maintained a gradient response to inducer concentration under aerobic and semi aerobic conditions, highlighting the system's modularity and robustness to changing chassis and nutrient conditions (Figure 3.6 and Appendix Figure A-5). The SRS circuit in *E. coli* Nissle 1917 exhibited tight control but a low response to different concentrations of aspirin, while the PAR circuit demonstrated a higher response to aspirin (Figure 3.6). The performance of the SRS, SRS*, and PAR circuits in *E. coli* Nissle 1917 was similar to that in *E. coli* DH5a, and consistent with the hypothesis of SalRr/SalRa competitive binding. Notably, when supplied with a limited amount of 0.4% w/v glucose, the expression profile of PAR showed less leakiness, implying that the circuit could function optimally under nutrient-limited conditions. This change might be due to unknown host interference, where cells experiencing glucose starvation alter their resource allocation [270]. Furthermore, it was noticed there was a slight growth advantage for transformed bacteria in the case of 50 μ M of aspirin than for control cases (0 μ M) (Appendix Figure A-5). This could be due to the fact that *E. coli* actively participate in the breakdown of aspirin and feed on the acetate metabolite [271]. Using a FeCl₃ quantification assay to determine the aspirin salicylate conversion [261], it was demonstrated that the presence of *E. coli* facilitates the aspirin breakdown (Appendix Figure A-6), which could be due to its non-specific esterase activity [272] (Appendix Figure A-7).

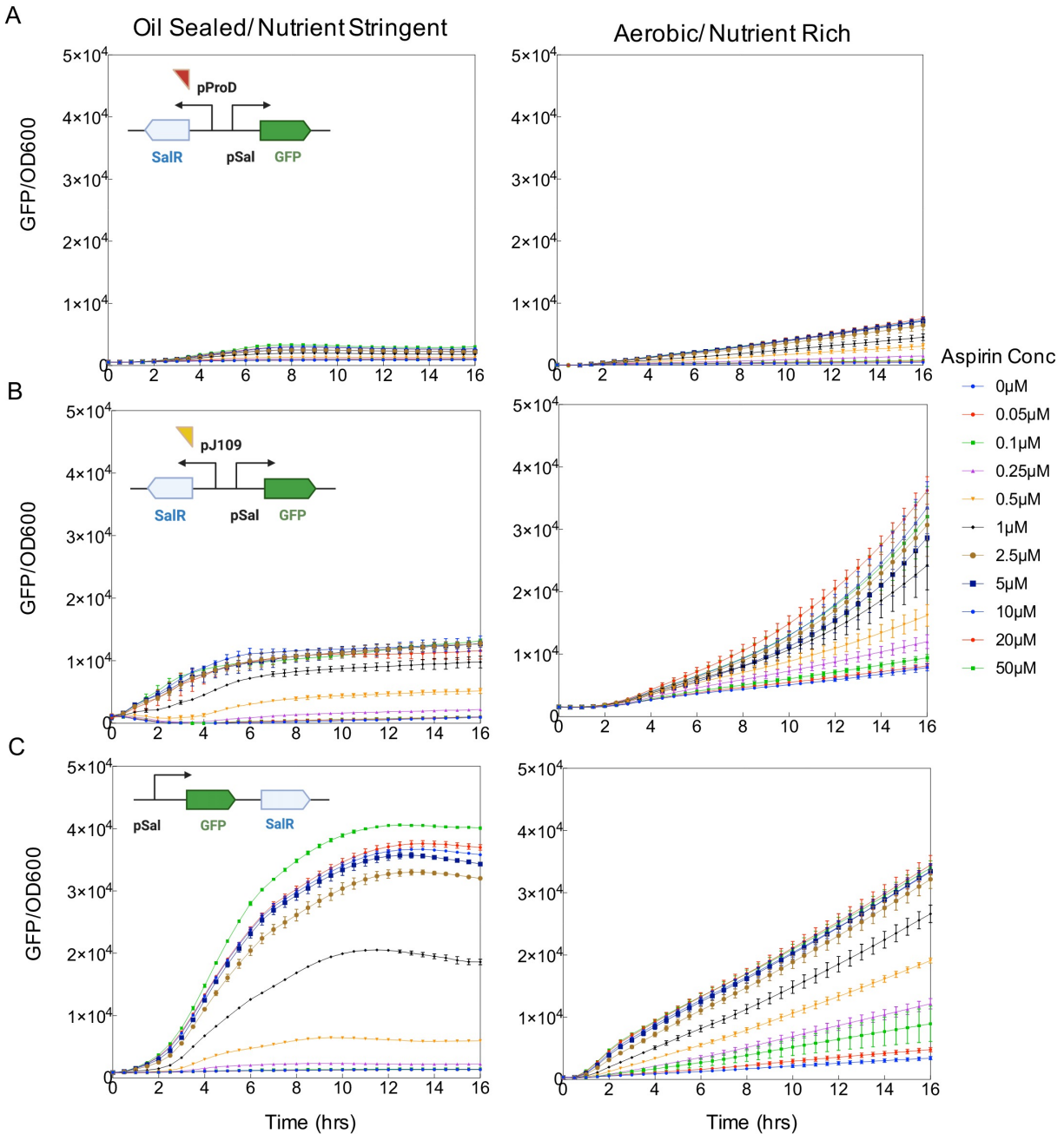


Figure 3.6. Induction kinetics of all three constructs in *E. coli* Nissle 1917

Circuit performance of the PAR format (P_{Sal} -gfp-salR) and the SRS format (P_{roD} -salR- P_{Sal} -gfp; P_{J109} -salR- P_{Sal} -gfp) were tested in *E. coli* Nissle 1917 strains. The induction kinetics were tested in two different medium conditions: an aerobic/nutrient-rich (LB) condition and a nutrient-stringent condition (M9 supplemented with 0.4% w/v glucose and oil sealed). Aspirin gradient used is (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50 μ M). N=3. The standard deviation and mean value of three biological replicates were plotted in each graph.

3.3.5 Constructing antisense RNAP collision to probe optimal level for SalR expression

To construct a system with both merits of PAR (high response level) and SRS (low basal expression), an antisense RNAP collision design was adopted and constructed according to [141] and characterised in EcN. To leverage the activator/repressor feature of SalR, I aimed to fine-tune and scan the expression ‘sweet spot’ using an inducible promoter. As shown in Figure 3.7 A, an P_{Bad} promoter with its regulator AraC was cloned in the convergent direction of *salR* orf. Upon induction of P_{Bad} promoter with L-Arabinose, the flux of forward RNAs (P_{SalR}) collides with the antisense RNAs (P_{Bad}), thus abolishing the transcription of sense mRNA and downregulating the target gene. The system with the addition of antisense promoter demonstrated a lower basal expression (13990 ± 414 RFU) compared to the PAR constructs (24681 ± 222 RFU) as shown in Figure 3.7 B. Upon induction under constant aspirin concentration ($50 \mu\text{M}$) with varying L-Arabinose concentration (0. 0.02% 0.2%w/v), the GFP response output of the AraC_Rev_RNAP construct decreased from $100,629 \pm 7584$ RFU to $85,122 \pm 4008$ RFU, while the original PAR constructs remained unchanged (Figure 3.7 C). The AraC_Rev_RNAP constructs also demonstrated a slight decrease in sensitivity (limit of detection = $0.25 \mu\text{M}$) compared to the PAR (limit of detection = $0.05 \mu\text{M}$), as outlined in Figure 3.7D. No apparent OD growth differences were observed in either case (Appendix Figure A-8).

To further verify and fine-tune the antisense constructs, the RBS strength was attenuated between the antisense P_{Bad} promoter and *salR* orf by inserting a series of AT repeats. The longer the insertion, the weaker the promoter strength [120], as shown in Figure 3.8 A and B. However, both attenuated RBS (RBS_3AT and RBS_5AT) demonstrated an increased basal expression compared to the original RBS. Upon induction using 0.2% w/v L-Arabinose and aspirin gradient (Figure 3.8 C), all three variants exhibited a pincer-like profile change (both featuring decreased

induced amplitude and increased basal expression) compared to the non-induced control, and a decrease in the system intensity as well as an increase in system leakiness.

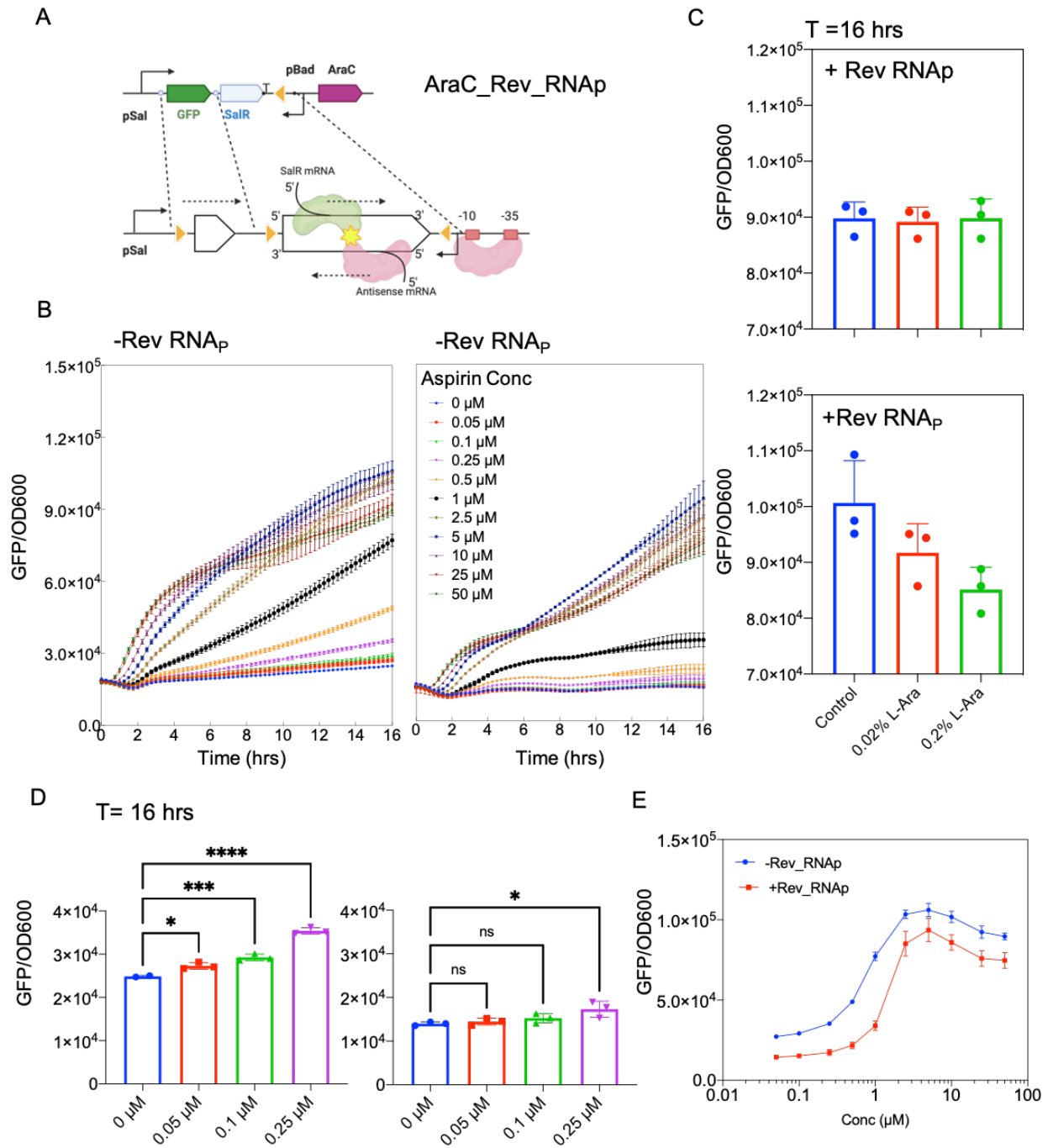


Figure 3.7. Using antisense P_{Bad} promoter to tune the expression level of SalR.

Upon induction of the antisense (AraC_Rev_RNAp) promoter, a flux of RNAp transcription is created in the opposite direction that cancels the forward flux of salR transcription. A) the design

and schematic mechanism of the RNap collision to fine-tune the expression level of SalR. B) Compared to the case without (-Rev RNap) and the case with antisense P_{Bad} promoter, +Rev RNap demonstrated a lower baseline. Both cases were tested in EcN strains and induced with a gradient of aspirin (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 μ M). N=3. C) +Rev RNap exhibited a reduced steady-state relative fluorescence (T = 16 hrs) under a saturated level of inducer (50 μ M) and responded to an increased level of L-Arabinose gradient. (0, 0.02%, 0.2% w/v). N=3. D) Limit of detection for the (-Rev RNap) and (+Rev RNap). Paired t-tests were carried out to determine the significance level. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. E) Dosage response curve using T = 16 hrs.

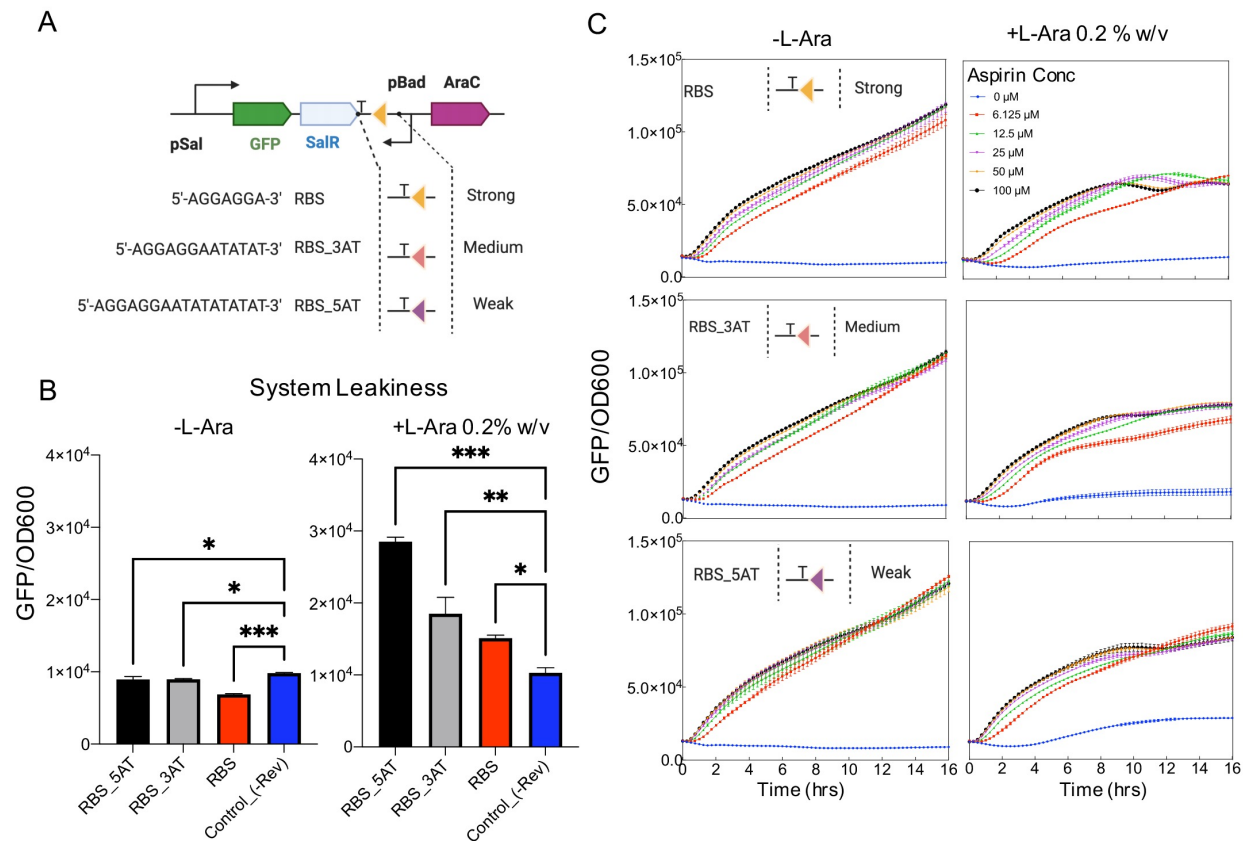


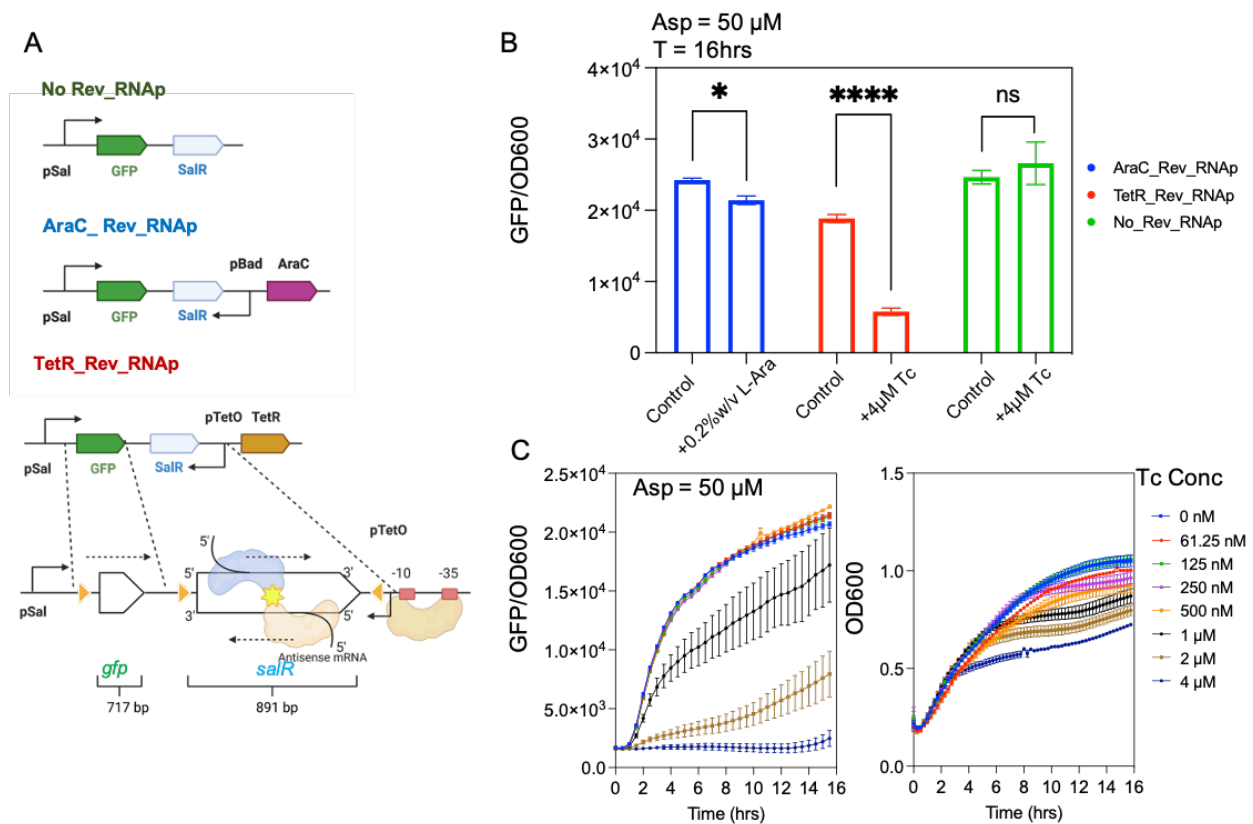
Figure 3.8. Changing the strength of antisense RNap flux through RBS swaps.

A) Ribosome binding strength can be adjusted via addition of the repetitive AT spacers. B) The system leakiness (T = 16 hrs) for all four cases with and without L-Arabinose induction. N=3. Paired t-tests were carried out to determine the significance. *** $p < 0.001$ ** $p < 0.005$ * $p < 0.05$. C) Induction kinetics profile for all three cases (Rev RNap + with different RBS) in EcN. Aspirin gradient used is (0, 6.125, 12.5, 25, 50, 100 μ M). N=3.

This could be explained by the activator/repressor features displayed via SalR, where reduction in SalR expression would likely affect both sides of the dynamical range. Nevertheless, the narrowing in dynamical range was not ideal and thus requires further optimisation.

3.3.6 Tight control using TetR-based RNAp antisense constructs

The introduction of antisense P_{Bad} and alteration of the RBS strength did not successfully repress the GFP basal leakiness. Therefore, to achieve tight control over SalR expression, the antisense P_{Bad} promoter was replaced with a P_{TetO} promoter based on the well-established (TetON) system. P_{TetO} has demonstrated both a broader dynamical range and higher inducer cooperativity compared to the P_{Bad} promoter in *E. coli* strains [273][274].



A) Schematic diagram for the original construct P_{Sal}-gfp-salR (No RNAp), AraC_Rev_RNAP (Antisense AraC_P_{Bad} attached) and TetR_Rev_RNAP (Antisense TetR_P_{TetO} attached). B) Relative fluorescence output (T = 16 hrs) at the saturated inducer level of 50 μ M aspirin. Under 4 μ M of tetracycline, the GFP expression can be fully suppressed. Paired t-tests were used to determine the significance. * $p < 0.05$ **** $p < 0.0001$. C) Induction kinetics (16 hrs) curve of TetR_Rev_RNAP under both aspirin 50 μ M and a gradient of tetracycline (0, 61.25, 125, 250, 500, 1000, 2000, 4000 nM). OD600 growth curve is shown on the right. N=3.

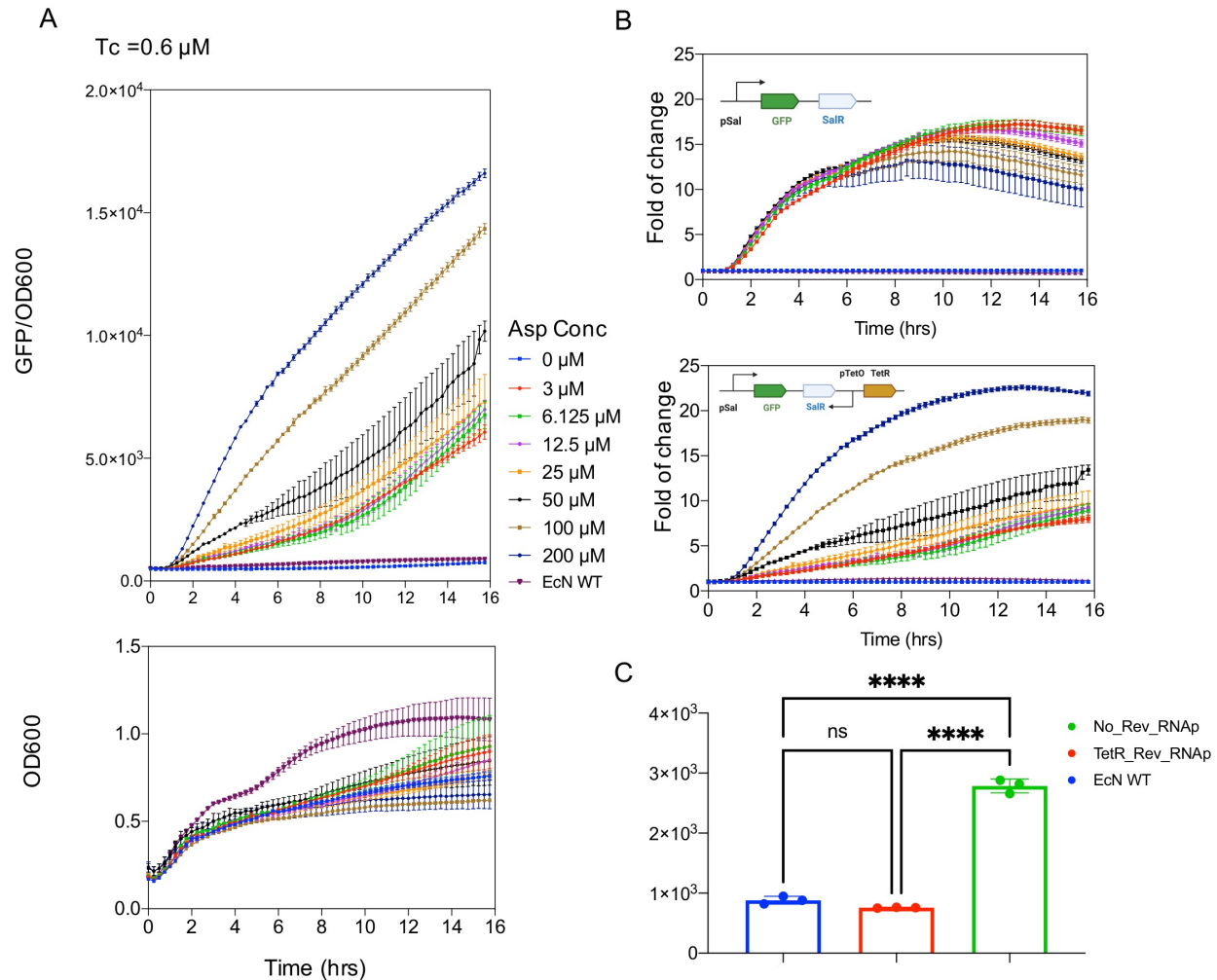


Figure 3.10. Induction kinetics of TetR_Rev_RNAP under aspirin gradient.

A) Induction kinetics of TetR_Rev_RNAP and No_Rev_RNAP in EcN under 0.6 μ M of tetracycline and a gradient of aspirin (0, 3, 6.125, 12.5, 25, 50, 100, 200 μ M). OD growth of the induction kinetics is shown at the bottom. The addition of 0.6 μ M tetracycline did not affect the growth rate of EcN. B) Induction fold of TetR_Rev_RNAP compared to No_Rev_RNAP. C) Leakiness (T = 16 hrs) compared with the case with the control -Rev RNAP, wild type EcN and TetR_Rev_RNAP. Intrinsic fluorescence was removed using LB medium. Paired t-tests were used to determine the significance. **** $p < 0.0001$.

As shown in Figure 3.9 A, a TetR_Rev_RNAP system was constructed with the same RBS site and spacer as the AraC_Rev_RNAP system. In Figure 3.9 B, under a fully saturated induction level of aspirin (50 μ M), full system (GFP) suppression can be achieved with the addition of 4 μ M of tetracycline.

In contrast to the marginal decrease observed in AraC_Rev_RNAP constructs, TetR_Rev_RNAP demonstrated a clear dose-dependent repression effect, ranging from 0 to 4 μ M tetracycline (Figure 3.9 C). A minor effect of the OD was observed, however in the first 3 hrs of induction, it was clear the system could be fully silenced without affecting the OD growth. In Figure 3.10, under supplement with 0.6 μ M of tetracycline, the basal expression level of non-induced constructs could be suppressed to the same level as wild-type bacteria. (Figure 3.10 A, C) while maintaining a functional dose response to aspirin gradients ranges. OD600 (Figure 3.10 C) indicated that the addition of 0.6 μ M tetracycline did not affect the cellular growth. The addition of the antisense promoter also broadened the operational range of the original PAR constructs. The TetR_Rev_RNAP system did not get saturated at 200 μ M compared to the PAR system previously shown in Figure 3.6 B. Due to the suppression of leakiness, TetR_Rev_RNAP also exhibited a higher fold of change upon full induction (~ 22 F.c), compared to the No_Rev_RNAP (~ 17 F.c).

Figure 3.11 demonstrated a two-dimensional heatmap of the original PAR (No_Rev_RNAP) and the optimised TetR_Rev_RNAP construct under induction with both tetracycline and aspirin. It is clear that TetR_Rev_RNAP can respond to dual input control of aspirin and tetracycline, and displayed a quasi-XOR gate between the threshold concentration of aspirin (50 μ M) and tetracycline (5 μ M); these thresholds are low enough to be considered safe for most medical applications.

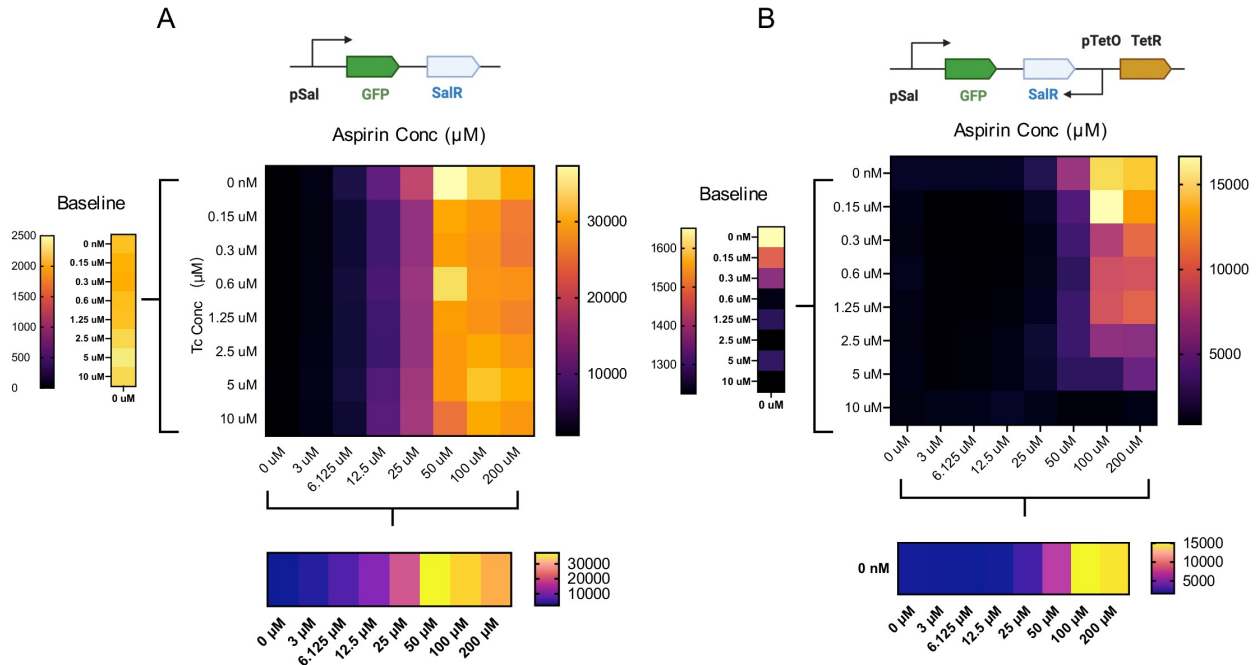


Figure 3.11. Induction Heatmap of original (PAR) and TetR_Rev_RNAP.

A) Heatmap of the relative fluorescence level of the original P_{Sal}-gfp-salR (No_Rev_RNAP), tested in EcN under a gradient of aspirin (0, 3, 6.125, 12.5, 25, 50, 100, 200 μM) and tetracycline (Tc) gradient (0, 0.15, 0.3, 0.6, 1.25, 1.5, 5, 10 μM). B) Heatmap of TetR_Rev_RNAP tested under the same conditions in EcN. Both cases with the single inducer of Tc or aspirin is extracted and shown on the right and bottom, respectively. The basal line of TetR_Rev_RNAP can be reduced with increasing tetracycline concentration.

3.4 Discussion

In this chapter, I addressed the current shortage of suitable control units in living medicine and characterised an aspirin-responsive circuit in the probiotic strain EcN. There is only a limited range of inducers that can effectively operate within the human body, as many inducers may adversely impact human health, or are broadly found in common dietary materials and should therefore be avoided. For example, antibiotic inducers might alter the structure of microbiota in the gut, and sugar inducers may trigger unwanted induction. However, aspirin is mainly absent from the common human diet, and its pharmacological safety and effectiveness have been demonstrated extensively through many clinical trials and *in vivo* studies [275][276]. For the most

sensitive SalR-based systems studied in this work (P_{Sal} -GFP-salR), the effective range of aspirin dosage required for activation is 0.05 - 10 μM , which is more than ten times lower than the human safe dosage of aspirin ($\sim 111 \mu\text{M}$). Furthermore, induced GFP expression in cells with SRS and PAR systems was observed to start after about 50 mins and reached a significant strength within 16 hrs (Figure 3.6 and Figure 3.4), which is of a comparable time-scale to aspirin's biological half-life.

An aspirin/salicylate-triggered NahR regulatory system was previously developed by Royo et al [79]. *Salmonella* spp was engineered for *in vivo* expression of 5-fluorocytosine to treat mice with fibrosarcoma. This regulatory circuit was a salicylate-inducible cascade expression system that was derived from a naphthalene degradation pathway in *Pseudomonas putida* [79]. It demonstrated effective drug delivery to minimise tumour size in mice, proving the concept of using salicylate/aspirin as inducers to trigger the synthesis of cytosine deaminase that catalysed the production of potent cytotoxic fluorouracil for cancer chemotherapy. In this case, the gene circuit configuration was a simple cascade expression without feedback, and the effective induction concentration of aspirin was 125 - 5000 μM [79]. Given that aspirin shows toxic effects at concentrations greater than 832 μM [277], sensitive gene circuits for aspirin have an advantage in clinical practice. In addition, the well characterised SRS and PAR systems are simpler gene circuits than the regulatory module *nahR/Psal::xylS2*, demonstrating a dosage response to the lower range of aspirin, 0.05-10 μM . The importance of feedback loops in genetic circuits is evident in their abundance in naturally occurring gene regulatory systems [130]. As a universal network motif in transcription interactions [278], feedback systems can provide several advantages including robustness to noise [279], improved temporal response [133] and stability in changing cellular contexts [280]. The present study not only compared two gene circuits SRS (no feedback)

and PAR (with feedback), but also successfully applied a mathematical model to simulate the performance according to LTTR transcriptional regulatory mechanism (Figure 3.4 and Figure 3.5). The hypothesised regulatory mechanism was further supported by altering the promoter strength and creating an intermediate SRS* system (no feedback and with a weaker promoter P_{J109}). This provides a useful modelling and design architecture for the largest sensory regulator family.

Another critical aspect of genetic circuit design for usage in bacteria therapy is tight control. Only a few genetic circuits demonstrated a near basal line leakiness with non-ideal inducer compounds such as benzoate [281] and organic dye [282]. Furthermore, chimeric promoter design often requires an in-depth understanding of the regulatory mechanism and may vary between family types. I explored using an inducible antisense promoter as a tuning strategy, which is widely adopted in natural organisms across different species (archaea [283], prokaryotes [137] and eukaryotes [284]). The system performance is validated in the probiotic strain EcN and demonstrated a near background level baseline with a supplement of tetracycline ($0.6 \mu\text{M}$) without incurring any additional metabolic burden to the host cells. Furthermore, the cargo expression could be fully silenced by induction of the reverse input under the tolerable concentration of tetracycline ($4 \mu\text{M}$). This tight control construct could be beneficial for expressing cargo proteins that are toxic or cell-burdening. Anhydrotetracycline could be further tested, which has been reported to demonstrate a higher dose tolerance used in human practice and a higher affinity towards TetR [242]. This provides a potentially universal and convenient method for creating tight control circuits regardless of the input.

The result from our investigation of the molecular mechanism of SalR regulation suggests that aspirin/salicylate could be transported [285] into *E. coli* and activate the gene circuits. In *A. baylyi* ADP1, a putative protein encoded by *salD* is presumably responsible for salicylate transport

across the membrane. This protein is homologous to the *E. coli* FadL membrane protein [258]. However, it is unclear how efficiently *E. coli* can transport aspirin/salicylate across the membrane. In *E. coli*, aromatic permeant acids such as salicylate and benzoate induce a large number of low-level multidrug efflux systems, governed by the *Mar* operon (*marRAB*) as well as additional, unidentified mechanisms. Since *E. coli* does not have a specific transport protein for aspirin/salicylate, it is likely the efflux system will remain on after the induction; it was hypothesised that there is a maximum equilibrium concentration of aspirin/salicylate inside the cell cytoplasm.

Notably, a few new questions emerge from the current observations that would require further investigation. For instance, it was shown that *E. coli* could facilitate the breakdown of aspirin into salicylate and acetate. However, it was unknown whether salicylate interacts with SalR through the same binding pocket via non-specific binding or if it is similar to that of BenM, also a lysR family member, which possesses two distinct binding sites for both its inducer molecules [286]. Furthermore, despite all three circuits (SRS, SRS*, PAR) exhibiting a functional dosage response in either nutrient-stringent micro-aerobic conditions or nutrient-rich aerobic conditions, the induction curves are significantly different with each one. This implies the global resource regulator and oxygen could have an unknown impact on the system dynamics. To fully investigate the system dynamics under anaerobic conditions, a suitable oxygen-independent reporter module could be used, such as gas vesicle formation (ARG system) [287] and the LacZ gene [81]. Further experiments using acetate as a sole carbon source could also be carried out to determine how different carbon sources would alter the dosage response.

Since many genera of bacteria naturally co-exist with tumours in clinical situations [288], probiotic bacteria such as EcN should be ideal chassis to construct anti-cancer agents due to their

designable features, and it is important to characterise modular genetic constructs in these species. EcN has also been shown to selectively colonise the necrotic tumour core over healthy tissues [41]. Overall, using aspirin as an external drug cue for the controllable and tight expression of gene circuits, combined with safe delivery and tissue penetration, would enable higher precision and systematic killing of tumorous cells. This chapter demonstrates that a toolbox of aspirin-inducible SalR systems validated in EcN exists that would be useful to speed up the medical translation of bacterial therapy.

Chapter 4 Spatial Control: P_{VRecA} based DNA damage sensor

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In the previous chapter, I demonstrated the construction and fine-tuning of an aspirin responsive circuit. To further expand the therapy tailored genetic tool box with a spatial control circuit, the SOS box of the *recA* promoter, P_{VRecA} from *V. natriegens* was characterised, cloned and tested into probiotic strain *E. coli* Nissle 1917. The constructed circuit was rationally engineered according to the predicted interactions between LexA repressor and the promoter P_{VRecA}. The redesigned P_{VRecA-AT} promoter with modified SOS box demonstrated a sensitive and robust response to DNA damage induced by UV and genotoxic compounds (Mitomycin C, deoxycholate, taurocholic acid and carcinogenic antibiotics). A HrpRS based transcriptional amplifier and a negative feedback loop were added to the optimised P_{VRecA-AT} to achieve a robust SOS system that is resistant to environmental fluctuations in parameters including pH, temperature,

oxygen and nutrient conditions. Long term stability was also assessed for over 40 hrs cultivation in a turbidostatic mini-reactor. The results of this chapter were published in [8].

4.1 Introduction

Naturally occurring microorganisms living in the human body present an opportunity for *in situ* bacterial diagnostics and therapy [59][36]. Despite advancements in treating gut inflammation [36], [59], [84], hyperammonaemia [59], obesity [226] and various types of cancer [43], [289], [290], bacterial therapy still lacks accurate spatial control systems that can act as “input modules”. Synthetic biology could contribute to the development of inexpensive, rapidly deployable bacterial diagnostics and therapy [291]. An ideal module for bacterial therapy should display the following traits: (i) high sensitivity so that it can be triggered by low dosage of inducers; (ii) a tight control that minimises the background expression; (iii) a strong dynamic response to ensure detectable outputs; (iv) resilient to nutrient and environmental fluctuations, (v) spatially controllable to enables localised activation. (vi) Finally, the host engineered with the modules should be clinically safe.

Some carcinogenic or DNA damaging compounds generated in the human body are responsible for cancers [292]–[294]. The SOS response system is a global response to such DNA damage and represents a universal regulation mechanism in bacteria [295][296], yeast [297] and humans [298]. The response mechanism of the SOS system shows significant overlap with few divergences in different prokaryotic organisms [295]. However, the wild-type *E. coli* SOS system is not sufficiently sensitive or rapid for the diagnosis of DNA damaging molecules or radiation cues. A hypersensitive yet stable system would be useful for creating a mild radiation-controlled bacteria therapy, and cargo expression can be sufficiently switched on using low radiation dosage

while is not harmful to the human body. Therefore, a sensitive, low background, strong responsive, stable and spatially controllable SOS system is required for the application of bacterial therapy and detection of carcinogenic toxicity.

The gram-negative and non-pathogenic *Vibrio natriegens* is so far the fastest growing bacterium that has been isolated [7]. First reported in the early 1960s, *V. natriegens* was found to have a doubling time of fewer than 10 mins under optimal conditions, which is less than half of the shortest reported doubling time of *E. coli* (30 – 40 mins) recorded in the literature [300]. Considering its fast growth rate [7] and living environments, it is expected that *V. natriegens* may be equipped with a robust and sensitive SOS system for DNA repairing due to errors of DNA replication. The expression of the universal SOS-response regulator, RecA, in response to UV radiation by *V. natriegens* was previously reported [301]. However, the response time and sensitivity of the *recA* promoter of *V. natriegens* to DNA damage remain unknown.

In this study, the LexA binding sites of *V. natriegens recA* gene promoter (P_{VRecA}) was firstly identified according to the predicted promoter-LexA interactions. The transcriptional sigma factor (σ), LexA binding affinity and interactions with the P_{VRecA} promoter were then characterised by mutagenesis after investigating the correlation between the SOS binding box and the expression dynamics. I rationally designed a spacer between the LexA binding sites in P_{VRecA} and tested its performance to sense DNA damage induced by carcinogenic chemicals and UV radiation. To demonstrate the promoter modularity and further strengthen the tightness of the control, a series of chimeric promoters were created. Finally, the performances of the redesigned SOS boxes were examined.

Natural heterogeneity, noise and variability of gene expression in constantly changing environments is a challenge for bacteria therapy. Furthermore, the physiological condition of

human tissue (pH, oxygen and nutrient availability) [147] can alter the dynamic range of synthetic circuits, thus undermining the performance of the engineered systems. Inspired by recent work on disturbance-resistant control systems [136], the stability and sensitivity of the SOS system were further improved through a designed negative feedback loop, which is an integrated HrpRS based transcriptional amplifier and TetR based repression system [14]. This operational feedback design was demonstrated to improve the robustness of the sensing circuit when subjected to different environmental changes. It was further demonstrated that our optimised promoter (P_{VRecA_AT}) could be used as an optogenetic tool. Optogenetic control units use light to control gene expression and have broad applications in synthetic biology. These systems are particularly attractive because of their ability to enable remote (i.e. non-contact), precise (predictable input-output response), and rapid actuation of gene expression [302][303]. This optimised SOS system can be precisely controlled by light-induced DNA damage in either the planktonic or biofilm phases.

All circuits were tested and validated in *E. coli* Nissle 1917 due to its established safety and efficacy in clinical applications [2][304] and genomic similarities with other *E. coli* variants [192]. Overall, a toolbox for the SOS gene-circuit was developed for the detection of DNA damage, which can detect a range of disease biomarker molecules and carcinogenic antibiotics sensitively and robustly.

4.2 Material and methods

Table 4.1 Strains used in Chapter 4.

Strains	Genotype	Ref
<i>E. coli</i> Nissle 1917 (EcN)	Wild type	Ardeypharm GmbH (Herdecke, Germany)
<i>E. coli</i> DH5a	<i>fhuA2</i> Δ (argF-lacZ) U169 <i>phoA</i> <i>glnV44</i> Φ 80 Δ (lacZ)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	NEB
<i>E. coli</i> BL21(DE3)	<i>fhuA2</i> [<i>lon</i>] <i>ompT</i> <i>gal</i> (λ DE3) [<i>dcm</i>] Δ <i>hsdS</i> λ DE3 = λ <i>sBamHI</i> Δ <i>EcoRI-B</i> <i>int::(lacI::PlacUV5::T7 gene1)</i> <i>i21</i> Δ <i>nin5</i>	NEB
<i>E. coli</i> JM109	<i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> (<i>r_k⁻</i> , <i>m_k⁺</i>), <i>relA1</i> , <i>supE44</i> , Δ (<i>lac-proAB</i>), [<i>F'</i> <i>traD36</i> , <i>proAB</i> , <i>laqI^q</i> Δ M15].	NEB

Table 4.2. Plasmids constructed in Chapter 4.

Short Abbreviation	Plasmids Name	Description	Ori
pP _{VRecA} _GFP	P _{VRecA} _AT-GFP	P _{VRecA} modified (Four AT repeats) promoter fused with orf GFP (pMK backbone); used as control for case without amplifiers.	p15A
pP _{VRecA}	P _{VRecA} _sfGFP	P _{VRecA} wild type promoter fused with orf sfGFP(pMK backbone)	ColE1
pP _{VRecA} _AT	P _{VRecA} _AT_sfGFP	P _{VRecA} modified (Four AT repeats) promoter fused with orf super fold GFP (pMK backbone)	ColE1
pP _{VRecA} _AA	P _{VRecA} _AA_sfGFP	P _{VRecA} modified (Eight A tandem) promoter fused with orf super fold GFP (pMK backbone)	ColE1
pLexA_RecA	P _{ELexA} - P _{ERecA}	<i>recA</i> and <i>LexA</i> orf cloned under its original promoter from <i>E. coli</i> Nissle 1917	ColE1
pUnified_LexA_RecA	P _{VRecA} _AT_sfGFP+P _E . LexA- P _E .RecA	P _{ELexA} -P _{ERecA} construct integrated with P _{VRecA} _AT_sfGFP	ColE1
pP _{VRecA} _Jungle_D onw	P _{VRecA} _Jungle_Down_sfGFP	P _{VRecA} modified (Double SOS) promoter fused with orf super fold GFP, additional SOS placed 7bp downstream (pMK backbone)	ColE1

pP ^{VRecA_Jungle_Up}	P ^{VRecA_Jungle_Up_sfGFP}	P ^{VRecA} modified (Double SOS) promoter fused with orf super fold GFP, additional SOS placed 7bp Upstream(pMK backbone)	ColE1
pP ^{VRecA_Jungle_Both}	P ^{VRecA_Jungle_Both_sfGFP}	P ^{VRecA} modified (Triple SOS) promoter fused with orf super fold GFP. Additional SOS placed both up & downstream (pMK backbone)	ColE1
pP ^{ERecA}	P ^{ERecA_sfGFP}	<i>E. coli</i> native recA promoter fused with orf super fold GFP (pMK backbone)	ColE1
pP ^{VRecA_α}	Mutant_α_P ^{VRecA_sfGFP}	P ^{VRecA} modified (fully truncated SOS & σ binding region) promoter fused with orf super fold GFP (pMK backbone)	ColE1
pP ^{VRecA_β}	Mutant_β_P ^{VRecA_sfGFP}	P ^{VRecA} modified (fully truncated SOS region) promoter fused with orf super fold GFP (pMK backbone)	ColE1
pP ^{VRecA_ε}	Mutant_ε_P ^{VRecA_sfGFP}	P ^{VRecA} modified (partial truncated (-35) SOS region) promoter fused with orf super fold GFP (pMK backbone)	ColE1
pP ^{VRecA_γ}	Mutant_γ_P ^{VRecA_sfGFP}	P ^{VRecA} modified (partial truncated (-10) SOS region) promoter fused with orf super fold GFP (pMK backbone)	ColE1
pP ^{VRecA_AT_SsrA}	P ^{VRecA_AT_sfGFP_SsrA}	P ^{VRecA} modified (Four AT repeats) promoter fused with orf super fold GFP attached with LVA fast degradation tag (pMK backbone)	ColE1
pP ^{VRecA_GFP_Amp}	P ^{VRecA_AT_HrpRS_P_hrpL_GFP}	P ^{VRecA_AT} promoter fused with HrpRS transcriptional amplifiers (pMK backbone)	p15A
pP ^{VRecA_GFP_Amp_TetR}	P ^{VRecA_AT_HrpRS_P_hrpL_sfGFP_TetR}	P ^{VRecA_AT} promoter fused with HrpRS transcriptional amplifier controlling both <i>sfGFP</i> orf and <i>TetR</i> orf (pMK backbone)	ColE1
n.a	P ^{Bad_tdtomato}	P ^{Bad} promoter fused with orf <i>tdtomato</i> fluorescent protein (pGEMT backbone); Source of <i>tdtomato</i>	ColE1
pP ^{tetO_HrpV}	P ^{TetO_tdtomato_HrpV}	P ^{TetO} promoter fused with orf <i>HrpV</i> and orf <i>tdtomato</i> (pGEMT backbone)	pSC101

Table 4.3 Primers used in Chapter 4.

Primer Name	Sequence	Related plasmids
Randomised_ P _{VRecA} Fwd	NNNNNNNNNNNNNNNNNNNNNTCTATACCTGTATAG AAAAAACTTTAGCTGATCTACGTATAACTTTGC	α, β, γ and ϵ mutant of P _{VRecA} _sfGFP
Randomised_ P _{VRecA} Fwd	NNNNNNNNNNNNNNNNNNNNNTAATAACTTTTCATTG CTGAGCGATTAAGTCTCAAGAAAAGTTTAATGAC	
P _{VRecA} _Vec_Fwd	ACTTTGCCGCGAGAGCTTATCACGCTCTAGAAGCGG CCGCGAATTCCAG	
P _{VRecA} _Vec_Rev	GATGAATAAATCGGAGAAAAGTATACTAGAGATTAA AGAGGAGAAATACTAGATGCGTAAAGGAGAAGAAC	pBW201_ P _{VRecA} _GFP
P _{VRecA} _GFP_Frag_Fwd	GAATTCGCGGCCGCTTCTAGAGCGTGATAAGCTCT GCGGCAAAGTTATACGTAGATCAGCTAAAG	
P _{VRecA} _GFP_Frag_Rev	TTTCTCCTCTTTAATCTCTAGTATACTTTCTCCGA TTTATTCATCTTTTTGGGCGACGAATAGT	
Rbs_SalA_Fwd	CTCTACAAATACTAGTAGCGGCCGCTGCAGCTCAA AGGAAATGAGTCGTGGG	P _{VRecA} _AT_sfG FP_SalA
Rbs_SalA_Rev	GGCAGTGAAAGGAAGGCCCATGAGGCCAGTTAAA TCGTTGCAACAGGTTGTATTG	
pMK_ P _{VRecA} _sfGFP_fwd	CTCATGGGCCTTCCTTTCACTGCCCGCTTT	
pMK_ P _{VRecA} _sfGFP_Rev	ACCCACGACTCATTTCCTTTGAGAACTTGAAGCGG CCGCTACTAGTATTTGTAGAGCTCATC	P _{VRecA} _AT_Hr pRS_P _{hrpL} _GF P
P _{VRecA} _gfp_amp_frag_fwd	CGCGGCCGCTTCTAGAGCGTGATAAGCTCTGCGGC AAAGTTATACGTAGATCAGC	
P _{VRecA} _gfp_amp_frag_Rev	CCTCTTTAATCTCTAGTATACTTTCTCCGATTTAT TCATCTTTTTGGGCGACGAATAGT	
P _{VRecA} _gfp_amp_vec_fwd	ATAAATCGGAGAAAGTATACTAGAGATTAAAGAGG AGAAATACTAGATGAGTACAGGCATCG	P _{TetO} _tdtomato _HrpV
P _{VRecA} _gfp_amp_vec_rev	GCCGCAGAGCTTATCACGCTCTAGAAGCGGCCGCG AATTCCAG	
pJKR_L_TetR_fwd	TAAGCGACTAAAAAATTGAATGTAG	
pJKR_L_TetR_rev	ATTATATCTCCTTCTTAAAGTTAAATTTAATG	P _{TetO} _tdtomato _HrpV
HrpV_fwd	TTTAACTTTAAGAAGGAGATATAAT ATGATTGAGGTAACGGAAAAGTCGG	
HrpV_rev	AAAAAACGGGTTTATTAGTGCATTTCTCGCTCA AATG	
tdTomato_fwd	AATGCACTAATAAACCCGTTTTTTTTGGGCTAGAAA TAATTTTG	P _{TetO} _tdtomato _HrpV
tdTomato_rev	CTACATTCAATTTTTTAGTCGCTTATTACTTGTAC AGCTCGTCCATGCCG	

UV302 nm and chemical induction

Evaluation of the genotoxicity biosensor was done in Corning Black 96 wells plate (flat, clear bottom): 5 μ l overnight bacteria culture was added into 190 μ l medium containing M9 minimal media (1x), glucose (0.4 %) and kanamycin (60 μ g/ml), and 5 μ l mitomycin-C stock to achieve a final concentration ranging from 0.05 μ M to 250 μ M. For UV induction, initial OD600 was set to be 0.1, this was done by adding 5 μ l overnight bacteria culture into 195 μ l medium containing M9 minimal media (1x), glucose (0.4 %) and kanamycin (60 μ g/ml), followed by 302 nm UV exposure from 0 to 10 seconds using transilluminator UVP Benchtop UV Transilluminators (ThermoScientific™). The plate was incubated in a BioTek plate reader at 37 °C for 16 hrs with OD600 and luminescence or GFP reading being measured every 15 minutes. All experiments were carried out in three technical repeats. The data points and error bars represent the mean and standard deviation, respectively. The processed data were plotted and analysed with Prism version 9.1.

Biofilm growth and UV induction.

For biofilm growth, 100 μ L of transformed overnight culture were aliquoted onto LB agar with 50 μ g/ml Kanamycin supplemented. This was allowed to grow into a circular lawn upon 16 hrs incubation at 37 °C. Subsequently, the fully developed biofilm was exposed to 302 nm UV for 10 sec and 20 sec. Fluorescent reads were taken 4 hrs after the exposure using Molecular Imager VersaDoc® imaging system by BioRad. Images were subsequently processed using ImageJ® with a consistent threshold.

Turbidostatic induction

For turbidostats experiments, the ChiBio experimental platform [305] was employed with culture volumes of 20 ml. M9 minimal media (1x), 5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% w/v thiamine, glucose (0.4% w/v) and kanamycin (60 $\mu\text{g}/\text{ml}$) were used, and experiments were run until substantial biofilms were observed to form in the culture chamber. Fluorescence measurements of sfGFP were performed each minute using excitation/measurements of 457 nm/510 nm, respectively. Cultures were grown to an OD600 of 0.3 and maintained constant via Turbidostats functionality for ~5 hrs prior to the first UV dose in each experiment. This first UV dose (i.e., 1 $\times$) was equal to activating a 10 mW (optical power) 280 nm LED for 2.4 sec. In each subsequent dose, the duration of LED activation was doubled. Peak height is defined as the difference between the maximum GFP reading in the five hours following a UV dose and the minimum UV reading in the preceding five hours (e.g., since the previous UV dose). For the sfGFP-ssrA experiment, fluorescence peaks were indistinguishable from measurement noise below UV doses of 16 $\times$.

4.3 Results

4.3.1 Cloning *recA* promoter from *V. natriegens* into *E. coli* Nissle 1917 for better response to SOS repair.

A schematic diagram of the SOS DNA repair mechanism is shown in Figure 4.1A, LexA dimer binds the SOS box and represses the promoter of genes related to DNA repair (Figure 4.1, Appendix Figure B-1), including the *recA* gene. Upon receiving DNA damage, the single-stranded (ssDNA) generated from mismatched DNA and the RecA protein form a complex (ssDNA-ATP-RecA), which induces the co-protease activity and auto-cleavage of the LexA dimer [306], resulting in de-repression of gene expression of the SOS machinery (Figure 4.1D). Alignment of the *recA* promoters in various bacteria shows that the binding sites of LexA dimer is highly conserved (CTGT-N₈-ACAG) and is flanked by -35 and -10 sigma binding sites (Figure 4.2 A). Sequence alignment of *V. natriegens* and *E. coli* indicated a high similarity in both conserved LexA and obinding sites (Appendix Table B-1). Sequence alignment of *Vibrio fischeri* (replication time~280 min) exhibited similar conserved regions but different upper/lower flanking sequences (Appendix Table B-2), which implicates that the flanking region of LexA binding motifs could affect the response time of SOS machinery. *V. natriegens* and *E. coli* Nissle 1917 share 72% homology in LexA protein sequence, and the peptide sequence of the putative binding sites are very similar (Appendix FigureB-1C) [306][307]. It suggests that LexA in both bacteria could recognise a similar SOS box in *Vibrio natriegens recA* (*vrecA*) gene promoter.

A plasmid carrying the P_{VRecA} (*V. natriegens recA* promoter, NCBI ref: NZ_CP016347.1) promoter was constructed to control the expression of a superfolder GFP (*sfGFP*) gene [308] in *E. coli* Nissle 1917. To make the performance of gene-circuits comparable, a basic P_{ERecA} promoter was constructed with native *E. coli* k12 promoter of *recA* gene using the same ribosome binding site (RBS, AAAGAGGAGAAA, BBa_B0030 [309] and the gap sequence GGTACC between

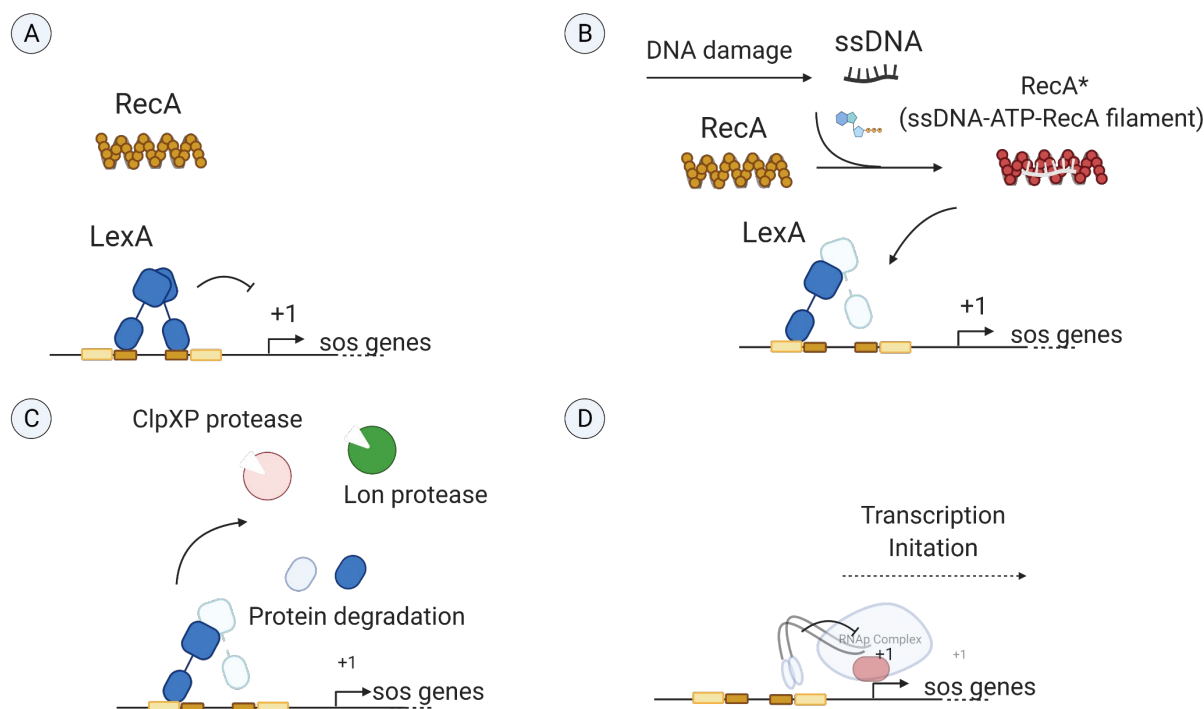


Figure 4.1. SOS DNA response mechanism.

Schematic diagram of the SOS DNA repair mechanism. A) In its native state, SOS machinery is dormant and repressed by LexA, while a low level of RecA is stored inside the cell in its inactive form. B) Upon DNA damage, generation of single-stranded DNA will activate RecA and form complexes of ssDNA-ATP-RecA filament. C) This subsequently triggers the self-cleavage of the LexA dimer and further degradation by the Lon protease and ClpXP. D) The repressor is fully released from the SOS binding sites, resulting in sequential activations of 40+ different DNA repair genes.

RBS and ATG start codon of *sfGFP* gene (Appendix Figure B-3). Figure 4.2 C shows that the P_{VRecA} promoter was more sensitive and had a higher expression amplitude than the *E. coli* native promoter P_{ERecA} . The P_{VRecA} promoter can be triggered by UV with exposure times as short as 2 sec, while the *E. coli* native *recA* promoter P_{ERecA} required at least 4 sec (Figure 4.2C). The fold change of P_{VRecA} promoter was significantly higher than P_{ERecA} (Figure 4.2 B). UV exposure durations up to 10 s did not affect cell growth compared to a control without UV exposure in the testing conditions (Appendix Figure B-4 and Figure B-7). However, P_{VRecA} also demonstrated a higher leakiness than the P_{ERecA} (Figure 4.2C).

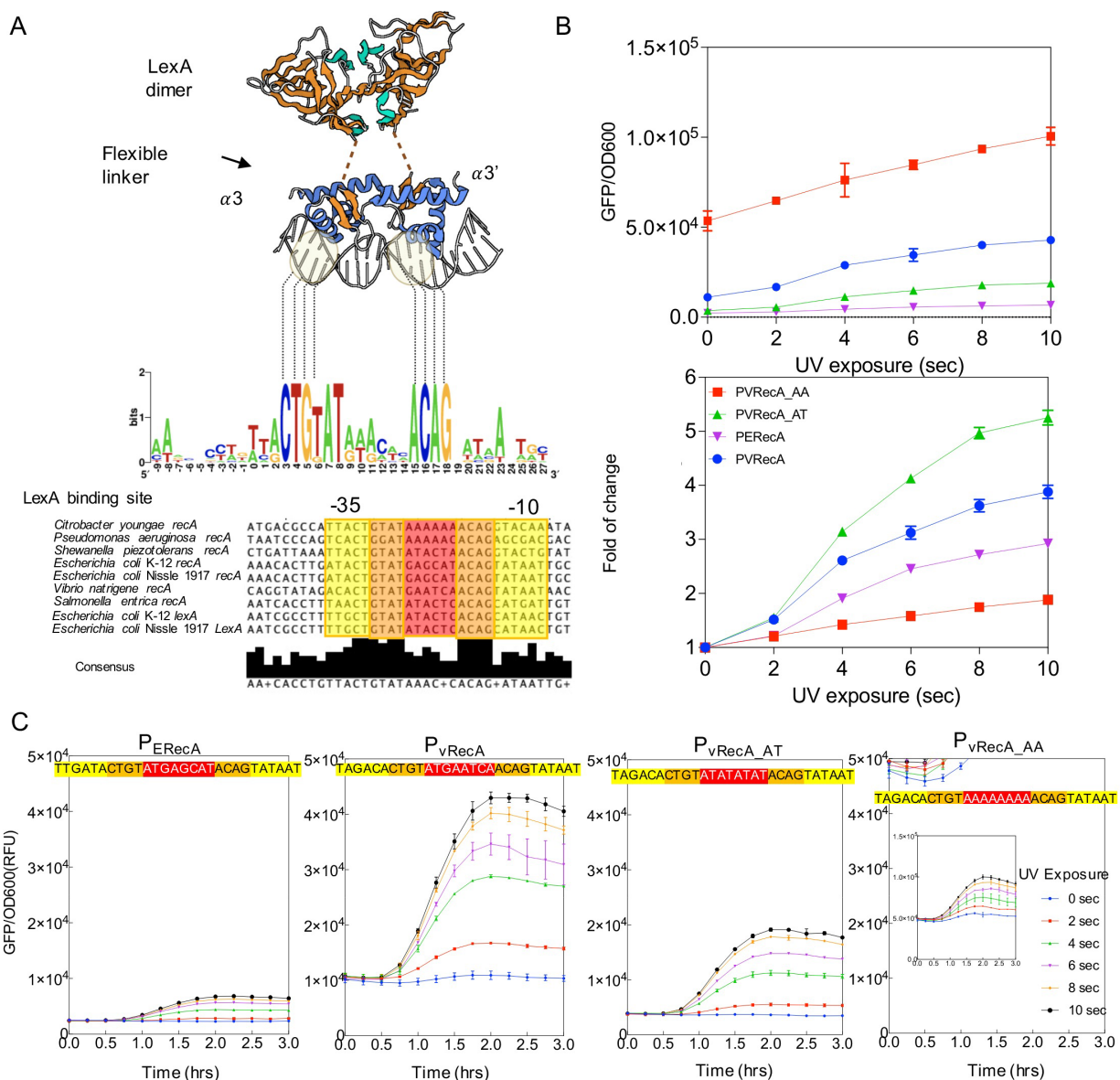


Figure 4.2. Binding of LexA and P_{VRecA} promoter with different SOS box.

A) Weblogo diagram of consensus LexA binding site compiled between six bacteria species, the consensus sequence for the SOS box is {CTGTN₈ACAG}. In the 3D structure, DNA base pair interaction with LexA dimer was highlighted in the orange box where each of the motif base-pair interacts with the α helix domain directly. The 3D crystal structure was acquired from (Uniprot ID P0A7C2). The below box shows the sequences alignment of the highly conserved region of *lexA* and *recA* binding site (Orange) and sigma factor binding site (Yellow) across six different species. B) Dosage response and induction fold change profile for four different constructs under the exposure of UV 302 nm. N=3. C) GFP induction of all three P_{VRecA} variants and *E. coli* P_{ERecA} under the exposure of UV 302 nm for 0,2,4,6,8,10 sec respectively. The GFP kinetics after exposure were shown for 3 hrs. Sigma factor binding site -35 (TAGACA) and -10 (TATAAT) was

highlighted in yellow and LexA binding site highlighted in orange, altered SOS flanking region highlighted in red.

Based on the predicted structure of the LexA-DNA complex [310], the LexA binding motifs of CTGT and ACAG are reverse complementary (Table 4.3). The AT-rich spacer sequence between CTGT and ACAG in the SOS box would be prone to twist due to the weaker hydrogen bond in AT pairs, which should enhance LexA-CTGT binding and repression. Molecular docking was performed using AutoDock vina and Oligo Calc to simulate the Gibbs energy required for LexA binding with different variants, AT-rich spacer (12.5 Kcal/mol) exhibited the most energetic favourable binding compared to AA repeats (14.9 Kcal/mol) and wild type P_{VRecA} (15.9 Kcal/mol) (Appendix Figure B-2). Therefore, such an AT-rich spacer may be able to reduce the baseline expression of P_{VRecA} promoter.

As shown in Figure 4.2C and Table 4.3, it was found that a spacer region of four AT repeats ($P_{VRecA-AT}$) (ATATATAT) indeed created a lower baseline expression level and a more significant fold change than the wild type promoter and AAAAAAAAA ($P_{VRecA-AA}$) mutant (Figure 4.2). Despite a slight decrease in the absolute intensity, compared to the wild type P_{VRecA} SOS (TGAATCAA), the baseline expression of $P_{VRecA-AT}$ was ~2.45 fold lower than that of P_{VRecA} , this suggests that $P_{VRecA-AT}$ allows tight binding of LexA dimer, enhancing repression (Figure 4.2). Interestingly, alternation of the spacer to A-tandem resulted in higher leakiness and less fold change (Figure 4.2), suggesting that twist angle and minor groove in the spacer might also affect the LexA binding. Alternatively, other factors other than LexA binding efficiency may be at play, which is worth further investigation in LexA deficient strains. The overall results indicated that the spacer with AT repeat should have a high affinity to LexA and lower dissociation constant K_d , consistent with our predictions and mutation study done in *E. coli* SOS mutant [306].

4.3.2 Characterisation and tuning SOS box of *V. natriegens* of *recA* promoter.

To further analyse the P_{VRecA} promoter, the LexA crystal structure and LexA-promoter interaction model [310] were used to annotate the P_{VRecA} promoter, the SOS box in P_{VRecA} promoter was identified, which is a 16-bp sequence (CTGTATGAGCATACAG) flanked by sigma factor binding sites, -35 (TAGACA) and -10 (TATAAT) (Table 4.3). Table B-1 shows that the structure of the SOS box in *V. natriegens* is very similar to that in *E. coli* Nissle 1917, and their LexA binding sites are identical, with recognising reverse complementary CTGT/ACAG with 8 bp spacer in between (CTGT-N₈-ACAG). A series of mutations and truncations were then created for the P_{VRecA} promoter of wild type *V. natriegens*, including a full -35, SOS and -10 deletion; partial SOS deletion; full SOS deletion with intact -35; and -35 plus SOS full deletion. This demonstrated that deletion, extension of SOS box, or removal of the -35 sequence completely silenced and disrupted the P_{VRecA} promoter in response to UV-induced DNA damage (Appendix Figure B-5), whilst leaving only the -35 site intact increase the background expression level. These results validated our annotation of the SOS box and -35 and -10 sites in the P_{VRecA} promoter, although previous reports proposed different annotations [311].

Table 4.4. SOS box design in the P_{RecA} promoter of *E. coli* and *V. natriegens*

Name	SOS sequence 5' → 3'
P_{ERecA}	TTGATACTGTATGAGCATACAGTATAAT
P_{VRecA}	TAGACACTGTATGAATCAACAGTATAAT
$P_{VRecA-AT-Repeats}$	TAGACACTGTATATATATACAGTATAAT
$P_{VRecA-A-Tandem}$	TAGACACTGTAAAAAAAAACAGTATAAT

Table 4.5. The performance of gene circuits in *E. coli* Nissle 1917.

Circuits	Dynamic range (rfu)	LOC (UV/MMC)	Inducer range /f.c (fold change)
P _{ERecA} -sfGFP	2292±40 -- 6738±47	1 sec	1 sec – 10 sec
P _{VRecA} _sfGFP	10941±767-- 42957±1075	1 sec	1 sec – 10 sec
P _{VRecA-AT} _sfGFP	3588±127--18844±501/ 2193±75 -- 13284±307	1 sec/0.1 µM	1 sec – 10 sec /0.1 µM - 10 µM
P _{VRecA-AA} _sfGFP	53527±2214 - 10068±1965	1 sec	1 sec – 10 sec
P _{VRecA_Jungle_Down_sf} GFP	2089±74 -- 7088±121	1 sec	1 sec – 10 sec
P _{VRecA_Jungle_Up} _sfGFP	1464±132 -- 6130±162	1 sec	1 sec – 10 sec
P _{VRecA_Jungle_Both} _sfGFP	1271±39 – 3428 ±281	1 sec	1 sec – 10 sec
P _{VRecA-AT} -GFP	2765±31 -- 5969±882/ 3682±55 – 22771 ±919	1 sec/0.5 µM	1 sec – 10 sec / 0.5 µM - 10 µM f.c =20
P _{VRecA} _sfGFP+P _{E.Lex} A-P _{E.RecA}	1268±47 – 7579 ± 124	16 nM	16 nM ~ 750 nM / f.c = 46.87
P _{VRecA} -Amplifier*	1361±186 -- 12032± 257	6.125 nM	6.125 nM – 200 nM / f.c = 32.65
P _{VRecA} -Amplifier+ Feedback ⁺	12864±261-- 26100± 1105	6.125 nM	6.215 nM – 100 nM / f.c = 16.32

P_{VRecA}-Amplifier*: P_{VRecA-AT} connected with HrpRS transcriptional amplifiers
(P_{VRecA-AT}-HrpRS-P_{hrpL}-GFP)

P_{VRecA}-Amplifier+ Feedback⁺: P_{VRecA-AT} connected with HrpRS transcriptional amplifiers linked
with P_{tetO}-tdtomato-HrpV repressor. (P_{VRecA-AT}-HrpRS-P_{hrpL}-sfGFP-TetR + P_{TetO}-tdtomato-HrpV)

4.3.3 Signal amplification through transcriptional amplifiers.

After obtaining an optimised SOS box (spacer AT repeats), three chimeric promoters were created with multiple LexA binding sites to further reduce the leakiness of $P_{VRecA-AT}$ promoter. Inspired by the EilR repressor promoter structure in the Jungle Express system [282], an additional SOS box with 5' complement sequence was inserted 7 bp downstream of the original SOS sequence to enhance repression. As shown in Figure 4.3, this additional SOS box reduced the system background level (12509 ± 573 GFP rfu) by ~ 6 fold to $2,089 \pm 127$ GFP rfu $P_{VRecA-AT}$ Jungle_Down (Figure 4.3), which could be potentially due to the formation of a hairpin loop between two SOS boxes or recruitment of additional LexA protein at the promoter region (Appendix Figure B-6). Upstream insertion of an additional SOS box ($P_{VRecA-AT}$ Jungle_Up) displayed a further reduction of baseline leakiness to ($1,464 \pm 40$ GFP rfu) with lower noise (Figure 4.3A). The insertions of both upstream and downstream SOS boxes ($P_{VRecA-AT}$ Jungle_Both) displayed the lowest leakiness ($1,271 \pm 32$ GFP rfu), despite such addition also lowered the amplitude of system output (Figure 4.3 A&C). All strains harbouring system variants demonstrated similar cell growth (Appendix Figure B-7). Overall, these results demonstrated that the $P_{VRecA-AT}$ promoter could be redesigned to generate different dynamic responses via the simple addition of SOS boxes. This modularity could be useful in creating other chimeric promoters with dual-chemical/spatial control input.

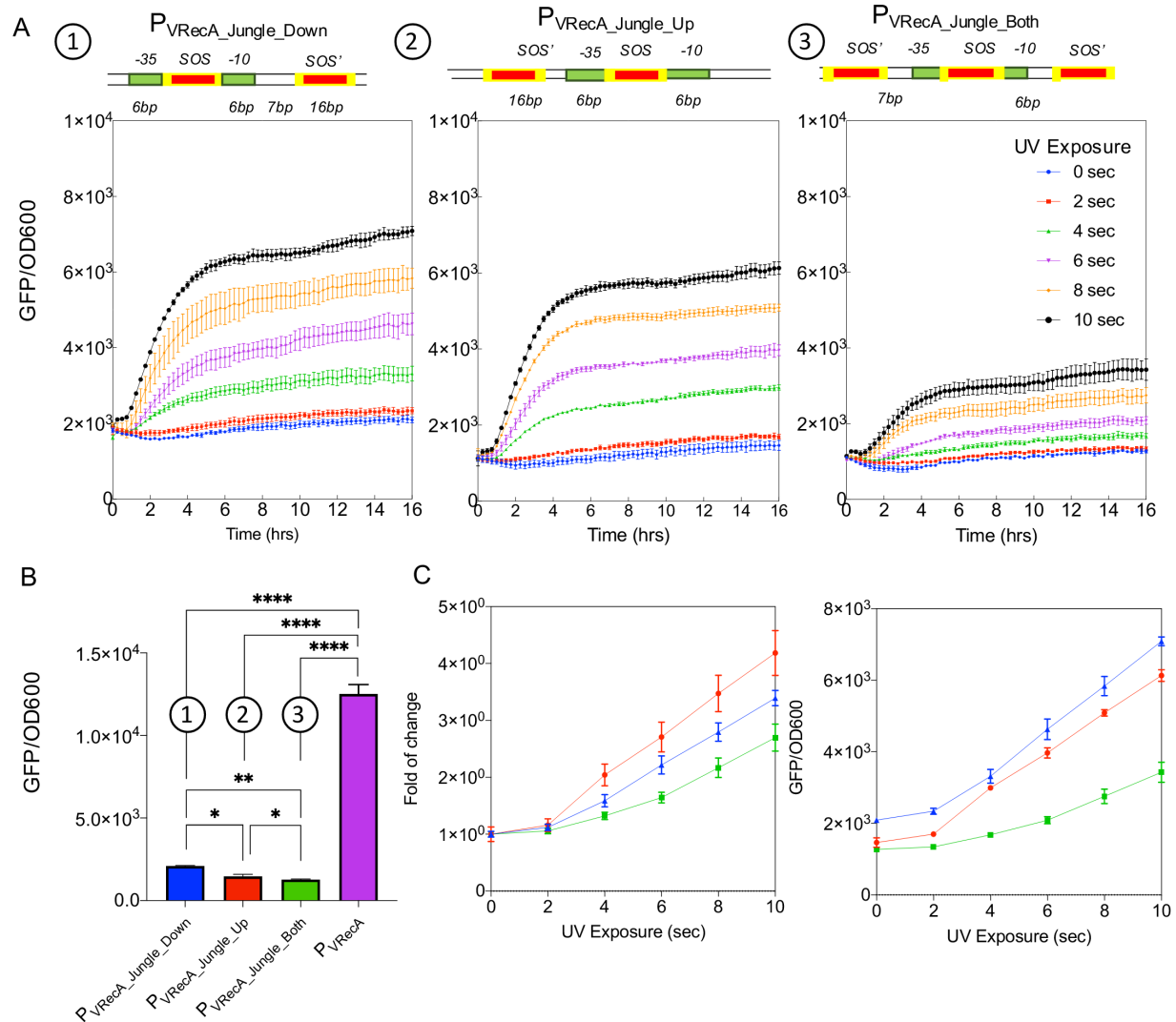


Figure 4.3. Characterisation of chimeric promoters with SOS boxes.

The design of each jungle promoter is shown on top. Green box indicates the sigma factor binding site -35 and -10. Yellow box indicates the σ binding sites, and the red box indicates the SOS box. Equal 7 bp spacing was preserved for each SOS insertion. A) Induction kinetics of different chimeric promoters under UV 302 nm exposure. B) A comparison of the baseline levels (T = 16 hrs) of different chimeric promoters. Paired t tests were carried out between each baseline level. $P < 0.05$ * $P < 0.005$ ** $P < 0.0001$ **** C) Dosage response and induction fold of change profile for each construct under the exposure of UV 302 nm light. The standard deviation and mean value of three bio-replicates were plotted in each graph. N=3.

4.3.4 Negative feedback gene circuit creating a robust performance

To enhance the sensitivity of the DNA damage sensing system, an HrpRS based transcriptional amplifier [14] was added to magnify the output signal of the optimised promoter ($P_{VRecA-AT}$).

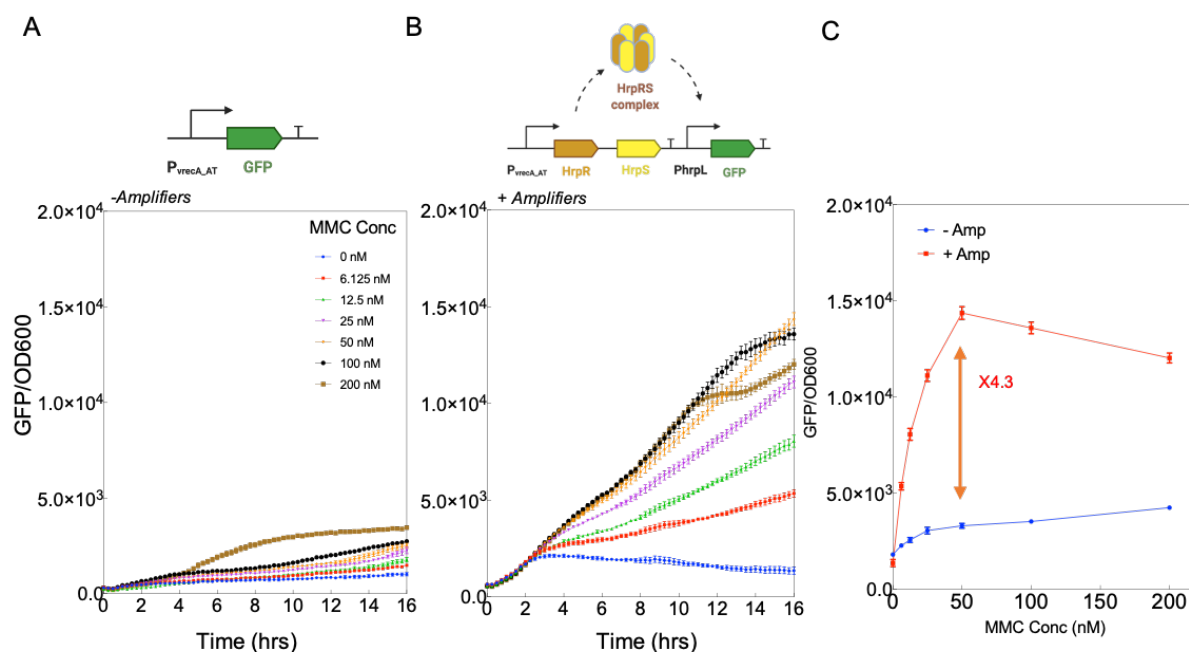


Figure 4.4. The amplification effect of P_{VRecA} promoter coupled with HrpRS transcriptional amplifier.

(A) Original $P_{VRecA-AT}$ promoter fused with GFP as an output. (B) $P_{VRecA-AT}$ promoter connected with the HrpRS transcriptional amplifier. The expression of HrpR and HrpS subunits subsequently activate the P_{HrpL} promoter with a higher flux of GFP output. Induction kinetics were tested with a concentration gradient of mitomycin C (0;6.125;12.5; 25; 50;100; 200 nM) over 16 hrs. (C) Dosage response of $P_{VRecA-AT}$ promoter with and without the amplifier. The maximum amplification achieved is 4.3-fold. The standard deviation and mean value of three bio-replicates were plotted in each graph. N=3

As shown in Figure 4.4, the sensitivity can be improved, allowing detection down to 6.125 nM of mitomycin C (MMC), which is by far the most sensitive genotoxic sensor reported [312]. The maximum amplification factor for $P_{VRecA-AT}$ promoter can reach 4.3-fold when coupled with

HrpRS based amplifier (Figure 4.4) without showing metabolic burden (Appendix Figure B-8). However, the introduction of this amplification system led to a significant delay in activation, with the response time extended to ~2.5 hrs. Compared to the original arsenic amplifiers construct, which also displayed a similar time of delay [14], the delay is likely to be independent of the biosensor target. Instead, this delay could be due to the global σ^{54} regulation of the HrpRS system and additional time required for the translation of the HrpRS complex in *E. coli* Nissle 1917. A number of nutrient depleting conditions have been tested, which have previously been shown to reduce response delay in σ^{54} dependent promoter [313][314]. It was noticed that there is a significant difference in response time when different carbon sources were used. Glycerol as the sole carbon source had a shorter delay (2.5 hrs) compared to glucose as the sole carbon source (4 hrs) (Appendix Figure B-9). Since little difference was observed in the OD, the delay might be related to the global catabolite repression in bacteria [315].

Although the amplifier constructs demonstrated high sensitivity with minimal leakiness (Figure 4.4), it lacks stability and robustness against environmental fluctuation, such as changes in the nutrient richness, pH and temperature. To improve the stability and robustness of our SOS sensing system, a tuneable TetR repressor was coupled with the transcription amplifier to make a negative feedback loop (Figure 4.5). This was achieved by adding TetR regulator protein under the control of P_{HrpL} and the counter repressor unit HrpV for HrpRS complex under the control of TetR regulating promoter (P_{tetO}). Upon the activation of the HrpRS amplifier, the flux of sfGFP and TetR expression going up, this led to an accumulation of inactive TetR which can only bind P_{tetO} promoter and induce downstream expression in the presence of anhydrotetracycline (aTc). Subsequently, the activated TetR induced the expression of HrpV and dissociated the formation of HrpRS complex which shut down the P_{hrpL} , thus forming a closed-loop negative feedback system.

To visualise the performance of the negative feedback system, a red fluorescent tdTomato was coupled to the downstream of HrpV. Upon activation of P_{tetO} , an increased expression of tdTomato with a decreased expression of sfGFP were observed (Figure 4.5C). Upon over induction of $P_{VRecA-AT}$, the promoter P_{hrpL} was induced to a high level, which also leads to a high expression of TetR. In the absence of aTc, expression of HrpV was repressed, the overall expression of sfGFP was high. In the presence of aTc, the expression of HrpV caused negative regulation through its interaction with HrpS [316], and the overall expression of sfGFP decreased. Once aTc reached between 6.25 and 12.5 nM, HrpV was likely to reach saturation, leading to significant repression of the overall sfGFP level (Figure 4.5). Overall, this negative regulation system can be controlled by the concentration of aTc (<12.5 nM), stabilising the overall gene expression.

To verify the performance of this negatively regulated amplifier system as a robust operational amplifier, the sensing circuits were subjected to several conditional changes including temperature, nutrient level, oxygen availability and pH (Appendix Figure B-10 and Figure B-11). The results demonstrated that in the presence of the negative feedback module (under the induction of 3 nM of aTc to allow the expression of HrpV) the $P_{VRecA-AT}$ sensing circuit exhibited system stability and robustness against environment fluctuations by maintaining the same level of induction fold at steady-state (16 hrs post-induction) (Figure 4.5 D). However, there was a significant decrease in the relative fold change and the operational range of the system, this could be due to the high affinity between the HrpRS complex and HrpV [316]. Future optimisation can be done using a lower affinity HrpV mutant or decreasing the half-lives of HrpV. The increased robustness demonstrated the potential of our $P_{VRecA-AT}$ sensing system with an additional feedback module for *in-situ* applications, where exposure to the real-world environment or gut environments can make it challenging for synthetic circuits to perform as predicted.

Overall, I described an ultrasensitive DNA damage sensor equipped with a functional genetic operational amplifier that combines a chimeric promoter as the noise canceller, a HrpRS-based construct as the signal amplifier and a P_{tetO} negative feedback system.

4.3.5 Optogenetic feature of SOS gene circuit in response to UV light(280~365nm)

To further demonstrate the optimised UV inducible gene-circuits (P_{VRecA_AT}) in the context of spatial control. As a proof-of-concept, *E. coli* Nissle 1917 loaded with P_{VRecA_AT} and P_{VRecA_AT} *Jungle_Down* system formed colonies on LB plates, which were activated by the 302 nm UV light to express sfGFP (Figure 4.6). The fluorescence of sfGFP-expressing colonies was imaged after UV exposure for 10 and 20 s respectively, following 4 hrs incubation at 37 °C. The sfGFP expression under chimeric P_{VRecA_AT} *Jungle_Down* promoter displayed a lower baseline but less sensitivity compared to that of the P_{VRecA_AT} system (Figure 4.6 A), the results from solid biofilm was consistent with the experiments done in bacterial liquid culture condition shown in Figure 4.2 and Figure 4.3. Interestingly, a ring-like structure was observed (Figure 4.6), this could suggest SOS system being induced to express sfGFP in newly grown cells more readily than the stationary cell in the centre.

To investigate the temporal dynamics and stability of the DNA damage sensor, I tested the transformed (P_{VRecA_AT}) system using a Chi. Bio turbidostatic platform [305], which can maintain a constant culture OD while measuring fluorescence and inducing DNA damage with 280 nm UV radiation. Two variants were tested (P_{VRecA_AT} with either sfGFP or sfGFP attached with *ssrA* fast-degradation tag), with each subjected to a UV dosing regime in which radiation dose intensity was doubled every 5 hrs (Figure 4.7). For the sfGFP variant, it was observed a log-linear relationship between UV dose and sfGFP expression, with the system demonstrated a reliable response over a

128-fold increase in induction level (Figure 4.7). In each cycle, a return-to-baseline took approximately 5 hrs which was potentially due to the time necessary for GFP dilution during growth. The sfGFP-ssrA (LVA- BBa_K1399001) construct (in which the fluorescent marker is degraded on a faster timescale than cellular growth) supported this hypothesis, demonstrating that the optimised $P_{VRecA-AT}$ reporter system can be used to generate short (~1 hr) pulses of gene expression in response to UV induction. The plasmids of both circuits were sequenced to check for any mutation after 40 hrs, the results indicated no mutation occurred on the plasmid level. (Appendix Figure B-13).

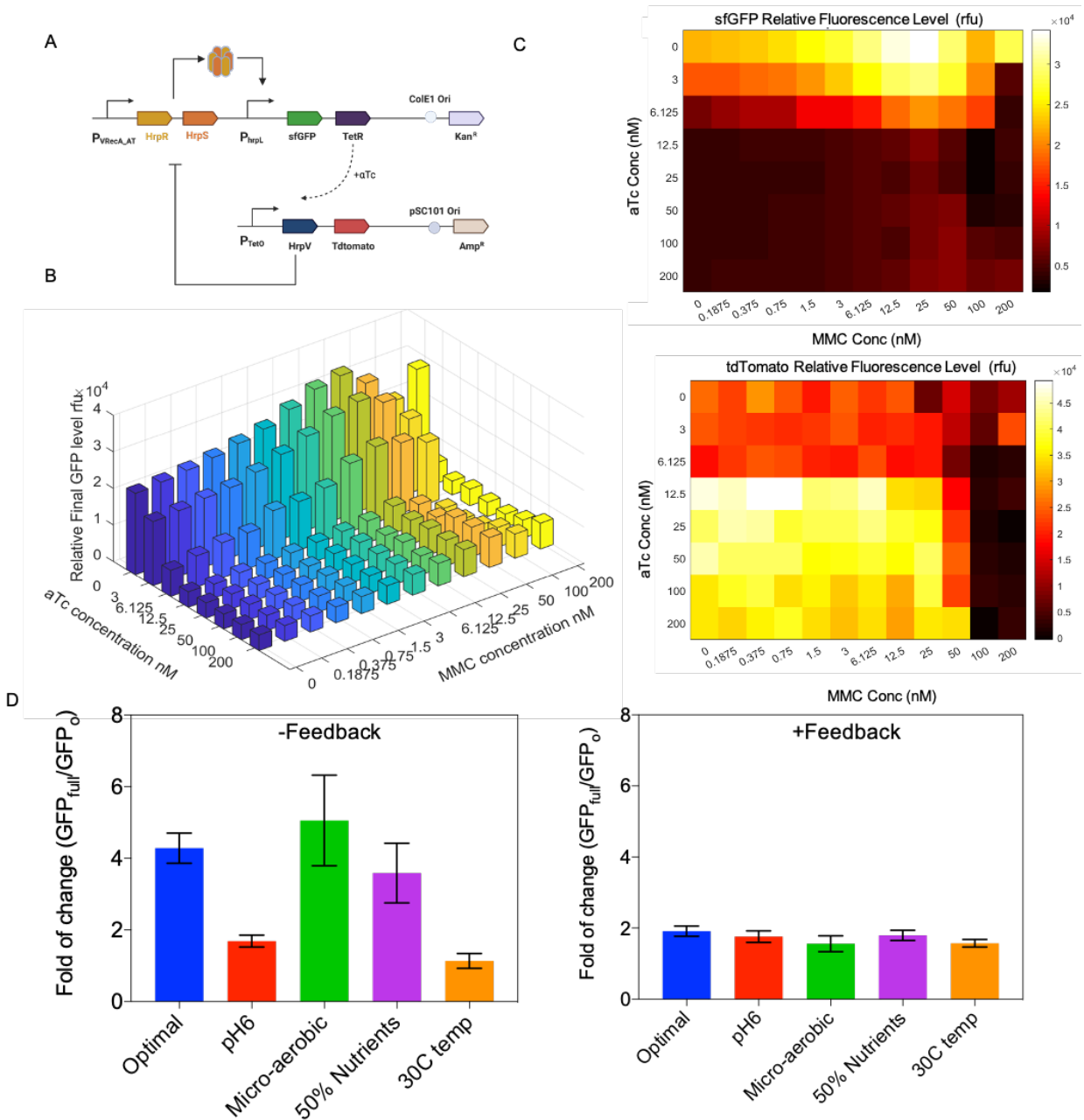


Figure 4.5. Performance of $P_{VRecA-AT}$ promoter with amplifier and negative feedback loop.

(A) Schematic diagram of the genetic operational amplifier with a negative feedback loop. A plasmid consists of the optimised $P_{VRecA-AT}$ promoter fused with *hrpRS*, *sfGFP* gene and *tetR*; Another plasmid consists of a negative regulator P_{HrpL} and Tdtomatoes reporter. (B) Expression profile under the induction of both aTc gradient (0 to 200 nM) and MMC gradient (0 to 200 nM). (C) Heatmap of *sfGFP* and Tdtomatoes expression corresponding to different concentrations of inducers. (D) Comparison of the fold change of $P_{VRecA-AT}$ amplifier gene-circuits with and without the negative feedback loop. The steady-state of aTc = 3 nM and MMC = 50 nM at the end of 16 hrs time course were used to calculate, optimal condition indicates as pH7, oxygen available, 37 °C, M9 supplemented with 2% w/v glucose. pH6 was calibrated using HCl titration, the micro-

oxygen environment was created using oil sealing over each well. 50% nutrient indicated 1% w/v glucose was supplemented instead of 2% w/v used in optimal condition. The standard deviation and mean value of three bio-replicates were plotted.

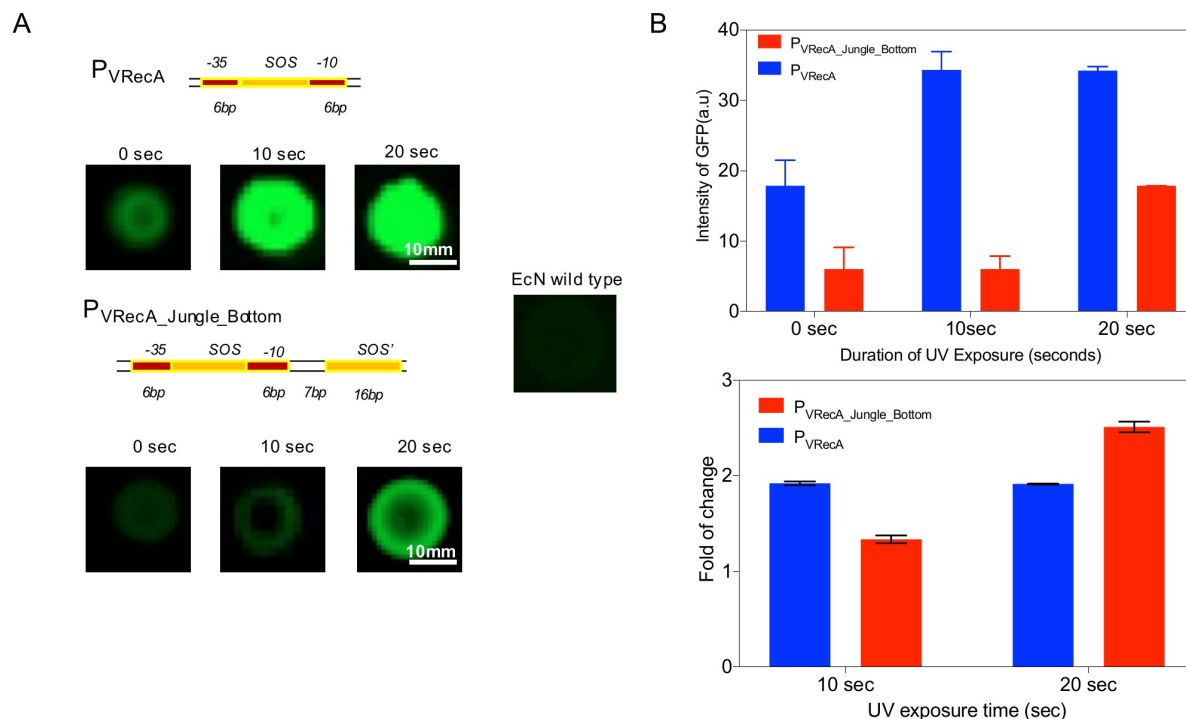


Figure 4.6. System performance as optogenetics module & biofilm engineering.

Optogenetic induction using macro-colonies. A) Top: Images of EcN colony transformed with optimised P_{VRecA}_sfGFP circuits followed by exposure to 302 nm UV for 10 sec, 20 sec, respectively. Bottom: Images of EcN colony transformed with optimised P_{VRecA_Jungle_Down}_sfGFP (Jungle_Bottom) following the same procedures. Images were taken under channel FITC. Intensity inverted for better visualization and pseudo colour applied. B) Calculated mean intensity of green fluorescence (T = 4 hrs) were normalised against wild type control. Both images were processed using the same setting using ImageJ. N=3

The sfGFP induction kinetics was then analysed using a microplate reader to assess circuit behaviour in a different physiological context. In Appendix Figure B-12, upon system induction with 2 sec of UV exposure, EcN transformed with sfGFP-ssrA was able to generate a short GFP pulse with fast degradation, 2.5 hrs are required for the emerging peak to return to ground state. In comparison, the original sfGFP showed similar pulse behaviour but did not return to ground zero

within 16 hrs. Collectively, the $P_{VRecA-AT}$ promoter induction in *E. coli* Nissle 1917 can be triggered within < 2 hrs and the duration of the downstream expression is between 2.5 – 5 hrs and the strength of SOS expression can be adapted to reach significantly higher levels after intermittent stimulation and continuous cultivation.

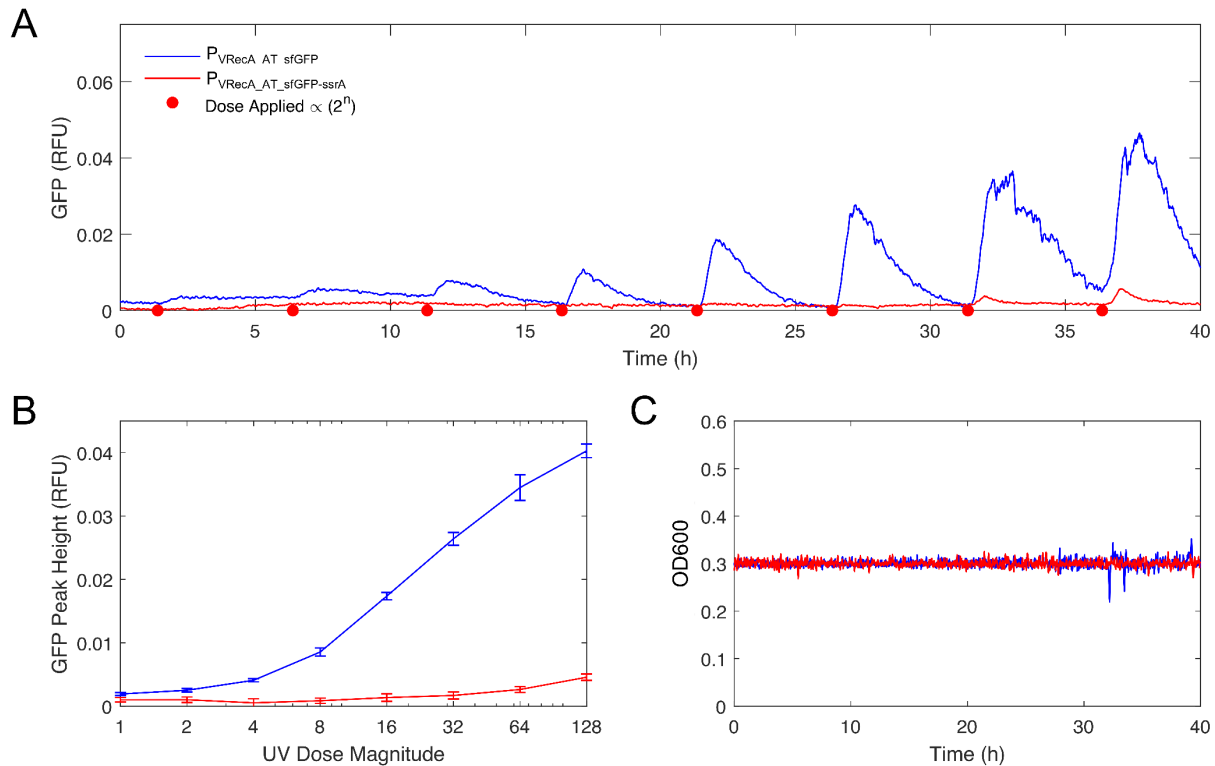


Figure 4.7. Turbidostats experiment for checking long term stability.

Adaptation of *E. coli* Nissle 1917 with $P_{VRecA-AT_sfGFP}$ and $P_{VRecA-AT_sfGFP-ssrA}$ in turbidostats over 40 hrs with interval UV exposure. (A) Dynamics of sfGFP expression under periodic UV exposure. Every 5-hrs is a cycle. The first UV dose was 10 mW 280 nm for 2.4 seconds and the UV doses doubled in the next cycle. Small fluorescence expression was observed for $P_{VRecA-AT_sfGFP-ssrA}$ as rapid degradation of the fluorescent protein when coupled with the ssrA tag. (B) Relationship between applied UV dose and fluorescence peak height. Error bars are the standard deviation of ten reads in fluorescence taken in the 10 minutes window entered on each peak. Each experiment has four replicates. (C) OD was maintained constant in the turbidostats during the experiment.

4.3.6 Application of SOS biosensors for gut toxin and antibiotics

Out of the optimised toolbox, I applied the optimised SOS sensing system $P_{VRecA-AT}$ that displayed the highest fold of change and fastest responding time towards different DNA damaging compounds. One application is the *in-situ* detection of genotoxic biomarkers that are associated with colon cancer; which includes bile salt derivatives: deoxycholate, taurocholic acid [293][317], and other DNA damaging compounds related to diet such as acetaldehyde [294]. As shown in Figure 4.8, the DNA damaging sensing circuit of $P_{VRecA-AT}$ can detect genotoxic effects of 0.5 mM deoxycholate, which is a common secondary bile salt derivate metabolised by gut microbiome [318]. The normal gut concentration of deoxycholate ranges from 450~700 μ M [319], high concentrations of deoxycholate have been correlated with colon cancer and could induce DNA breakage [318]. Furthermore, the sensing system demonstrated a detection threshold of 9 mM taurocholic acid, which is within the range of estimated local concentration in the gut [320] [292]. This is the first report to directly quantify taurine conjugated bile salt using a whole-cell bacteria biosensor. It has been demonstrated that acetaldehyde [321] ($> 450 \mu$ M) is also detectable using $P_{VRecA-AT}$ sensing system (Figure 4.8 C). It has been shown that bile salts are able to damage the membrane and require the bacterial membrane transport system to get into cells [322], which might explain the delay of response in deoxycholate, taurocholic acid (Figure 4.8 A & B). Overall, by using the gene circuit of SOS sensing system loaded by probiotic-based *E. coli* Nissle 1917 strains, a broad spectrum of human health-related carcinogenic can be detected. Our approach has clear merits compared to existing methods, which often rely on animal testing [323] and costly mammalian cell-based assays such as AMEs test [324] or UMU assay [325].

To compare the strength of genotoxic effect of deoxycholate, taurocholic acid and acetaldehyde, the induction kinetics of mitomycin C was also examined, which is a widely used

chemotherapeutic drug that causes cancer-specific DNA cross-link during cell replication [326]–[328]. A dosage guideline for mitomycin C was provided in Appendix Table B-3, where the concentration can be varied between 12 μM to 24 μM depending on the target tumours. Within the testing concentrations of these carcinogenic compounds, there was no impact on the cell growth of *E. coli* Nissle 1917 with sensing plasmid $P_{V\text{RecA-AT}}\text{-sfGFP}$ (Appendix Figure B-14) except the case when deoxycholate was used as an inducer, it was evident that *E. coli* cells could break down deoxycholate and used as a carbon source [329].

To further extend the application spectrum in food security, I have also tested several fluoroquinolones derived antibiotics (Levofloxacin, Enrofloxacin and Ciprofloxacin) which represent some of the most commonly prescribed antibiotics for bacterial infections [330]. Despite their efficacy and high penetrability, these antibiotics show severe carcinogenic effects on mammalian cells, including irreversible nerve damage and tendon rupture [331][332]. Although the prescription of fluoroquinolones to human patients has been warned against by the FDA [333], fluoroquinolones are still commonly used in animal feedstock which poses an enormous risk in food security [334].

Existing methods to detect the presence of fluoroquinolone commonly involved micellar electrokinetic chromatography (MEKC) [335], high-performance liquid chromatography (HPLC) [336] and mass spectrometry (MS) [337]. These instruments lack portability and are costly. In contrast, the optimised $P_{V\text{RecA-AT}}$ sensing system could detect fluoroquinolone down to 0.97 $\mu\text{g/L}$, which is far below the usage limit of fluoroquinolones for animal feedstock (1.5 - 15 mg/L) [338] (Figure 4.9). Similarly, metronidazole is another type of antibiotic that is also heavily used in animal feedstock and veterinary treatment. However, the $P_{V\text{RecA-AT}}$ sensing system showed no response, which might be due to the fact that the cytotoxicity of metronidazole only arises

following reduction by the liver [339]. The OD graph (Appendix Figure B-15) confirms our suggestion that metronidazole does not affect cell growth, but all three other antibiotics were found to stall cell growth at a concentration higher than 125 $\mu\text{g/L}$.

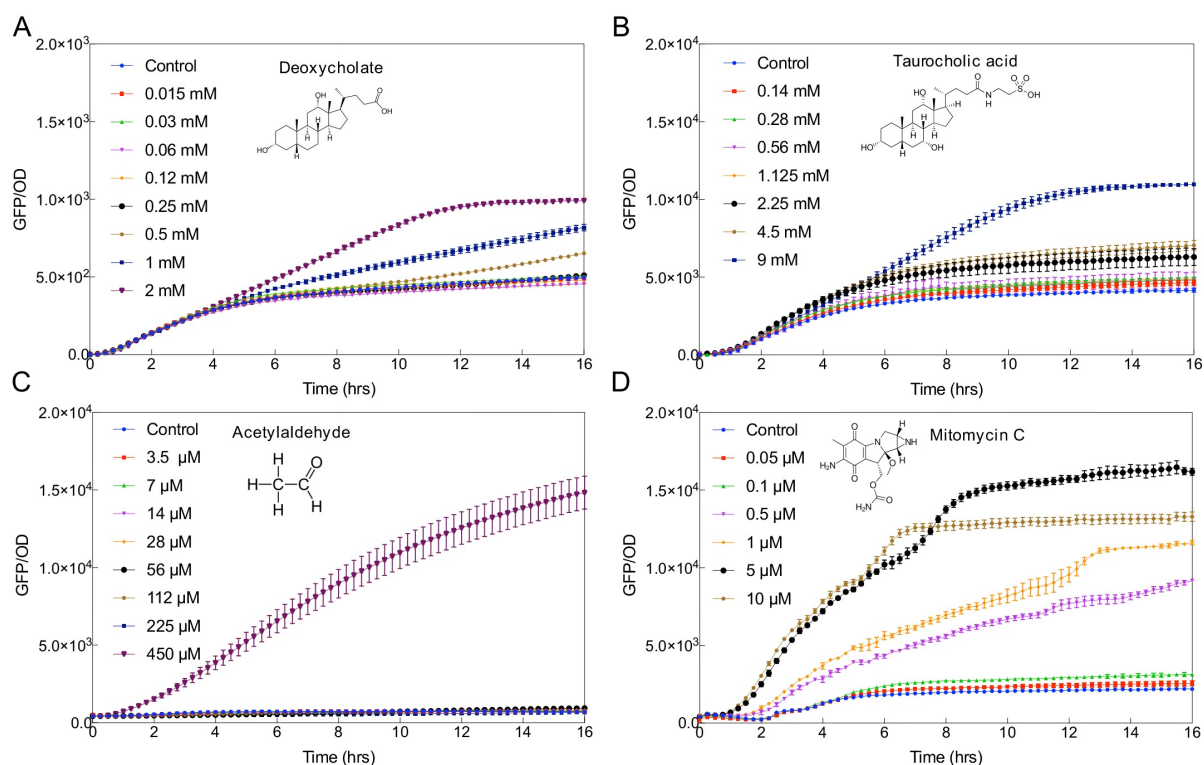


Figure 4.8. Application of $P_{VRecA-AT}$ -sfGFP for the detection of bile salt derived carcinogens:

Transformed EcN with $P_{VRecA-AT}$ -sfGFP to detect human diet-related carcinogenic compounds. Bile salt derived (A) Deoxycholate and (B) Taurocholic acid, alcohol derived (C) Acetylaldehyde and well-established chemotherapeutic agents (D) Mitomycin C. The standard deviation and mean value of three bio-replicates were plotted in each graph.

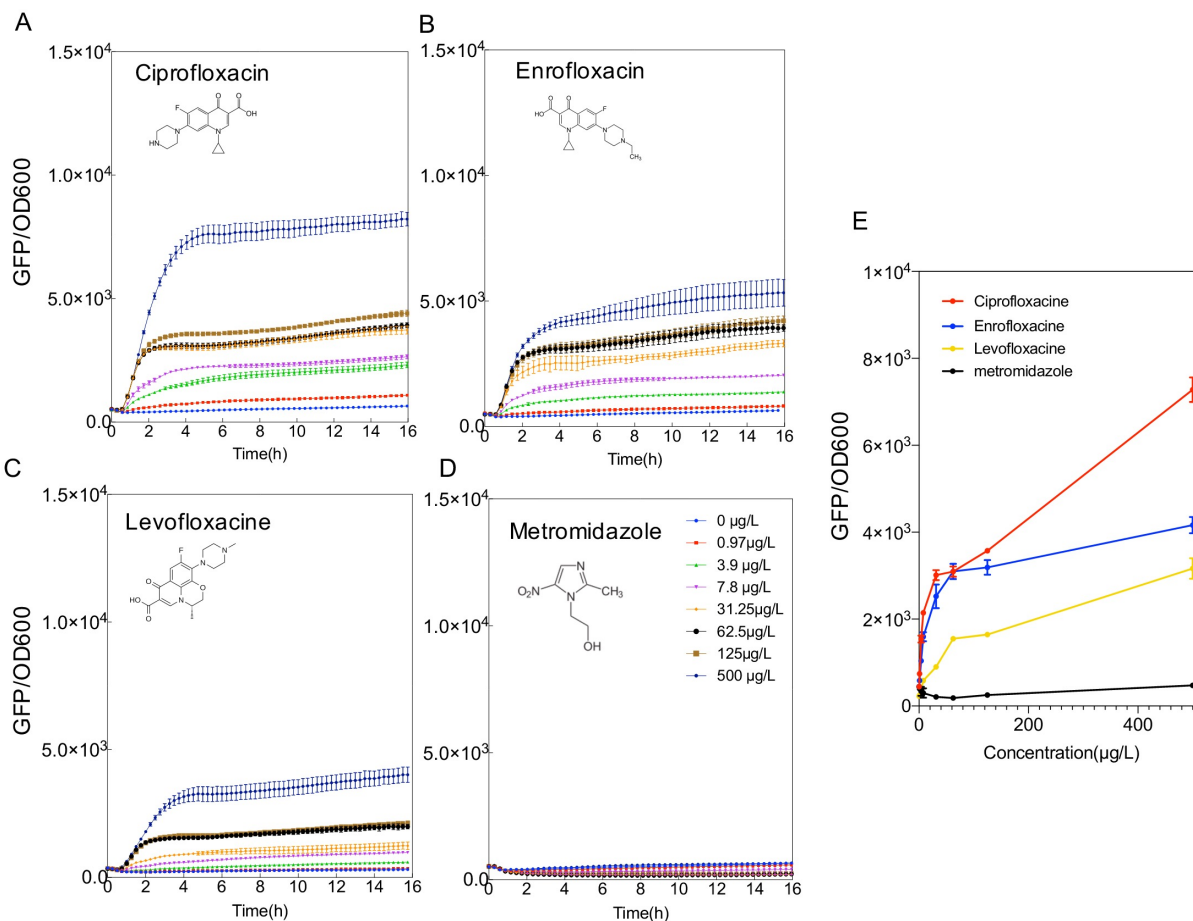


Figure 4.9. Application of $P_{VRecA-AT}$ -sfGFP for the detection of antibiotics derived carcinogens:

Transformed EcN with $P_{VRecA-AT}$ -sfGFP to detect carcinogenic antibiotics used in the animal feedstock. (A) Ciprofloxacin, (B) Enrofloxacin, (C) Levofloxacin and (D) Metronidazole. (E) Dosage responses of different antibiotics. The standard deviation and mean value of three bio-replicates were plotted in each graph.

4.4 Discussion

In this chapter, I analysed the *recA* promoter in *V. natriegens*, identifying the σ binding sites (-35 and -10) and SOS box in order to develop an ultra-sensitive, rapid and robust DNA damage sensor. Although there is no direct evidence to support a correlation between SOS response time and bacteria growth rate, several studies reported links between SOS and DNA replication [340], modulating growth pattern [295] and growth fitness [300]. It would be ideal for testing the optimised circuit in its native context of *V. natriegens* and a Δ *lexA* variant *E. coli* to further investigate the correlation between replication speed and SOS response time.

In this chapter, it was found that P_{VRecA} from fast-growing *V. natriegens* has higher sensitivity and broader dynamic range than *E. coli* P_{ERecA} , I then engineered the P_{VRecA} promoter through a rational and systematic approach to reduce its background noise and amplify its sensitivity. Compared to the conventional *ad hoc* method, our approach provided an example of bottom-up engineering.

Based on the structure of the LexA protein and its quantified interaction with the *recA* promoter [341][342], I identified the precise location of the SOS box and optimised the AT composition of the SOS box to create a $P_{VRecA-AT}$ promoter with low leakiness and broader dynamic response. This engineering process also showed that the SOS box is a modifiable biological part with high flexibility that could be fitted into the synthetic gene circuit design process [343].

An ideal synthetic gene circuit should have the capacity to withstand stochastic and environmental perturbations while maintaining a high signal-to-noise ratio. To improve the robustness of the circuits, I employed a design motif from the previously reported Jungle Express system [282], which could incorporate our optimal SOS sequence. Meanwhile, by using a HrpRS based genetic amplifier system, I managed to increase the detection sensitivity and signal amplitude significantly with a minor increase of the system response time. To improve the stability

of our optimised $P_{V_{RecA}}$ sensing circuit, a negative feedback system was incorporated to achieve a more predictable operation upon changes in the environment. Nevertheless, low-level expression of HrpV reduced the system induction fold, therefore, the future improvements could include the addition of a fast degradation *ssrA* tag after the *hrpV* gene to alleviate the inhibition. Together, I proved that our DNA damage sensing circuit confers robustness and predicted function in response to changes in temperature, nutrient level, oxygen availability and pH. In essence, I engineered a functional SOS responsive operational amplifier by combining a noise canceller, a signal amplifier and a negative feedback system within a synthetic gene circuit. One merit of this DNA damage sensing system is its potential to perform under real-world stochastic environments such as the human gut, another merit of using irradiation over molecular triggers as it is not restricted by diffusion limitations. The detailed annotations of the promoter provide the engineering room for creating user-defined dynamics and potential chimeric promoter, these features allow such circuits to be used in a plug-and-play fashion.

It was demonstrated that the DNA damage sensing system could be used as an optogenetic actuator through the induction of UV light. In the experiment where I exposed the macro-colonies transformed with our DNA damage sensing system with UV light, the results showed that a 10-sec temporal difference could alter the phenotype of the macro-colonies. As a proof-of-concept, the sensing system can be extended as a valuable tool to study signalling dynamics, generation of mutation and acquisition of antibiotic resistance within a biofilm [344]–[345]. For instance, using the system to monitor the UV damage and its corresponding biofilm formation. Despite the system activation being different to canonical optogenetic systems such as the Cph8/OmpR system and BphP1[346] which commonly involve a light-sensing domain, I proved that our ultra-sensitive DNA damage sensing system is adaptable and functional upon UV induction without altering the

growth of the bacterial host. For long-term stability, although the UV dose of P_{VRecA-AT}-sfGFP gene circuit was increased 128-fold in 40 hrs, no mutation on the plasmid P_{VRecA-AT}-sfGFP and P_{VRecA-AT}-sfGFP-ssrA was found during the entire course of the experiment, according to DNA sequencing. It has been reported that bacterial gene regulation systems can be adapted in response to the environment without DNA mutation [347]. However, a whole-genome sequence would be necessary to check any chromosome changes after prolonged exposure. Similarly, transcriptomics study could be carried out to determine how the host SOS machinery is regulated to cope with the gradual increment of UV exposure. Nevertheless, since the sensing system uses transcriptional machinery to detect UV light, the operational timescale could be longer than translational sensors such as a riboswitch [124][348] or two components system with membrane receptors [124].

Finally, I demonstrated the potential of this optimised P_{VRecA-AT} promoter for its real-world deployment. The key application of this sensing system is to detect carcinogens, such as genotoxic compounds remaining in agricultural or dairy products and secondary metabolites generated by the human microbiome. Although it is difficult to distinguish the specific type of carcinogenic compounds, it can still be used as a broad-spectrum indicator. The DNA damage sensing system can potentially be further developed into a Point of Care (PoC) detection device or used as a programmable input for living medicine. Compared to currently existing tools for carcinogen detection such as the Comet Assay [349] and the Ames (Bacterial reverse mutation) test [350], the optimised sensing system offers a cost-effective, fast and easy-to-operate solution for such measurements [349].

Chapter 5 Disease marker control: NorR based nitric oxide sensor

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In the previous chapters, I developed two transcriptional systems that can be triggered via either an ingestible drug molecule or a spatial DNA damage cue. In this chapter, nitric oxide (NO) responsive circuits were constructed based on the NorR and its regulating promoter P_{NorV} isolated from the *norVW* operon of *E. coli* Nissle 1917. Nitric oxide (NO) is a disease biomarker molecule that is often found in many chronic inflammatory bowel diseases [9] and tumour progression [82] with elevated concentration. An ideal nitric oxide sensor would not only allow *in-situ* diagnosis and condition-triggered drug release but also can be used to decipher the precise dynamics within host-microbiome interaction. Through adopting a similar combinatorial tuning approach as demonstrated in the previous chapter, the optimised NorR sensor demonstrated a broad dynamic

range and sensitive detection of 15 μM of an NO donor DETA/NO. Certain host interference associated with this σ^{54} dependent system were also investigated and solved using mini SimCells. The results of this chapter have been submitted to ACS synthetic biology and are currently under review.

5.1 Introduction

Nitric oxide (NO) is a water-soluble (solubility 1.87 mM at 20 °C) [351], free radical gaseous molecule that plays an essential role in human physiological functions and diseases [352]. An increased level of nitric oxide is often detected in many chronic inflammatory bowel diseases [9] and tumour progression [82]. It also plays an essential role in host signalling between the gut microbiome and immune system [353]. Conversely, it exhibits protective effects in the early development of various arterial diseases. [354]. Because of its prevalence and important biological role, a nitric oxide sensor would have the potential for *in-situ* disease diagnosis and condition-triggered drug release. It could also assist to further decipher the precise dynamics within host-microbiome interaction and enable *in vivo* tracking of disease progression. Among existing transcriptional nitric oxide sensing pathways such as *soxR*, *oxyR*, *fur* and FNR [355][356], P_{NorV} and its NorR regulator in the *norVW* operon system has been reported to show the highest sensitivity ($\sim\text{nM}$), specificity and degree of change in response to nitric oxide. [357][358]. Memory circuits have been developed based on the NorR regulatory system to record gut inflammation in mouse model [84]. These circuits demonstrated a sensing range of 30~100 μM in response to nitric oxide donor DETA/NO (diethylenetriamine/nitric oxide) and functionally validated in inflamed mouse ileum explants [84].

Although a plethora of studies characterised the activation mechanism and the effects of NorR binding site modification [359][360], few investigations focus on rational gene-circuit

designs, the effect of NorR expression level and integration host factor (IHF) binding site of the P_{NorV} promoter. In this study, I characterised how the expression level of NorR can affect the sensory response, followed by fine-tuning the dynamic range through promoter and feedback loop design. Based on the experimental data, a similar competitive binding mechanism developed previously was used to explain the NorR transcriptional regulation and simulate the NO sensing performance. The redesigned NorR gene circuit is able to detect NO in a medical relevant range of 26 nM ~ 13.5 μ M, which was converted based on its operational range of 15 μ M ~ 500 μ M DETA/NO. Furthermore, by examining and challenging the system with various nutrient conditions (different carbon and nitrogen source), strong nitrogen dependent host interference and memory behaviour was observed. I also demonstrated that the host interference can be bypassed upon loading into chromosome-free mini SimCells, with improved robustness, sensitivity and safety.

5.2 Material and methods

Table 5.1 Strains used in Chapter 5.

Strains	Genotype	Ref
<i>E. coli</i> K12 Δ norR	<i>F'</i> proA+B+ lacIq Δ (lacZ)M15 zzf::Tn10(TetR)/fhuA2 glnV Δ (lac-proAB) thi-1 Δ (hsdS-mcrB)5 Δ norR	-
<i>E. coli</i> Nissle 1917	Ardeypharm GmbH	-
<i>E. coli</i> DH5 α	fhuA2 Δ (argF-lacZ) U169 phoA glnV44 Φ 80 Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	NEB
<i>E. coli</i> MC1000 Δ minD	F- λ - Δ (ara-leu)7697 [araD139]B/r Δ (codB-lacI)3 galK16 galE15 e14- mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r-m+) Δ miniD:kanR	[198]

Table 5.2. Primers used in Chapter 5.

Primers	Sequence 5'→3'	Template
IHFNative_fwd	GCCGTCAAGGCCGCATTAAATAGCGTGGTGATC AGGCC	pGEMT_P _{NorV_β} _sf GFP_norR
IHFNative_rev	AGTTCTTCTCCTTTACGCATCTAGTATTTCTCC TCTTTCGGTACC	
pnorV-sfGFP-NorR- fwd	ATGCGTAAAGGAGAAGAAC	
pnorV-sfGFP-NorR-rev	TTTAATGCGGCCTTGACG	
DeletionIHF-fwd	GCCGTCAAGGCCGCATTAAATAGCGTGGTGATC AGGCC	pGEMT_P _{NorV_null} _sf sfGFP_norR
DeletionIHF-rev	GTTCTTCTCCTTTACGCATCCTAGTATTTCTCC TCTTTCGGTACC	
pnorV-wt-sfGFP-fwd	GATGCGTAAAGGAGAAGAAC	
pnorV-wt-sfGFP-rev	TTTAATGCGGCCTTGACG	

Table 5.3 Plasmids used in Chapter 5.

Short abbreviation	Plasmids	Description	Ori
pPnorV	pGEMT_P _{NorV} _sfGFP_norR	PnorV_sfGFP_norR with double IHFs site promoter <i>pnorV_alpha</i>	ColE1
pPnorV β	pGEMT_P _{NorV_β} _sfGFP_norR	PnorV_sfGFP_norR with single IHF site promoter <i>pnorV_beta</i>	ColE1
pPnorVnull	pGEMT_P _{NorV_null} _sfGFP_norR	PnorV_sfGFP_norR with deleted IHFs site promoter <i>pnorV_null</i>	ColE1
pProA	pGEMT_P _{roA} _norR_PnorV_sfGFP	<i>norR</i> orf under control of constitutive promoter <i>proA</i> , no feedback.	ColE1
pProC	pGEMT_P _{roC} _norR_PnorV_sfGFP	<i>norR</i> orf under control of constitutive promoter <i>proC</i> , no feedback.	ColE1
pProD	pGEMT_P _{roD} _norR_PnorV_sfGFP	<i>norR</i> orf under control of constitutive promoter <i>proD</i> , no feedback.	ColE1

Chemical & Medium recipe

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Nitric oxide inducer is purchased under CAS number 146724-94-9. Full chemical name: Diethylenetriamine/nitric oxide. The same antibiotics working concentration was used as in Chapter 3 and Chapter 4. For making M9 medium and PBS medium, CSH protocol was used (<http://cshprotocols.cshlp.org/>):

M9 medium:

Reagent	Amount to add (for 100 mL M9 x1)
M9 salts (5 x)	20 mL
MgSO ₄ (1 M) ^a	200 uL
CaCl ₂ (1 M) ^a	10 uL
H ₂ O	78 mL

^aAutoclave and store at room temperature

PBS medium:

Reagent	Amount to add (for 1000 mL PBS x1)
NaCl	8 g
KCl	200 mg
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	245 mg
H ₂ O	1000 g

Gene circuits synthesis & cloning

All protocols were carried out with enzymes obtained from New England Biolabs (NEBs), and all primers were synthesised by Sigma-Aldrich. Standardised backbone vector pGEMT (Promega) was used for all constructs with ColE1 Ori (15~20 copy/cell). A strong ribosome binding site (AAAGAGGAGAAA) (BBa_B0030) was used for both *sfGFP* orf and *norR* orf. A strong bidirectional terminator (BBa_B0020) was attached after the stop codon for insulation. All plasmids in Table 5.1 were designed and then synthesised using the GeneArt™ cloning service.

For IHF deletion of the P_{NorV} promoter, primers designed are shown in Table 5.2 and standard Gibson cloning was carried out using NEB Hi-Fi assembly kit. Original *norR* sequence and P_{NorV} sequence was retrieved from *E. coli* Nissle 1917 (GenBank No. CP007799.1). Sequence info and plasmids map are provided in Appendix info C.

To generate the *norR* mutant *E. coli* strains, molecular cloning was carried out as follows. P1 phage transduction method was used to generate *E. coli* MC1061 *norR* knockout strain, and the *ΔnorR* strain from Keio collection was used as a donor for the gene knockout cassette. Kanamycin resistance marker was then excised from the host-strain chromosome using the method described previously [361]. Briefly, transduced strains were made electrocompetent and transformed with the plasmid, pCP20. Transformants were grown overnight at 30 °C in Luria Bertani (LB) medium supplemented with 100 µg/mL ampicillin. The resulting cultures were plated on LB agar medium and grown at 42 °C. Individual colonies were tested for ampicillin and kanamycin sensitivity. The gene deletions and subsequent kanamycin marker removal were verified by the polymerase chain reaction

Nitric oxide induction and time kinetics experiment

Transformed EcN was first inoculated in an LB medium with supplemented antibiotics (Kanamycin 60 µg/ml; Carbenicillin 100 µg/ml) for 16 hrs 37 °C 250 rpm. An OD₆₀₀ of 0.1 was used for initial induction. This was done by diluting the overnight culture roughly 40 times and (5 µl) was added to a total of 200 µl plate reader induction volume. PCR film was applied to avoid gaseous NO escape from the induction wells. For kinetic reads, TeCan Spark® was used to take GFP measurement at a 15 mins interval over the course of 16 hrs with temperature maintained at 37 °C. 10 secs shaking was set to avoid any clump formation before each read. Optical density at

600 nm was monitored as reads for bacterial growth. The results were analysed and plotted using Prism® 9.1 software.

Medium shifting experiment investigating cellular memory

Single fresh colony of transformed EcN was picked and inoculated in LB or M9 supplemented with 2% w/v Glycerol for 16 hrs 37 °C 250 rpm. Next, the OD600 measurement was used to calibrate to ensure an equal number of cells (OD600 ~0.1) were seeded for the next stage. Cells were washed with PBS and diluted 100x to ensure no medium carried over during the induction stage. Saturated inducer concentration (Aspirin 100 μ M. Mitomycin C 500 nM and DETA/NO 1 mM) was used to induce each corresponding circuit. P_{Sal} -gfp-salR, P_{VrecA_AT} -sfGFP and $P_{NorV\beta}$ -sfGFP-norR were used for this experiment. The same plate reader setup was used as shown previously.

Lyophilization of mini SimCells and revival

Lyophilization (Freeze dry) protocol for preparation and revival are shown in Figure 5.8. Freshly transformed cells (EcN) were grown under the optimal condition (LB, 37 °C 200 rpm) until it reaches an OD600 of 0.4~0.5. Next, the cells were washed with PBS medium three times under 1500 rcf for 10 mins to remove any growth medium. Cell pellets were resuspended with an equal volume of protection buffer (recipe is shown below). The resuspended cells were then aliquoted into a 1.5 ml Eppendorf tube and freeze in the -80 °C fridge overnight. Martin Christ ® Alpha 1-4 LSC basic freeze drier was used for the lyophilization process, the sample holder and inner chamber were prechilled to -20 °C to prevent any thawing of the bacterial samples. Upon fully sealing the vacuum chamber, 0.1 mPa vacuum was applied for 12 hrs until all samples dried

and displayed a light-yellow colour. For bacteria revival, 1 ml of SOC medium was added to the cell powder and incubated for 15 mins at 37 °C. Next, this was carried for plate reader induction.

For purified mini SimCells, PBS medium was used instead.

Protection buffer

Reagent	Total amount (100 ml)
Bovine Serum albumin powder	5g
Sucrose	6g
Trehalose	6g
Dextran	1.5g
Deionised water	-

5.3 Results

5.3.1 Competitive binding mechanism of NorR regulated gene circuits

P_{norV} promoter is a σ^{54} dependent promoter, regulated by NorR and induced by nitric oxide (NO). Unlike the classic transcription activation in the σ^{70} class, σ^{54} regulated class often formed a stable complex upon binding and is transcriptionally inert [357]. It requires activated regulator NorR and ATP hydrolysis to trigger subsequent conformation change and initiate transcription. In Figure 5.1A, NorR forms a homo-hexamer ring where N terminus binds the upstream bacterial enhancer binding site (bEBs) and the C terminus interacts with the σ^{54} holoenzyme. Notably, DNA bending ($\sim 160^\circ$) was achieved by the binding of two integration host factors (IHFs) that enabling close contact between the NorR hexamer and σ^{54} factors. In its off state, the central AAA+ domain which confers the ATPase activity of NorR is silenced (Figure 5.1B), upon nitric oxide binding with the non-haem iron centre of the GAF domain, AAA+ domain is exposed to allow further ATP hydrolysis and transcriptional activation [362][363]. The NorR regulator (100% similarity) and its corresponding promoter P_{NorV} located in the *norVW* operon is highly similar between EcN and conventional lab strains *E. coli* K12 (98% similarity) (Appendix Figure C-1). The NorR circuits were initially designed in a simple regulatory and open-loop format (Figure 5.2A), which P_{NorV} promoter was connected with superfolder *gfp*, the regulator gene *norR* cloned from EcN was placed under the control of constitutive promoters with different strength (P_{roA} , P_{roC} and P_{roD}) [262] (Figure 5.2A). Due to the gaseous nature of nitric oxide, a well characterised nitric oxide releasing donor diethylenetriamine/nitric oxide (DETA/NO) was used as an inducer [364]. The gene circuits were activated by NO released from DETA/NO in the range of $1.5 \mu\text{M} \sim 0.5 \text{ mM}$ DETA/NO and a minimal detection limit of $1.5 \mu\text{M}$ was observed (Figure 5.2B).

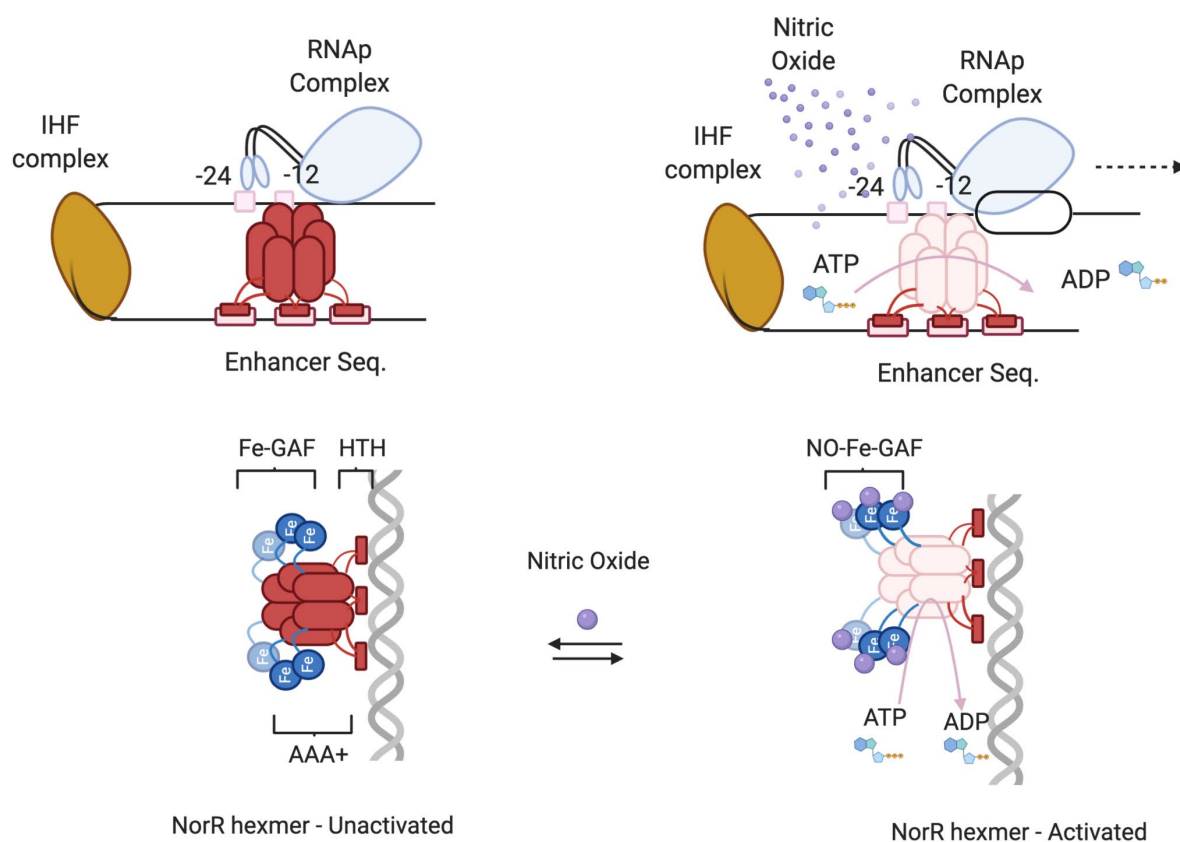


Figure 5.1. Molecular mechanism of NorR mediated σ^{54} dependent transcriptional activation.

A) P_{NorV} promoter in its off state. Integration host factors (Yellow) bends the DNA with a near 160° sharp angle to allow NorR (Red) homohexamer contact with σ^{54} factor at its binding site (-24) & (-12). NorR binding with upstream enhancer sequence is showed in the pink block. In the absence of an inducer, RNAP complex is closed from transcription. Upon nitric oxide binding, NorR exposes its AAA+ ATPase domain and activates σ^{54} into its active form for DNA opening and transcriptional initiation. B) The way NorR interacts with nitric oxides is through its non-haem iron centre (Blue), which signals a reversible conformation change that opens up the ATPase domain upon binding.

As demonstrated in Figure 5.2B, a strong constitutive promoter strength and high level of NorR expression led to a decreased amplitude of the induced sfGFP expression (Figure 5.2B). The strongest promoter (P_{roD}) produced the lowest induction amplitude in comparison with the middle strength promoter (P_{roC}) and the weak (P_{roA}) promoter (Figure 5.2B and C).

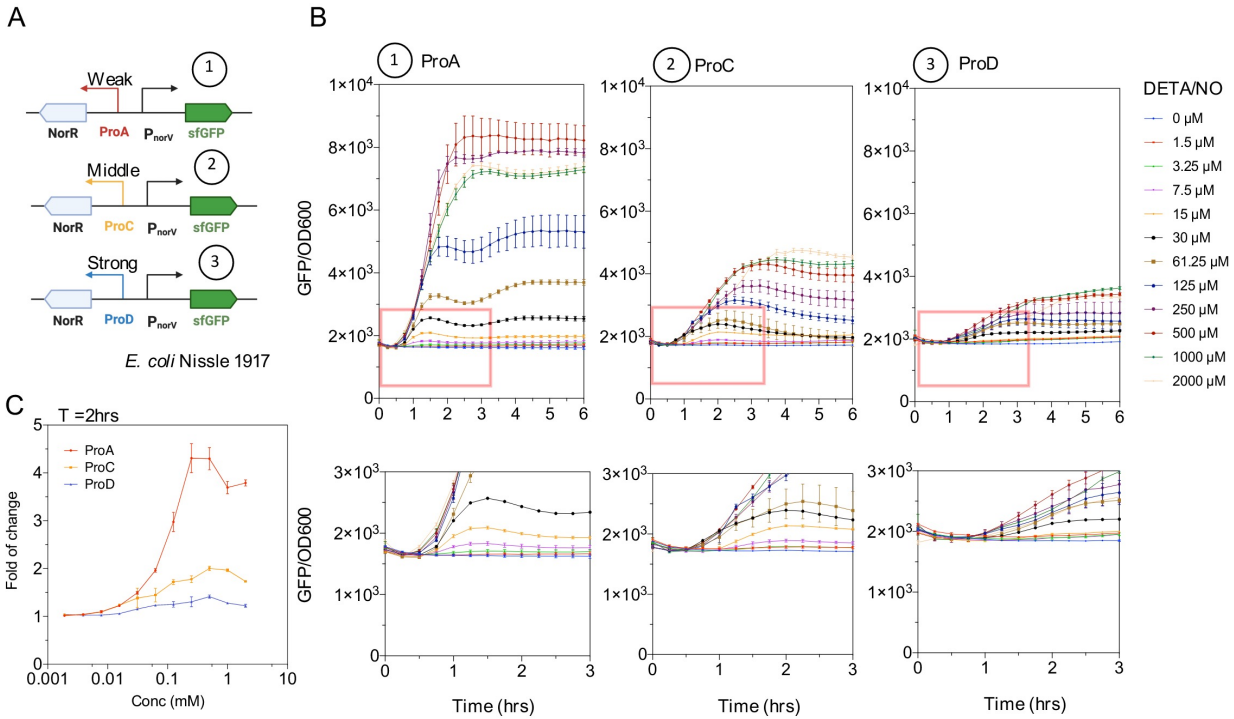


Figure 5.2. Performance of NorR circuits in open-loop format with different promoters

A) Schematic diagram for the open-loop sensor circuits with constitutive promoters of different strengths B) System induction kinetics (6 hrs) and the first 3hrs were magnified to show the limit of detection for each circuit. DETA/NO gradient used is (0, 1.5, 3.25, 7.5, 15, 30, 61.25, 125, 250, 500, 1000, 2000 μ M). C) Dosage response curve of each construct at steady states. (T=2 hrs) The standard deviation and mean value of three bio-replicates were plotted in each graph.

There is no apparent difference between OD growths in all three cases, as shown in Appendix Figure C-2, this ruled out the possibility that the decrease in GFP output was caused by any additional burden posed by NorR overexpression. These results are consistent with the previous report on SalR regulated aspirin response [6], where the overexpression of the regulator leads to dynamic binding competition with the promoter and suppress the overall amplitude. In this study, the overexpression of NorR produced a high level of inactivated NorR and reduced the possibility of binding by NO activated NorR (Figure 5.2B, C). Hence, the gene circuit with the strongest constitutive P_{roD} promoter had the lowest amplitude (Figure 5.2), compared to P_{roA} and P_{roC} which are 33.3 and 3.59 times weaker than P_{roD} . However, in the weak NorR expression, there

was no detectable increase in the baseline level, this could be due to the formation of transcription inert complex [357]. The σ^{54} RNAP complex requires both the activated NorR and ATP hydrolysis to trigger subsequent conformation for initiating transcription (Figure 5.1). Therefore, this extra step in ATP hydrolysis ensured a tightly regulated system. Many σ^{54} dependent promoters intrinsically have a minimal baseline expression, for instance, the well-characterised toluene promoters P_{XylR} [365][313] or pathogenicity regulon P_{HrpI} [316].

5.3.2 Mathematic model of competitive binding mechanism

To further validate the proposed competitive binding mechanism, a conceptual model was constructed to simulate the competitive binding behaviour of the circuits in Figure 5.2. Similar to the model previously reported [6], the models were built to describe the time kinetics of each protein through two sets of ODEs. $[NorR]$ denotes the concentration of the regulator, and $[GFP]$ denotes the fluorescence protein output. Most members of σ^{54} based transcriptional systems are tightly regulated and display intrinsically low baseline [281][313], thus, the leakage term was not included in this model.

For NorR construct made in the simple regulated format, the constitutive promoter strength is denoted as α_1 (P_{roD}) α_2 (P_{roC}) and α_3 (P_{roA}) respectively [262]. δ_1 and δ_2 are separately the decay rates of $[NorR]$ and $[GFP]$. Given that NorR and GFP are stable protein, $\delta_1 = \delta_2$.and the decay rate is mostly due to dilution effect caused by cell division.

$$\frac{d[NorR]}{dt} = \alpha - \delta_1[NorR] \quad \text{Equation (1)}$$

α_{norV} denotes the P_{NorV} promoter production rate. The regulating term $S([NO], [NorR])$ describes the interaction between NorR, inducer nitric oxide and the P_{NorV} promoter, which can be expanded as:

$$S([NO], [NorR]) = \frac{Kr}{Kr + [NorR]} \left(\frac{[NorR]}{[NorR] + KA} \cdot \frac{[NO]^n}{[NO]^n + KA} \right) \quad \text{Equation (2)}$$

Hence, GFP expression in gene circuits can be described as:

$$\frac{d[GFP]}{dt} = S([NO], [NorR])\alpha_{norV} - \delta_2[GFP] \quad \text{Equation (3)}$$

The strength of each constitutive promoter has been previously characterised in *E. coli* [262] and the ratio between each promoter was used for the simulations. The initial delay caused via DETA/NO hydrolysis and NO accumulation was accounted for 30 mins. The conceptual model output demonstrated a qualitative pattern of the competition dynamics (Figure 5.3), confirmed that the overexpression of redundant regulatory proteins would lower the overall amplitude of gene expression in activated gene regulation. More details can be found in Appendix C.

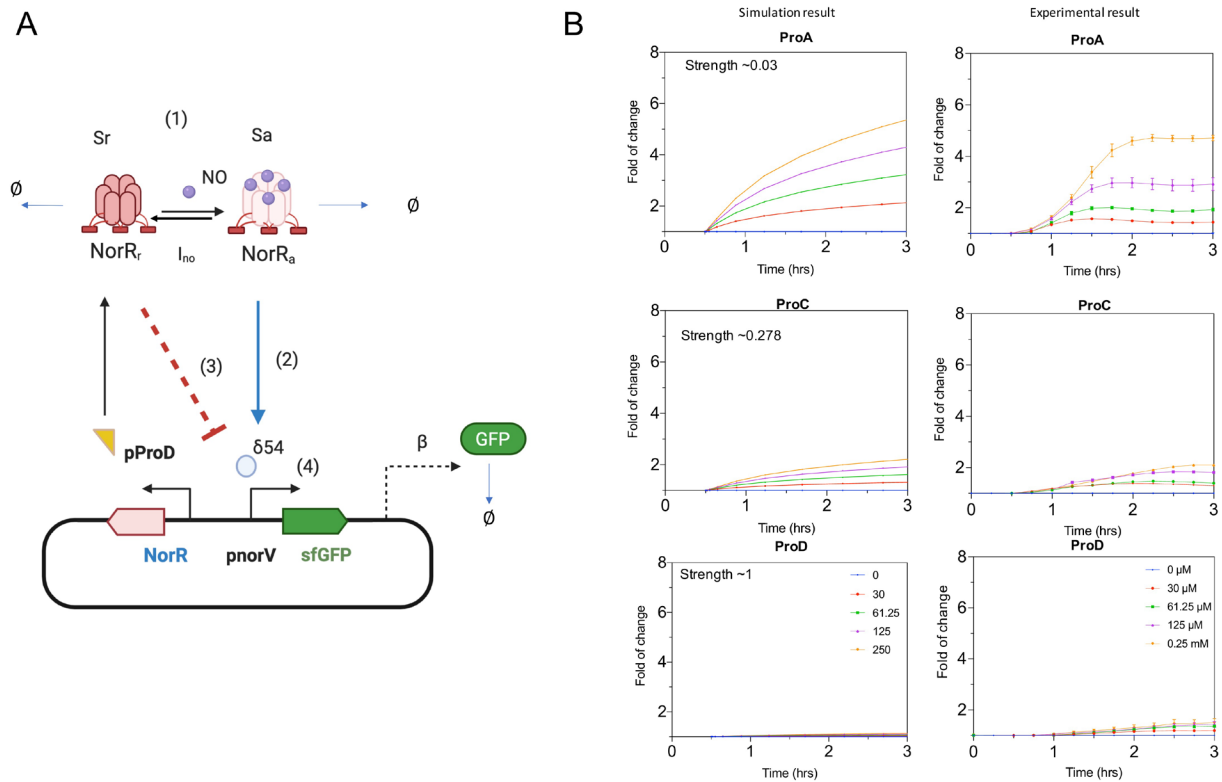


Figure 5.3. Simulation results for the competitive binding mechanisms of NorR.

A) Model architecture of the competitive binding mechanisms. ODEs were used to simulate 1) Ligand activation of NorRr 2) Activation of the P_{NorV} promoter and 3) Repression caused via non activated NorRr and 4) The sum expression of sfGFP. B) The relative promoter strength was characterised in *E. coli* and directly used to simulate the induction kinetics. Data normalised into

the fold of induction (GFPinduced/GFPcontrol) for both experimental results and simulation results. Time delay caused via DETA/NO breakdown accounted as the initial 30 mins delay.

5.3.3 Architecture change of the gene circuits significantly improve the performance

An autoregulatory format with a positive feedback loop was created to fine-tune the expression of NorR, which automatically tunes the level of NorR expression in response to NO concentration. To obtain a higher fold change and output amplitude, the P_{NorV} promoter was coupled with *norR* to form a positive feedback circuit. As shown in Figure 5.4, the NorR gene circuit with the positive feedback using the same P_{NorV} promoter displayed a much higher fold of induction (F.c~7) and higher amplitude ($20,801 \pm 592$ RFU) (Figure 5.4B), compared to the best case in open-loop format (P_{roA}) which only achieved a fold of induction (F.c~4.5) and amplitude (8344 ± 1040 RFU) (Appendix Figure C-3) (Figure 5.2 C). Similarly, the positive autoregulation system reaches its steady-state within 2 hrs, this restriction is likely contributed by the dilution effect of cell division.

After obtaining the optimal construct layout, the binding site components were modified in P_{NorV} . According to the promoter annotation [359][355], P_{NorV} promoter has two integration host factors (IHF) (Figure 5.4, Appendix Figure C-4). IHFs is a class of regulatory proteins involved in maintaining DNA architecture and allows site-specific DNA bending [366]. The features of two IHFs were investigated by removing one/both of the IHFs sites near the transcriptional start site. As shown in Figure 5.4B, upon deletion of both IHFs sites (P_{NorV} null), the gene-circuit significantly reduced its sensitivity and was only induced in mM (1 mM) range of DETA/NO.. However, when one IHF before σ^{54} binding site -12/-24 was deleted ($P_{NorV}\beta$), the relative fluorescence output increased by 25.3% in comparison with the native promoter (P_{NorV}). The baseline level of $P_{NorV}\beta$ promoter was increased by ~ 10% (3337.5 ± 67 RFU), compared to the

native promoter P_{NorV} (3031 ± 50 RFU). These results confirmed that IHFs participates in both NorR based transcriptional activation and repression, the second IHF site had minimal impact on the activation of the promoter P_{NorV} , similar to the previously reported evidence that the second IHFs binding site displayed a lower affinity towards the IHF binding [355], and inhibits the transcription initiation in its absence [367].

It has been reported that alteration of spacer or base pair of any NorR binding sites leads to catastrophic failure for system induction [357][368], thus, mutagenesis studies were not carried out for the NorR binding site. A summary of the dynamic profile was shown in Table 5.3. Overall, a simple architecture change using positive feedback exhibited similar sensitivity and dosage response at a low range of inducer 1.5 μ M to 61.25 μ M, but the overall output amplitude increased by 5-fold in construct pPnorV β . Compared to construct pProD From Figure 5.4C, it was also observed that both systems displayed inducer inhibition when induction concentration exceeded 1 mM of DETA/NO, similar to the case shown in construct pProA (Figure 5.2 C).

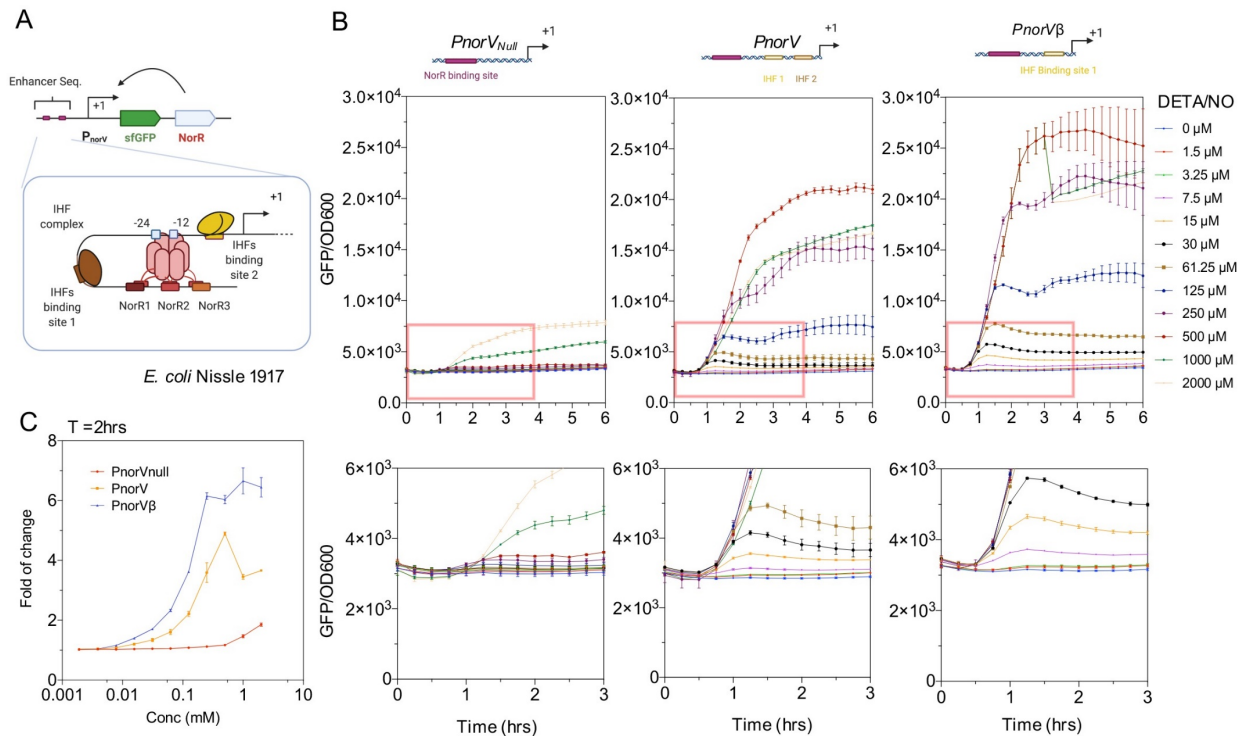


Figure 5.4. Performance of NorR circuit with positive feedback design and the effect of IHF spacers.

A) Schematic diagram of modified construct in positive feedback format. The P_{NorV} promoter annotation with two IHFs sites and NorR binding site were shown in box. B) System induction kinetics (6 hrs) and the first 3 hrs was magnified to show the limit of detection for each circuit. DETA/NO gradient used is (0, 1.5, 3.25, 7.5, 15, 30, 61.25, 125, 250, 500, 1000, 2000 μ M) C) Fold of change for each circuit at steady state. (T=2 hrs) The standard deviation and mean value of three bio-replicates were plotted in each graph.

Table 5.4. Dynamical property of characterised NorR circuit in this study.

Circuits Name	Dynamical range (RFU)	Operational range (DETA/NO μ M)	Limitation of detection (DETA/NO μ M)	Strains tested	Response time (mins)
pP _{norVnull} -sfGFP-norR	2989 $\pm$ 128—5540 $\pm$ 172	1000~2000	1000	EcN	~60
pP _{norV} -sfGFP-norR	2847 $\pm$ 36--13931 $\pm$ 193	1.5~500	1.5	EcN	~25
pP _{norVβ} -sfGFP-norR	3118 $\pm$ 35--20762 $\pm$ 2326	1.5~500	1.5	EcN/ DH5 α	~25
pP _{roD} -norR-P _{norV} -sfGFP	1847 $\pm$ 8--2257 $\pm$ 79	1.5~500	1.5	EcN	~25
pP _{roC} -norR-P _{norV} -sfGFP	1728 $\pm$ 35--3465 $\pm$ 136	1.5~500	1.5	EcN	~25
pP _{roA} -norR-P _{norV} -sfGFP	1614 $\pm$ 82--8220 $\pm$ 816	1.5~500	1.5	EcN	~25

5.3.4 Host interference and circuit performance in $\Delta norR$ strains

Since NorR regulation involves the dynamic competitive binding mechanism, it was hypothesised that NorR from native chromosomal expression could also compete with NorR from the synthetic gene circuit and further reduce the output amplitude (Figure 5.5A). The impact of the native chromosomal *norR* on the performance of synthetic gene circuit was then investigated by comparing construct pPnorV β in *E. coli* DH5 α and its $\Delta norR$ mutant. As presented in Figure 5.5 B, the results indicated that an increase in GFP output amplitude by 69% in the $\Delta norR$ mutant compared to *E. coli* DH5 α . The baseline leakiness also increased from 1825 ± 8 RFU (wild type) to 3128 ± 124 RFU ($\Delta norR$) in the first 3-hrs of induction. The system exhibited a much higher fold of change (F.c ~ 12) in $\Delta norR$ mutant than in wild type strains. (F.c ~ 5.4) (Figure 5.5C). In both cases, minimal sensitivity to DETA/NO was the same 30 μ M (Figure 5.5B). This result demonstrated that host native expression of NorR could compete to bind P_{NorV β} promoter and act as a repressor to the expression of sfGFP (Figure 5.5C). Overall, these results further validated the proposed competitive binding mechanism of NorR regulation that it can act as both a repressor and an activator.

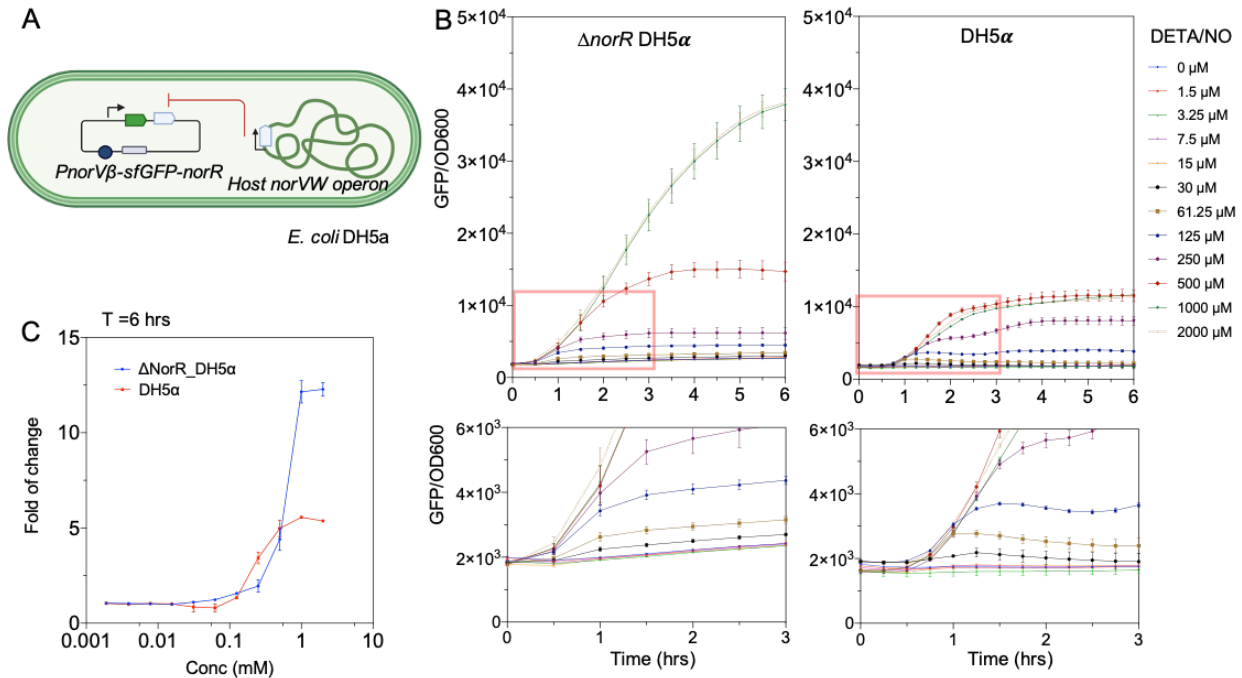


Figure 5.5. Host interference and circuits performance in $\Delta norR$ *E. coli* strains.

A) Graphical representation of the potential impact caused by the endogenous copy of NorR. B) System induction (6 hrs) dynamics in both *E. coli* DH5 α and *E. coli* DH5 α $\Delta norR$. The first 3 hrs was magnified to indicate the limit of detection DETA/NO gradient used is (0, 1.5, 3.25, 7.5, 15, 30, 61.25, 125, 250, 500, 1000, 2000 μ M). C) Fold of change curve at the steady-state (T=2 hrs). The standard deviation and mean value of three bio-replicates were plotted in each graph. N= 3

5.3.5 The performance of the optimised circuits in mini SimCells

In order to bypass host regulation and create a safe sensing system, I examined the performance of the gene circuit in purified mini SimCells. I cloned the optimised pPnorV β construct into *E. coli* MC1000 $\Delta minD$ strain which can generate mini SimCells due to the disruption of the *minD* gene. The abnormal division generates one cell with duplicative chromosomes and an anucleate minicell. Minicells generated from $\Delta minD$ strain contained plasmids with designed gene circuits have shown dose response in various systems [198][6]. For clarity, the minicells contained synthetic constructs will be termed mini SimCells.

Figure 5.6 shows that the chromosome-free mini SimCells were able to express sfGFP by DETA/NO induction in a dose-dependent manner. Through using the optimised protocol (outlined

in Chapter 6), I was able to retrieve highly purified mini SimCells culture with a yield of 5×10^9 mini SimCells/ml. Both OD600 measurement and plate assay confirmed that the purified mini SimCells did not contain any parent cell contamination. Plate assay was carried over 16 hrs and 48 hrs to rule out any slow-growing parent cells (Figure 5.6A). The relative fluorescence intensity (sfGFP/OD) in the purified mini SimCells ($19,076.67 \pm 519.3$ RFU) was approximately 8.6-fold less than that in the parent cell ($164,100 \pm 8161$ RFU), this was due to the small volume of mini SimCells which was about a quarter of normal *E. coli* cells and much less than the filamentous *E. coli* $\Delta minD$ parent cells (5~40 μm). Furthermore, using an established formula for minicell numeration [206], fluorescence per cell per volume for mini SimCells ($3.47 \times 10^{-6} \pm 9.4 \times 10^{-8}$ /cell volume) was comparable to that of the parent cells ($3.14 \times 10^{-6} \pm 5.4 \times 10^{-8}$ /cell volume) (Figure 5.6). Interestingly, the relative response fold in mini SimCells and parent cells were similar, but mini SimCells displayed a lower detection limit (1.5 μM) than parent cell (15 μM) (Figure 5.6A). As demonstrated in Figure 5.6C, compared to a delay of 1.25 hrs in the parent cells, mini SimCells showed faster induction (0.5 hrs). This indicates that mini SimCells could facilitate faster response time potentially due to its large surface-volume ratio. Overall, mini SimCells contain sufficient machinery for inducible gene expression of the σ^{54} dependent NO responsive circuits.

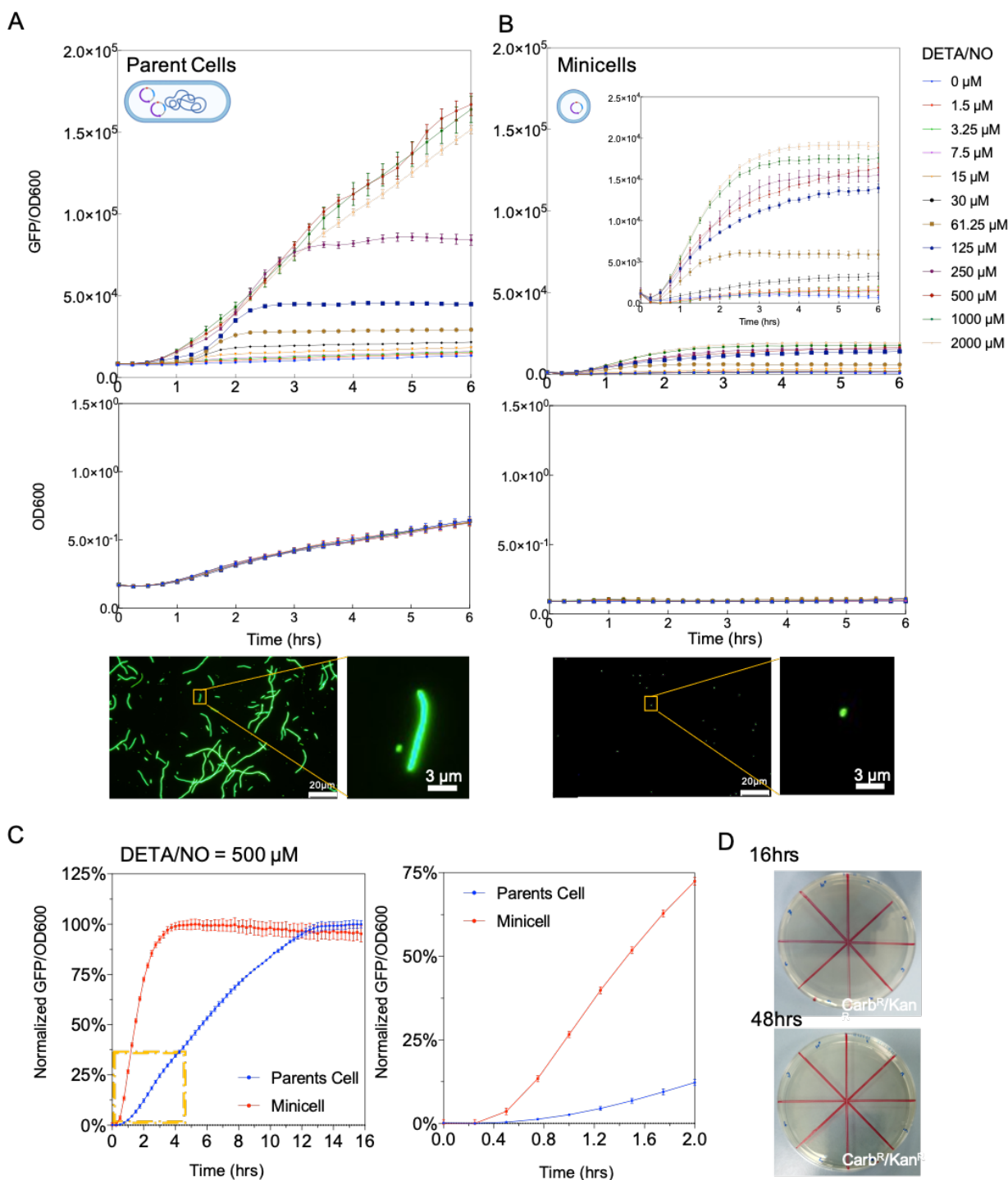


Figure 5.6. Optimised circuit testing in purified mini SimCells and parent cells.

NorR system induction kinetics and OD600 reads of A) parent cell and B) mini SimCell under DETA/NO gradient (0,0.75,1.5,3,7.5,15,30,61.25,125,250,500,1000 μM . C) Normalised induction kinetics for parent cell and mini SimCell under 500 μM (fully induced concentration) to show case difference in response time. The first 2 hrs (yellow square) is enlarged and shown on

the right. D) Plate assay was done 16 hrs and 48 hrs to confirm the contamination of parent cells. The standard deviation and mean value of three bio-replicates were plotted in each graph.

5.3.6 Mini SimCells bypassed physiology limitation of parent cells

From previous literature in the characterisation of σ^{54} dependent promoter family, it has been reported that σ^{54} related gene was commonly related to stress response, biofilm formation and flagellar movement [369]. In *E. coli*, almost half of the σ^{54} related gene is related to nitrogen assimilation [370] pathway and upregulated in nutrient starved condition [371][314]. To investigate how the nitrogen depletion impacts the synthetic circuits, EcN transformed with construct pPnorV β was tested in both PBS and M9 medium. From Figure 5.7, the circuits showed an apparent delay of 2 hrs when PBS medium was used during DETA/NO induction in parent cells. However, DETA/NO induction of parent cells in the M9 medium showed a faster response to DETA/NO, similar to that in LB. (Figure 5.2 and Figure 5.4). Interestingly, DETA/NO induction of mini SimCells showed functional dose-response, independent of the medium type, as the response time for sfGFP expression was similarly fast in both PBS and M9.

Further testing in different carbon sources did not significantly alter the response time or the dynamic range (Appendix Figure C-6). Despite the fact that little difference was observed in OD growth (Appendix Figure C-5) between parent cells in two different media, this delay could have been due to the lack of a nitrogen source in PBS. Since P_{NorV} is a σ^{54} dependent promoter and σ^{54} is related to nitrogen metabolism, it can be reasoned that under a relatively fixed number of RpoN factors in both log phase or stationary of the cells [372], upregulation of host nitrogen assimilation gene could have competed for the same transcriptional factor RpoN pool with the introduced gene circuits, thus leading to a time delay. Since RpoN (σ^{54}) only contributed 8% of the total transcriptional holoenzyme (σ^{70} ~78%) [373], σ^{54} -dependent promoters might be more

sensitive towards fluctuation of external nitrogen than other sigma dependent promoters. As shown case in Appendix Figure C-7, in contrast with previously developed SalR and vRecA system, NorR induction kinetics is more vulnerable to medium shift (LB→M9) and displayed a strong dependence of the initial inoculation condition. Interestingly, once the same gene circuit was introduced in mini SimCells, as shown in Figure 5.7B, nitrogen-free PBS had no impact on DETA/NO induced sfGFP expression. The gene circuits can be triggered equally fast in both PBS and M9 medium with a greater magnitude of induction ~ 10 , this could have been due to the absence of competition of RpoN pool in mini SimCells, which had lost the host chromosome.

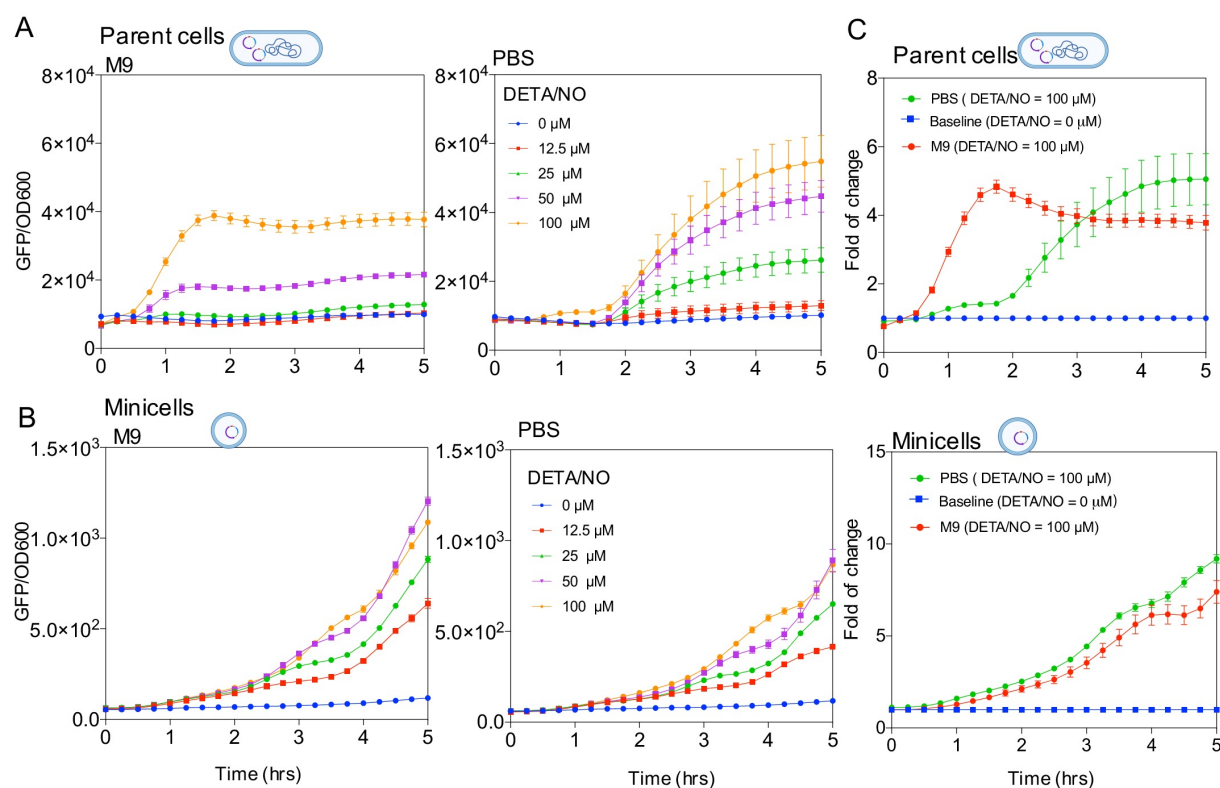


Figure 5.7. Mini SimCells bypass host interference.

A) Induction kinetics of $P_{NorV\beta}$ -sfGFP-norR circuits function in *E. coli* MC1000 $\Delta minD$ under nitrogen depleted condition (PBS) or nitrogen supplied condition M9 condition. Each medium condition was supplemented with 2% w/v glycerol as the sole carbon source. B) Induction kinetics of the purified mini SimCells loaded with the same construct. DETA/NO gradient used is (0, 12.5, 25, 50, 100 μ M). C) Normalised fold of induction changes in both cases. (GFP fully induced/ GFP

no induce) The standard deviation and mean value of three bio-replicates were plotted in each graph. N=3.

5.3.7 Long-term storage of mini SimCells loaded with sensory constructs

Lyophilization (freeze-drying) is a common approach for preserving biological compounds at room temperature for long-term storage and transportation [374]. To further explore the potential application of mini SimCells, the purified mini SimCells was lyophilised with the optimised nitric oxide sensing circuits (pPnorV β) and kept them at room temperature ($\sim 25^{\circ}\text{C}$). After storage for two months, the freeze-dried parent cells and mini SimCells were revived and examined. Figure 5.8A demonstrated that both mini SimCells and parent cells maintained the detection sensitivity of $15\ \mu\text{M}$ DETA/NO after revival using SOC medium. The freeze-dried parent cells exhibited a ~ 24 -fold lower intensity ($6,298 \pm .100$ RFU) than the freshly transformed cells ($151,700 \pm .2615$ RFU) (Figure 5.6 A). In contrast, mini SimCells exhibited ~ 11 -fold reduction in the output intensity ($1,668 \pm 32$ RFU), compared to the freshly transformed mini SimCells (Figure 5.8). This can be further calculated as $3.33 \times 10^{-7} \pm 6.32 \times 10^{-9}$ RFU/cell volume (μm^3) for the mini SimCells intensity and $2.01 \times 10^{-7} \pm 3.1758 \times 10^{-9}$ RFU/cell volume for the parent cells. However, the induction of sfGFP expression could still be triggered within 2-hrs at an induction fold of $F.c \sim 10$ for mini SimCells and $F.c \sim 15$ for parent cells (Figure 5.8C). Furthermore, mini SimCells still showed slightly faster response than the parent cells under saturated induction level. Appendix Figure C-8 demonstrated a direct comparison of the induction kinetics between the freshly transformed and lyophilised whole-cell biosensors versus mini SimCells biosensors. The reduction in fluorescence induction fold of mini SimCells might have been due to the loss of ATP regeneration machinery after long term storage, which would require further optimisation.

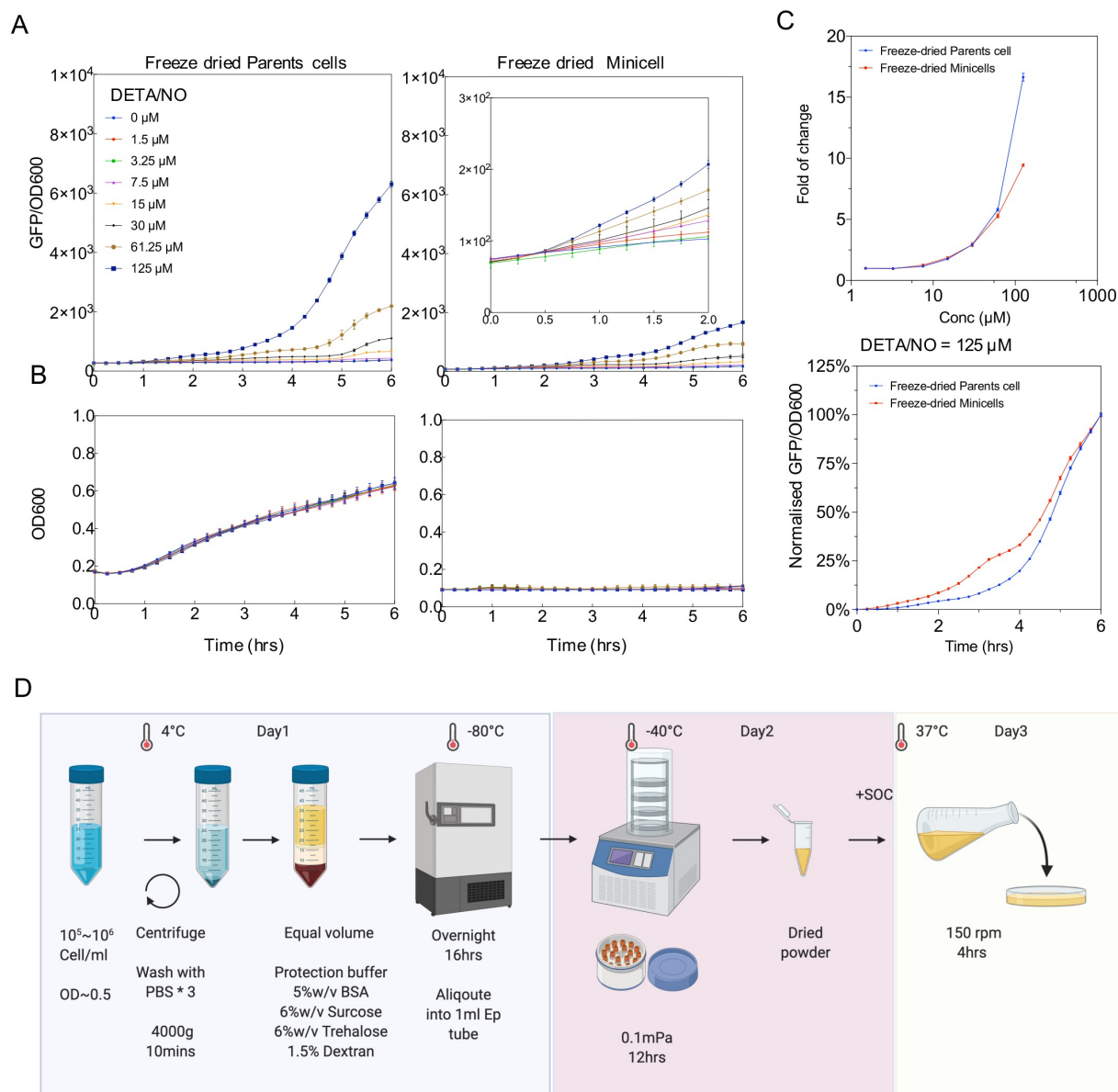


Figure 5.8. Function maintenance of freeze-dried mini SimCells biosensors.

A) Parent cells (*E. coli* MC1000 $\Delta minD$) and mini SimCells were transformed with optimised nitric oxide sensing circuits ($P_{NorV\beta_sfGFP_norR}$) and carried out the lyophilization process. After two-month storage at room temperature, cells were revived using SOC medium and tested for induction performance. DETA/NO gradient used here is (0, 1.5, 3.25, 7.5, 15, 30, 61.25, 125 μ M). B) OD at 600 nm was also recorded to indicate any parent cell contamination. C) Fold of induction (T = 6 hrs) and normalised response time. The standard deviation and mean value of three bio-replicates were plotted in each graph. N=3. D) Illustration of the freeze-drying protocol of mini SimCells.

5.4 Discussion

In this chapter, a set of nitric oxide biosensors have been constructed from *E. coli* Nissle 1917, based on its *norVW* operon and regulator tuning, it was successfully demonstrated that the competitive binding mechanism could also explain σ^{54} -dependent NorR regulatory system, similar to the σ^{70} -dependent SalR [34]. The binding of a regulatory protein and a promoter is a dynamic process, depending on the interaction and relative abundance between the regulatory protein and the inducer, the regulatory protein can be in its activated/induction (NorRa) or inactivated/repressive state (NoRr). The competitive binding mechanism proposed assumes that both activated and inactivated regulatory proteins are able to bind the same site of the promoter. This is supported by previous studies [362] where an iron cluster truncated NorR mutant failed to respond to NO level yet still repressed the promoter baseline level. The transport of NO into *E. coli* is mainly through diffusion [375], however, *E. coli* has multiply highly efficient NO scavengers such as nitrogen dioxygenase (NOD) and flavorubredoxin (NorV) that protect the cells against oxidative damage [376]. Upon overexpression of the regulatory proteins under limited intracellular inducers, the non-bound form of NorRr actively competes and occupy the binding site of the promoter, so the frequency of subsequent gene activation was repressed. No additional cellular burden was noted during the overexpression of the regulator. In summary, the competitive binding mechanism provides a potential universal tuning strategy applicable to different transcriptional regulations for modulating the dynamic range by merely rearranging architecture and the strength of regulator expression.

The detection limits of parent cells and mini SimCells gene circuits was 15 μM and 0.75 μM of DETA/NO (up to $\sim 500 \mu\text{M}$), respectively. Based on the release dynamics model in [364], the detection limit of the actual NO released to trigger pPnorV β construct was estimated to be

25.56 and 1.28 nM in parent cells and mini SimCells respectively. The operational range of the optimised circuits (25.56 nM ~ 13.51 μ M) fitted the medical relevant pathogenic threshold detected in active ulcerative colitis (UC) (>17.3 μ M), inflammatory bowel disease IBD (>13.65 μ M) and chronic inflammatory diseases (>14.01 μ M)[9][377]. This was in good agreement with previous reports of NorR response to nM scale of nitric oxide [378]. Furthermore, the initial lag in response (30 mins ~ 1 hrs), as observed in circuits activation, could have been due to the time required for NO release from DETA. Therefore, the true activation time for NorR regulatory system could have been much shorter. More experiments would be required for measuring the actual *in vitro* NO release kinetics from DETA/NO. Alternatively, other NO donors with faster release kinetics such as DEA NONOate (half-life 2 mins) could be tested [379].

To address how other promoter modules could affect the overall response dynamics, the promoter architecture and the effect of the IHFs site was investigated further. By rationally redesigning the IHFs site, it was demonstrated that the modified 2nd IHFs site is not essential to the induction performance. Instead, the deletion enabled a 25.3% increase of the GFP amplitude with no significant baseline increase. In contrast, the 1st IHF was found to be critical for system induction. It was evident that the 1st IHF is critical for bringing close contact of the NorR regulator with σ^{54} core enzyme [362]. Consider the 2nd IHF displayed a much lower affinity than the 1st IHFs site [355], and the previous evidence suggests its role in transcription inhibition [367], the results confirmed this *in vitro* and provided a minimal version of the P_{NorV} promoter (P_{NorV} β) with ideal induction characteristics. It is worth mentioning, the unique DNA bending feature displayed via IHF factors might be useful for rationally designing tightly regulated chimeric promoters for the future.

The results of this study confirmed that the optimised NorR constructs could be triggered by 15 μM of DETA/NO within 30 mins. Such a rapid response time and high sensitivity confirm its potential in applications such as an *in-situ* disease triggered sensor when loaded in EcN. Because of the importance of NO as a signalling molecule in human-microbiome communication [353] and plant pathogen response [380], the NO biosensor could be used as a direct probe in studies of interspecies communication, which rely on the S-nitrosylation [381]. The sensor developed here is effective at sensing DETA/NO within a range of 15 μM ~ 500 μM , a wider working range compared to the most recent published one that has a range of 30 μM ~100 μM [84].

Host interference is one of the significant bottlenecks in whole-cell biosensor applications, the fluctuation in biosensor expression as a consequence of changing medium conditions (carbon/nitrogen) has been previously reported for other σ^{54} -dependent systems [313][382]. Previous studies characterising *Pu* promoter, which is also an σ^{54} dependent promoter[313], have highlighted that such systems suffered from intrinsic exponential silencing when placed in a rich medium. However, this is not the case in a cell-free state when supplied with separate IHF, σ^{54} and core RNAPs [383]. The NorR system detailed here did not display such a delay in rich medium (LB). However, the system exhibited a response delay when induced in nitrogen depleted medium (PBS), this might be due to a competition for the limited σ^{54} pool, which correlates well with the fact that all the nitrogen assimilation gene are upregulated (*Ntr* regulon)[384] during nitrogen starvation and the fact *norVW* transcription is entirely dependent on σ^{54} [358]. Due to σ^{54} factor only contributed a small sharing pool (~8%) of the total transcriptional factors, this makes the NorR circuit activation strongly dependent on the cellular contexts when σ^{54} is limited. Such explanation is also supported in other studies where the promoter sequence spanning -12/-24 (σ^{54}

binding site) was found to be the rate limiting step and involved in growth condition coupling [385][386].

To validate this proposed hypothesis of resource competition and potentially overcome such nitrogen dependence, one method is to supply extra copies of σ^{54} on plasmids, however, this might lead to unexpected global physiology changes to the host cells. As a safer alternative solution, to overcome such limitations in cellular context-dependence and make the system more robust. The *E. coli* adapted mini SimCells with preloaded construct demonstrated increased sensitivity and induction fold. Such a strategy is advantageous in handling complex organisms which have developed many evolution redundancies that do not fit the application niche. Finally, the long-term storage of freeze-dried mini SimCell was tested and verified. Despite a significant drop in relative fluorescent intensity, the results demonstrated an acceptable self-life with much room to improve. Further optimisation steps could adopt different strategies for increasing the intracellular concentration [387] of trehalose or, in the case of mini SimCells, supply of plasmids-based glycolysis pathway for ATP regeneration [388]. Overall, the mini SimCells loaded NO sensors exhibited enhanced safety, robustness and sensor performance.

Chapter 6 System implementation in mini SimCells

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In the previous chapters 3, 4 and 5, I constructed and optimised three sets of transcriptional regulatory systems that allow different control routes for bacteria therapy. This chapter aims to transform and test the optimal candidate of each system into chromosome-free minicells to further expand their safety in potential clinical practice and validate the potential of minicells as a modular chassis when loaded with different transcriptional systems (σ^{70} and σ^{54}). For clarity purposes, only minicells with intact transcriptional, translational machinery and a preloaded plasmids system will be termed mini SimCells. Firstly, I thoroughly characterised the size distribution, Raman signal and motility profile of the minicells. An optimised purification protocol was also developed with

enhanced purity and yield. The results of this section outline the feasibility of minicells as a programmable chassis platform for loading transcriptional controls and performing a multi-input logic gate. The potential design constraints were also outlined and discussed for future improvement.

6.1 Introduction

Mainstream research on constructing artificial cells can be largely categorised into two approaches: 1) top-down and 2) bottom-up. As outlined in Chapter 2.1, despite the remarkable results each approach has achieved, each method demonstrates different drawbacks and challenges. For top-down approaches such as deleting non-essential genes to obtain a minimal genome [31], it is difficult to scale when considering the vastly diversified bacteria types and arguable that such elimination methods could derive a universal cell chassis. Furthermore, adaptive robustness conferred by a regulatory network may be lost in the process of removing non-essential gene regulatory networks with unknown functions or interactions. On the other hand, the bottom-up approach for synthesising artificial cells is challenged by the complexity involved in recreating a multifunction cellular network. For instance, a single prokaryotic cell such as *E. coli* contains a total of $10^6 - 10^7$ proteins [389]. This is regulated by approximately 131 transcriptional factors [390] and an RNA network accounting for 20% of the dry weight of the cell. The level of complexity involved in building a transcriptional/translational active cell from scratch is enormous, not including the later stage of fine-tuning. That being said, recent efforts of cell-free approaches in creating modular energy regeneration [388], cellular division [391] and signal passaging systems [392] have contributed to making modular parts with specific functions. By combining the merits of these two approaches, a middle angle ground for generating chromosome-free mini

SimCells is employed in this work. This is a convenient and robust way to leverage the intact cellular machinery while simultaneously bypassing issues associated with growth and host interference.

As previously outlined in Chapter 2.8, the existing medical usage of minicells demonstrated their capacity in terms of different loading material ranges from shRNA to small molecules at higher loading capacity [202][44] and their ability to trigger a CD8⁺ specific T cell response by maintaining functional T3SS machinery for surface expression of antigen [203]. Few studies have focused on the programmability of the minicells; furthermore, there is no systematic characterisation of their motility, size distribution and any profile change (chemical/physical) caused by the loss of DNA.

Through using a *minCDE* truncated *E. coli* strain and plasmid with constitutive GFP, I characterised the size distribution of mini SimCells, their motility and Raman signal for the DNA/RNA presence. Then, I explored alternative methods of generating mini SimCells through FtsZ overexpression. To obtain a large quantity of pure mini SimCell culture, I developed an antibiotic cocktail based on different cell wall lysis antibiotics and fully eliminated the contaminated parent cells. Further optimisation included cell enlargement using enriched growth broth and two-stage membrane filtration (0.45 μm and 0.22 μm pore size), achieving high purity with satisfactory yield (5×10^9 mini SimCells/ml). A SalR-based aspirin σ^{70} dependent sensor and a NorR-based σ^{54} dependent nitric oxide sensor were both tested and demonstrated functional dosage response with increased response time (24 ~ 48 mins) compared to the parent cells. Further experiments showed that mini SimCells contain sufficient cellular machinery to process the SalR, TetR-based antisense XOR logic. These results demonstrated that mini SimCells could indeed be used as a chromosome-free chassis for multi-input transcriptional control.

However, experiments using DNA damage-sensing vRecA circuits pointed out certain design constraints of min SimCells, and additional regulators and protease modules might be required for functional promoter activation. Further debugging of the vRecA system led us to an unexpected finding in signal amplification with the addition of regulator LexA and RecA in the whole cell *E. coli* Nissle 1917. To circumvent the failed response in mini SimCells and to construct a medically applicable construct based on the modified vRecA system in probiotics, I characterised and connected the modified circuits with a therapeutic cargo SalA (salicylate hydroxylase) and demonstrated its ability to convert aspirin into catechol. Catechol is a chemical compound that has been proven to produce cancer-specific toxicity and has an anti-inflammatory effect in several clinical studies [393][394][32].

6.2 Material and methods

Table 6.1 Strains & plasmids used in Chapter 6.

Short abbreviation	Plasmids	Description	Backbone
pP _{Sal} _PAR_Amp ^R	P _{Sal} _salR_GFP_Amp ^R	Autoregulated aspirin sensor with ampicillin resistance inserted.	ColE1
pP _{Bad} _FtsZ	P _{Bad} _ftsZ_tdtomatoes	FtsZ overexpression and red fluorescent protein tdTomato under control of L-arabinose inducible P _{Bad} promoter.	ColE1
n.a	P _{Bad} _tdtomatoes	P _{Bad} promoter controlling the expression of red fluorescent protein tdTomato.	ColE1
pP _{NorVβ}	P _{NorVβ} _sfGFP_norR	P _{NorV} _sfGFP_norR with single IHFs site promoter.	ColE1
pTetR_Rev_RNAP	P _{Sal} _SalR_P _{TetO} _tetR	Autoregulated aspirin sensor with antisense P _{TetO} promoter.	ColE1
pP _{VRecA} _AT_SalA	P _{VRecA} _AT_sfGFP_salA	P _{VRecA} promoter with AT repeat SOS box controlling <i>sfgfp</i> orf and <i>salA</i> orf.	ColE1
pP _{VRecA} _AT	P _{VRecA} _AT_sfGFP	P _{VRecA} promoter with AT repeat SOS box controlling <i>sfgfp</i> orf.	ColE1
pUnified_LexA_RecA	P _{ELexA} _lexA_P _{RecA} _recA_P _{VRecA} _AT_sfGFP	pLexA_RecA constructs fused with P _{VRecA} _AT_sfGFP constructs.	ColE1
pLexA_RecA	P _{ELexA} _lexA_P _{RecA} _recA	<i>E. coli</i> <i>lexA</i> orf and <i>recA</i> orf controlled under its native promoter P _{LexA} and P _{RecA} .	ColE1
pUnified_LexA_RecA_SalA	P _{ELexA} _lexA_P _{RecA} _recA_P _{VRecA} _AT_sfGFP_salA	pUnified_LexA_RecA constructs with additional <i>salA</i> orf.	ColE1

Table 6.2. Primers used in Chapter 6.

Primers	Sequence 5' → 3'	Related plasmids
Rbs+FtsZ_fwd	AACTCTCTACTGTTTCTCCATACCC GAGGCCGACGATGATTACGGCCTC	P _{Bad} _FtsZ_tdtoma toes
Rbs+FtsZ_rev	CTTCTTAAAGTTAAACAAAATTATTAATTCCAGTCAATT CTTAATCAGCTTGCTTACGC	
pBad_tdtomatoes_fwd	AATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG	
pBad_tdtomatoes_rev	GGGTATGGAGAAACAGTAGAGAGTTG	
pGEMT_PvrecA_sfG FP_SalA*_fwd	CAAGGCGATTAAGTTGGGTAAC	pUnified_LexA_ RecA_SalA
pGEMT_PvrecA_sfG FP_SalA*_rev	TCGCCCTTCCCAACAGTTG	
RecA_LexA_rev	TACCCAACTTAATCGCCTTG GTTGGGATATCGGCAACTAG	
RecA_LexA_fwd	GCAACTGTTGGGAAGGGCGACGTCTTGTGTATCAAACAA G	
rbs-SalA_fwd	TCAAGTTCTCAAAGGAAATGAGTCGTGGGT	P _{VRecA_AT_sfGFP} _ SalA
rbs-SalA_rev	AAAGCGGGCAGTGAAAGGAAGGCCCATGAGTTAAATCGT TGCAACAGGTTGTATTGCTGC	
Pmk-lkd-v.recA- sfgfp_fwd	CTCATGGGCCTTCCTTTTCACTGCCCCGCTTT	
Pmk-lkd-v.recA- sfgfp_rev	ACCCACGACTCATTTTCTTTGAGAACTGAAGCGGCCGC TACTAGTATTTGTAGAGCTCATC	

Table 6.3 Strains used in Chapter 6

Strains	Genotype	Ref
<i>E. coli</i> DH5 α	<i>F</i> – <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF) U169, hsdR17(rK–mK+), λ–</i>	NEB
<i>E. coli</i> Nissle 1917	Ardeypharm GmbH	-
<i>E. coli</i> MC1000	<i>K-12 F</i> – λ – Δ (<i>ara-leu</i>)7697 [<i>araD139</i>] <i>B/r Δ(codB-lacI)3 galK16 galE15 e14– mcrA0 relA1 rpsL150(StrR) spoT1 mcrB1 hsdR2(r–m+)</i>	-
<i>E. coli</i> MC1000 Δ <i>minD</i>	MC1000 Δ <i>minD</i> : KanR	-
<i>E. coli</i> S17	<i>TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc: Mu: Tn7 λpir.</i>	CGSC
<i>E. coli</i> BL21(DE3)	<i>E. coli str. B F</i> – <i>ompT gal dcm lon hsdSB(rB–mB–) λ (DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+] K-12(λS)</i>	NEB

Cell tracking and image processing

Image analysis was performed using ImageJ. Colony counting and size estimation were carried out using the inbuilt colony counter function. Mini SimCells movement tracking was performed using the inbuilt function Trackmate, random diffusion/active motion trajectory was analysed using TraJ Classifier. Prior to taking the footage, induced cells were placed in 4 °C fridges for 25 mins to slow down the parent cell movement for better imaging. Video and images were taken using an Olympus® BX53 fluorescent microscope.

Raman imaging and DNA/RNA signal detection

Overnight cell culture was washed three times with deionised water using a centrifuge at 4000 g for 10 mins per cycle. Prior to the Raman measurements, 2.5 μ l of the washed cells were dropped onto the aluminium-coated slides and left to dry in a laminar flow hood for 2 hrs, the charged couple device (CCD) was cooled to -60 °C. Raman signals were acquired using WITec alpha300® equipped with a 532-nm neodymium aluminium garnet laser. The laser power of 6 mW

was used for all samples. Objective magnification of 100x was used on a single cell with a spot size of $\sim 0.5 \mu m^2$. 12 replicates were taken for each sample type (three biological sets, each composed of four technical replicates). Spectre reads were acquired from 280 cm^{-1} to $3,891 \text{ cm}^{-1}$ with a 600 grooves-per-mm diffraction grating. All reads were processed by polyline baseline fitting and spectre normalization using ProjectFive® (WiTec) software.

Mini SimCells purification and collection

An optimised protocol for mini SimCells purification and extraction is shown in Figure 6.7. A freshly transformed bacteria colony was picked into 2 x concentrated LB medium (with no additional NaCl) and inoculated for 16 hrs at 37°C 200 rpm, followed by centrifugation at 4000 g for 15 mins to remove large cell pellets. Next, the supernatant (containing mini SimCells) was reinoculated with an antibiotic cocktail for 1 hr at 37°C , 250 rpm to lyse any active growing parent cells. This was then stored under 4°C overnight to eliminate any left-over or slow-growing parent cells. All centrifuge and liquid transfer procedures were kept at 4°C or on ice to avoid any heat damage and prevent the energy loss by mini SimCells. Further separation of mini SimCells was done by gradient centrifugation (4000 g, 3000 g, 2000 g, and 1000 g) with 10 mins intervals using ultracentrifuge (Beckman Coulter® Avanti JXN-26). The top and middle layers of the supernatant (2/3 of the liquid volume) were filtered using a $0.45 \mu m$ pore-size mixed cellulose ester (MCE) ThermoFischer® membrane homogenisation. The collection of mini SimCells was done using $0.22 \mu m$ pore-size MCE membrane and resuspended in PBS with an antibiotic cocktail for further plate reader experiments.

Quantification of mini SimCells yield

Using previously established formulas, we estimated the cell number of the parent cell and mini SimCells yield based on [395] and [206]:

$$N_{minicell} = OD_{600} \times 5 \times 10^{10} \quad \text{Equation(1)}$$

$$N_{Progenitor\ cell} = OD_{600} \times 7.8 \times 10^{10} \quad \text{Equation(2)}$$

N denotes as cell count per mL volume.

Medium recipe

2 x LB

Reagent	Total amount (1L)
Deionised water	950 mL
Tryptone	20g
NaCl	10g
Yeast Extract	10g

Antibiotics cocktail stock

Reagent	Total amount (10 mL)
Ceftazidime	1g
Cefotaxime	1g
Penicillin G sodium	1g
EtOH 100%	7 mL
Deionised water	-

6.3 Results

6.3.1 Characterisation of mini SimCells (size distribution, motility and Raman signal)

The previously reported size of minicell produced via *E. coli* lies in the range of 250 ~ 400 nm diameter [396]. This potentially allows for better penetrability in entering the porous (140 ~ 3000 nm) surface displayed on various tumour types [397]. To confirm and characterise the size distribution of mini SimCells, I used both FACS and image analysis to count the relative population contributed by parent cells and mini SimCells. As shown in Figure 6.1 A, due to the polypoid chromosome of the parent cells and abnormal division, the elongated parent cells can be often found with length > 20 μm . Conversely, mini SimCells are generally smaller than 1 μm . Using image processing based on the size cut-off, I estimated the size distribution of mini SimCells. It was found that mini SimCells that were 300 ~ 400 nm in diameter (Figure 6.1 B) contributed 43.6% of the total counts, while the remaining 100 ~ 300 nm contributed 56.4%. Dead cells or cell debris were ruled out due to the fact that only bioactive mini SimCells or parent cells can produce a GFP signal. These results are in good agreement with what has been reported previously [396].

The relative cellular volume has also been quantified and shown in Figure 6.1 C. Despite the fact that a few (5.4%) enormous parent cells had a volume in the range of ~300 μm^3 , the majority of the parent cells had an average volume of 23.8 μm^3 , 53 times larger than that of the mini SimCells (0.45 μm^3) and five times larger than a typical *E. coli* cell (~5 μm^3) [389]. From the microscope images, mini SimCells contributed 36% of the total events while parent cells constituted the remaining 64%. In Figure 6.2, I further analysed the population contributed via parent cells and mini SimCells in FACS. In contrast to normal *E. coli*, DH5 α transformed with the identical constitutive GFP plasmids (P_{roD} -GFP). *E. coli* MC1000 ΔminD displayed a subpopulation with 10-fold fewer side scatter reads (SSC-H) but with the same fluorescent level. This can be regarded as the mini SimCells population. As shown in Figure 6.2 B, mini SimCells contributed

33% of the total population after background subtraction, while parent cells contributed 65.7%, which was similar to the results obtained via image counting (Figure 6.1 C).

Another critical question for mini SimCells is whether they retain any motor function. Although numerous cryo-SEM studies on the minicell confirmed the integrity of its chemotaxis sensory array and motor machinery [398], few studies have characterised the dynamical behaviours of its motion. As shown in Figure 6.3 A, the parent cells were fixed by incubating them at 4 °C for 15 mins. The parent cell ceased to freely move around while mini SimCells were left unaffected (Movement track labelled). 120 secs of fluorescent video footage were recorded and split into 360 frames. A total of 311 tracks of mini SimCells cells were identified using ImageJ (frame gaps larger than five frames were rejected). As shown in Figure 6.3 B, it can be seen that the majority (~ 96.5%) of identified mini SimCells can travel at speeds ranging from 0.1 $\mu\text{m}/\text{sec}$ to 1.30 $\mu\text{m}/\text{sec}$. A further subpopulation of the slow mini SimCells (speed range from 0.01 ~ 0.42 $\mu\text{m}/\text{sec}$) contributed 62.8% of the mini SimCells population, and the fast-moving subtype (speed range from 0.5 ~ 1.3 $\mu\text{m}/\text{sec}$) contributed 34.5%. Interestingly, only ~ 11.5% of the mini SimCells could migrate over a net displacement of 30 μm . The majority of mini SimCells (88.5%) could only travel a short distance from 2.5 μm to 22 μm . Considering the relative size of mini SimCells lying in the range of 100 nm~ 400 nm, a displacement of 20 μm would mean that a mini SimCell contains sufficient energy to travel 50 ~ 200 of its body lengths within 120 secs. In comparison, normal *E. coli* of size 3 ~ 5 μm generally travel at a speed of 20 ~ 30 $\mu\text{m}/\text{sec}$ [399], equivalent to ~ 6 body lengths/sec. These findings mean mini SimCells have intact motor machinery and can swim at a lower speed of 1~2 body lengths/sec than typical *E. coli*.

I summarised and clustered the movement tracks. From Figure 6.4 and Figure 6.4 C, mini SimCells can display both active migration or random diffusion. Six individual trajectories were

shown as an example in Figure 6.4 C. Mini SimCells can not only swim and tumble over a considerable distance in different directions but can also randomly diffuse with little net displacement. For better clarity, in Figure 6.4, a single mini SimCell trajectory is provided with the fixed parent cell as a reference on its side. Its direction changes randomly within a total of 38 secs frames but displays both motion trajectories (tumble and swim). In summary, mini SimCells retain both flagellum and cilia structure and exhibit intact motility function. The movement is not affected by environmental stimuli such as temperature, perhaps due to the lack of chromosomes and corresponding temperature-responsive modules.

It was anticipated that mini SimCells should contain much less genetic material than normal cells or parent cells due to the lack of host chromosomes. Therefore, Raman spectrum-based cell sorting can be used as a label-free option for differentiating mini SimCells from the normal parent. To confirm this, I measured the single-cell Raman spectrum (SCRS) and the results are shown in Figure 6.5. After normalization and background subtraction of the SCRS reads, signal peaks at 785 cm^{-1} , 831 cm^{-1} and 1575 cm^{-1} , which commonly correspond to the presence of DNA, are missing [400]. This finding is useful in providing an alternative method for mini SimCells isolation using Raman-based high throughput cell sorting in the future [401].

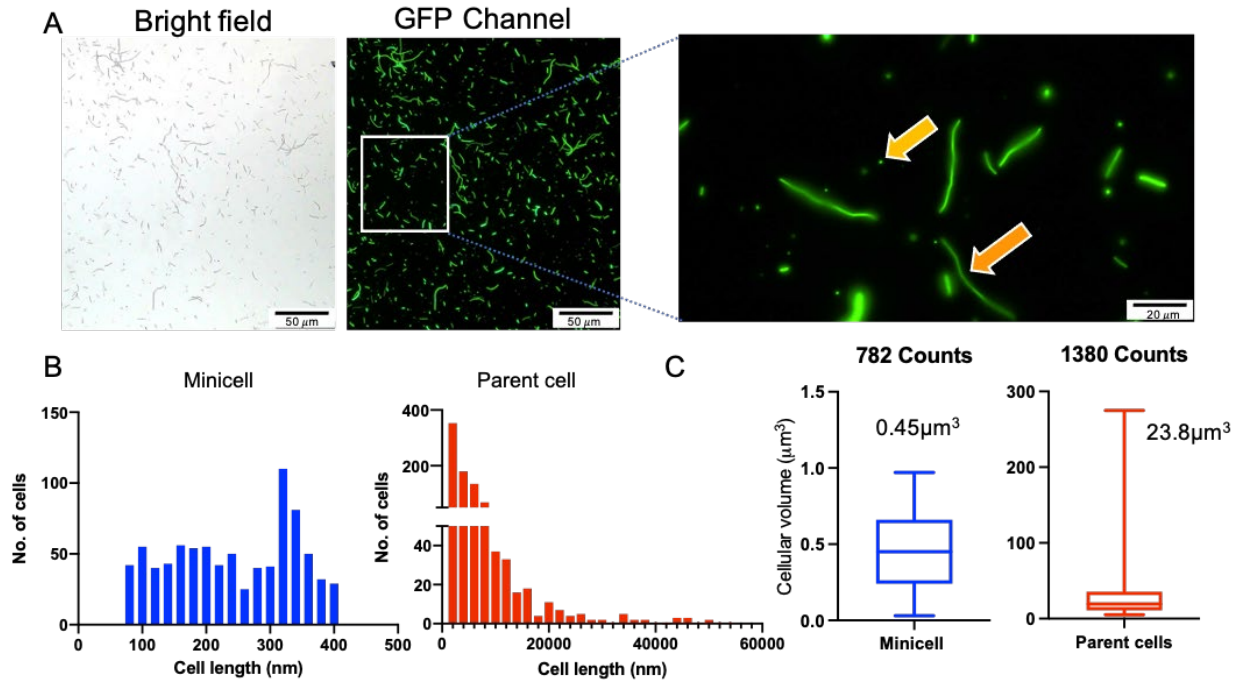


Figure 6.1. Characterisation of the size distribution of mini SimCells.

Disruption of *minD* orf will lead to mini SimCell production and elongated parent cell. A) *E. coli* MC1000 $\Delta minD$ transformed with constitutive expressed sfGFP plasmid (P_{roD} -sfGFP), elongated polypoid parent (orange) and mini SimCells (yellow) are indicated by the arrow. B) The size distribution of mini SimCells (Bin size = 10 nm) and parent cell (Bin size = 400 nm). C) Whisker box plot of the average volume of mini SimCells and parent cells. The top and bottom of the bar represent the max and min of the value. Medium value was marked in the plot with total counts labelled on top.

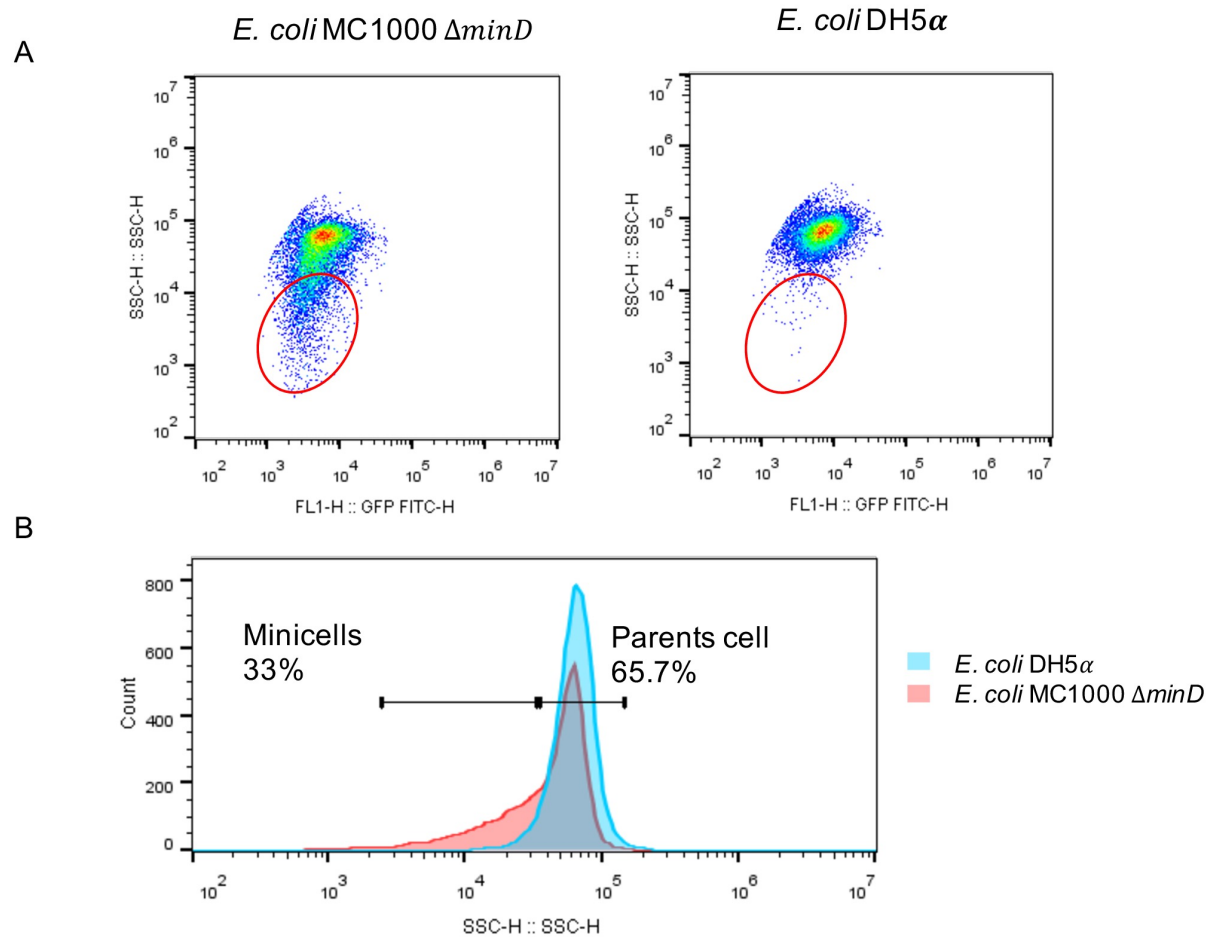


Figure 6.2. FACS profile of *E. coli* MC1000 $\Delta minD$

A) Flow cytometer reads of *E. coli* MC1000 $\Delta minD$ and *E. coli* DH5 α . Events count = 30,000. Background removed using LB medium and sonicated cell debris. B) Relative population distribution of mini SimCells (33%) and parent cells (65.7%) quantified based on SSC difference. Debris and background (1.3%) not shown.

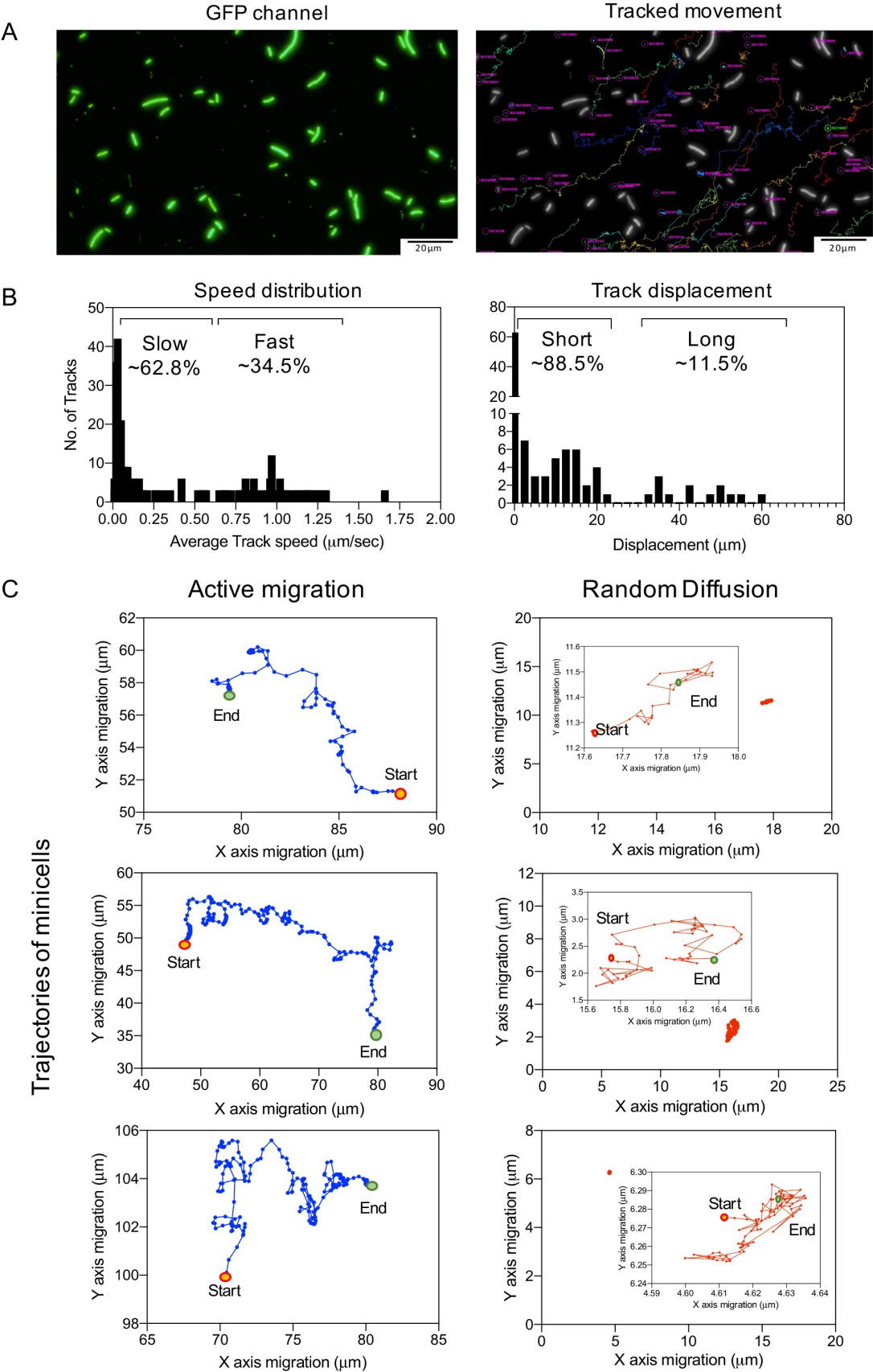


Figure 6.3. Characterisation of mini SimCells motility.

The single-cell trajectory was monitored under a fluorescent microscope for a total duration of 120 secs. Parent cells were fixed. A) Images (360 frames) were processed and 311 tracks were isolated for measurements. B) The distribution of average speed and track displacements. C) Two different modes of motion displayed by mini SimCells were shown, each with three examples. The start (yellow) and end (green) of the trajectory were marked with a coloured circle.

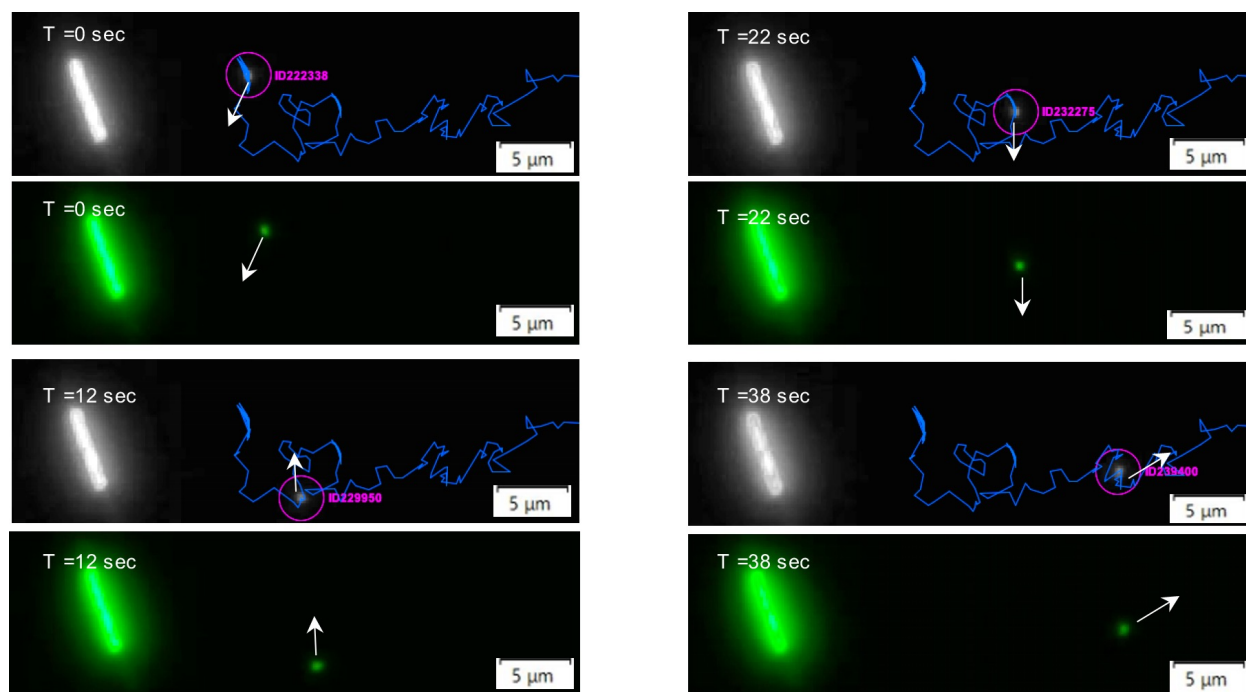


Figure 6.4. Tumble & swim displayed via mini SimCells.

Overlay of processed mini SimCells trajectory (white scale) and green fluorescent channels. Mini SimCells displayed tumbling and running modes similar to that of normal bacteria. Mini SimCells were initially moving downward between T 0 to T 12 sec. Subsequent random tumbling takes place between 12 sec – 22 sec and actively migrated away from parent cells during 22 sec – 38 sec. No attractant gradient was applied. A fixed parent cell is shown on the left as a reference.

Raman analysis of wild type DH5 α . A) *E. coli* MC1000 $\Delta minD$ -derived parent cells and mini SimCells. B) Box plot of normalised peak reads corresponding to the Raman spectrum of DNA. One-way ANOVA tests were carried out. A significant difference between mini SimCells and parent cells was found. ****P<0.0001, ** P<0.005. C) SCRS spectrum of parent cells, mini SimCells and wild type *E. coli*. Each line represents an average of 30 single cells. The Raman signal was normalised and peaks responsible for DNA (785 cm⁻¹, 831 cm⁻¹ and 1575 cm⁻¹) were shaded.

6.3.2 Overexpression of FtsZ promotes segmentation of the cell

To investigate other non-chromosome editing methods to generate the mini SimCells phenotype, I explored a strategy of overexpressing FtsZ to cause mini SimCell production and multiple septa formations, as previously reported [402][199]. To validate such an approach, the *ftsZ* orf from EcN was cloned and constructed into an overexpression plasmid under the control of P_{Bad} promoter, and *tdtomato* orf was also cloned downstream of the *ftsZ* orf. Using strains with chromosomally inserted P_{Bad_GFP} [403], I intend to validate the mini SimCells production efficiency via FtsZ overexpression (Figure 6.6 A). The design should allow a colour distinguishment of mini SimCells (red fluorescence) and parent cells (red and green fluorescence) under L-Arabinose induction. As shown in Figure 6.6 B, there is no sign of mini SimCells exhibiting red fluorescence only.

To our surprise, only cells exhibiting both red/green fluorescence or only green fluorescence were observed. For cells that expressed both fluorescent proteins, multiple septa were also observed, as shown in Figure 6.6 B and Figure 6.6 C. In general, elongated cells with multiply 3~4 segmentation rings contributed 44.5% of the total counts (123 cells), while green-only fluorescent cells contributed the rest (55.3%) and showed no sign of lysis under carbenicillin (100 $\mu\text{g/ml}$) selection pressure. This might be due to the formation of multiple FtsZ rings. Consequently, cells were divided into several non-dividing offspring with partial chromosomes. Some of these non-dividing cells retained the region of P_{Bad_GFP} for green fluorescent production. Figure 6.6 D confirmed the functional induction for both chromosomes and plasmid-encoded circuits. Parent cells with both green and red showed an elongated phenotype ($\sim 1 \mu\text{m}$), which agreed with previously reported literature [199]. However, the contradictory finding of no mini SimCell production means that overexpression of FtsZ alone cannot cause mini SimCell phenotypes. Therefore, for further experiments, *E. coli* MC1000 $\Delta minD$ was used for mini SimCell production.

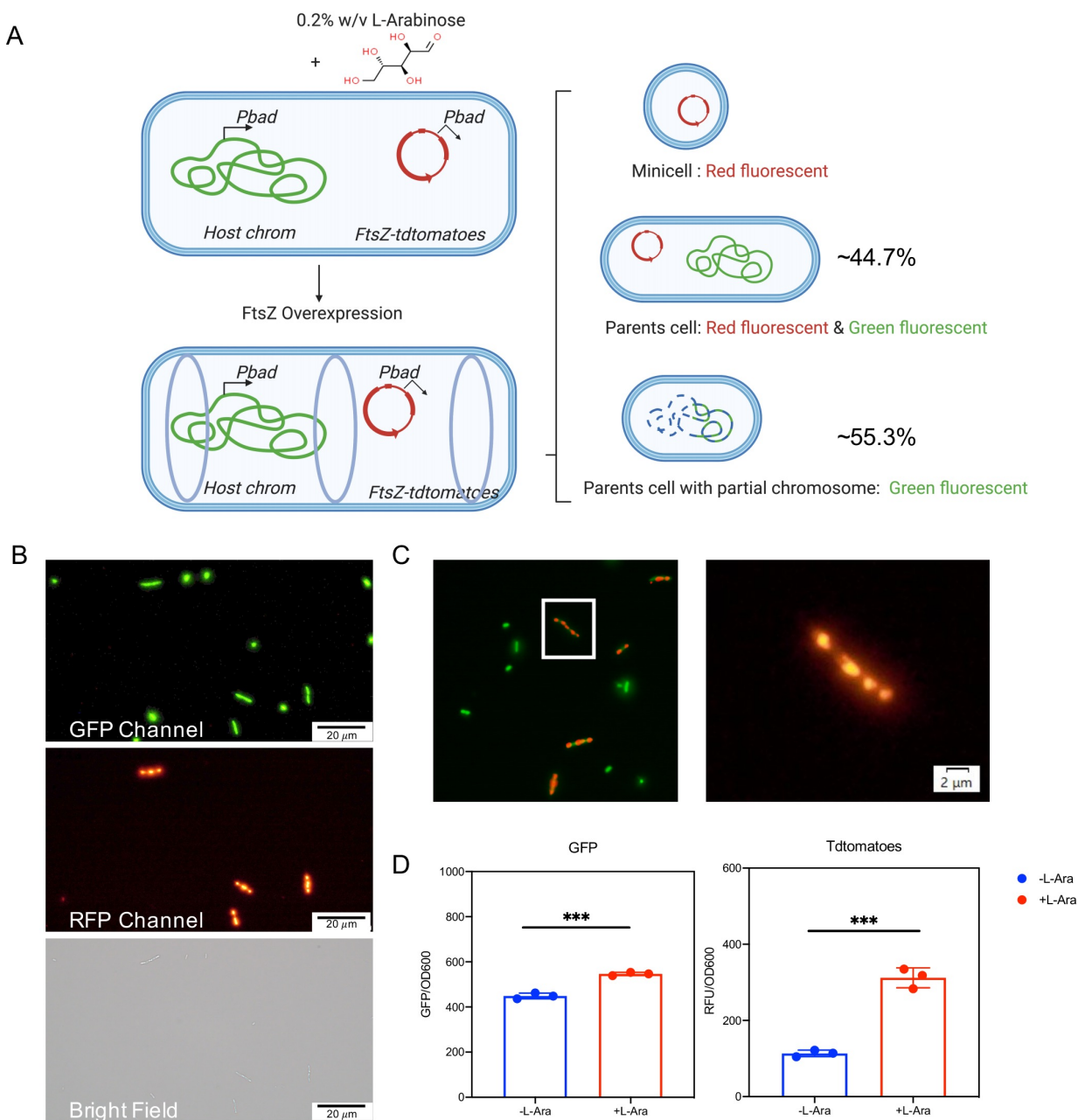


Figure 6.6 Cell segmentation via overexpression of FtsZ.

E. coli k12 with chromosomally inserted P_{Bad} -sfGFP transformed with plasmids P_{Bad} -ftsZ-tdtomatoes. A) Schematic diagram of plasmids and host system used and potential offspring phenotype. The relative population obtained is shown on the right. B) Images of GFP channel (sfGFP excitation/emission 480nm, 510nm) and RFP channel (tdtomato excitation/emission 554nm, 581nm). C) Overlaid pictures indicating cells with plasmids exhibiting segmented cellular structure and multiple Z-ring formations. D) Induction profile of both chromosome GFP and plasmids tdtomatoes using 0.2% w/v L-Arabinose for 4 hrs. N=3. Paired-t tests were used to compare the expression level. *** $P < 0.001$.

6.3.3 Purification and extraction of mini SimCells

To quantify the sensor performance in mini SimCells, pure culture of mini SimCells must be purified then isolated from the mixing pool. In general, existing methods for purifying mini SimCells can be largely categorised into four types: (i) Gradient centrifugation, (ii) Glycerol gradient, (iii) antibiotics elimination, and (iv) filtration. Previous methods often combined and used more than one method to form an overall purification strategy [206][223][203][198]. However, there is no systematic review to identify the sensitive step during mini SimCell purification and extraction. therefore, I optimised the overall process based on the previous in-lab protocol for mini SimCells [198] with additional steps to alter the 1) initial growth condition, 2) chemical lysis of the parent cells, and attempts for 3) mini SimCell homogenisation. As a guide for the selection, the antibiotic should not affect the transcription and translation function of mini SimCells. To screen for an optimal candidate, as shown in Figure 6.7, I transformed the *E. coli* MC1000 $\Delta minD$ with P_{Sal} -gfp-salR (aspirin sensor), purification of the transformed mini SimCells were then carried out and tested with four different antibiotics.

Three different types of cell-wall-inhibiting antibiotics derived from the β lactam family (Penicillin G sodium, Ceftriaxone and Cefotaxime) were tested for parent cell elimination, while Mitomycin C (transcription inhibiting) was used as a negative control. Appendix Figure D-1 indicated that all four cases showed negligible OD growth and maintained a functional dosage response of aspirin when using penicillin G sodium and Ceftriaxone. However, the plate counting assay of the mini SimCells filtrate (Appendix Figure D-1) demonstrated that none of the antibiotic candidates could achieve complete parent cell elimination over 16 hrs. This could be due to some of the slow-growing cells that were insensitive towards to the cell wall lytic antibiotics or were simply mutated under antibiotic selection pressure. To increase the overall elimination efficiency,

I further expanded the library with stable and more potent antibiotics according to (i) those previously used in literature for mini SimCell purification or (ii) new generation of cell wall synthesis-inhibiting compounds of the 4th generation β lactam-derived compounds, cephalosporin showed the most substantial potency (25 μ g/ml MIC) towards parent cell elimination. (Appendix Figure D-2) Therefore, ceftazidime, cefotaxime and penicillin G sodium were chosen for the next stage to make an antibiotic master mix. In Figure 6.7, it was shown that under the treatment of 100 μ g/ml of the antibiotic master mix (equal molars of cefotaxime, ceftazidime and penicillin G sodium), complete parent-cell removal could be achieved even without centrifugation steps. Under 50 μ g/ml of the master mix, there was a clear indication of in the drop of OD reads for cell lysis within the first 2 hrs. To have a better simulation of the actual steps taken in the protocol and to avoid the bias caused by growth phase difference, I tested the killing efficacy of the master mix when directly applied to the overnight culture (stationary phase cells) and incubated overnight at 4 °C. The cell culture became clear and cell-lysed after 4 hrs. It is clear that with multiple types of cell-wall-inhibiting/lysing antibiotics, total elimination of the slow-growing escaper can be obtained. To enlarge the parent cells further, I used an enriched medium with twice concentrated LB, and results indicated parent cells could elongate much longer compared to just using the medium in SOC, LB or M9 (Appendix Figure D-3).

To further remove any cell debris and homogenise the mini SimCell population, one extra step of using a 0.45 μ m pore-mixed cellulose ester membrane (MCE) was added prior to the final collection. The final results with the additional filtration step are shown in Figure 6.8. A 0.45 μ m pore can indeed block cell debris and further homogenise the mini SimCell product. From the DAPI/PI dead–alive staining of the 0.45 μ m filtrate, an estimate of 67.6% yield loss (GFP cells 295/DAPI cells 436) was found. This yield loss can be regarded as a trade-off to achieve higher

lived mini SimCells purity (93%) at the final 0.22 μm collection (GFP cells 80/DAPI cells 86). This is important for the downstream function testing since only mini SimCells with an intact membrane could be induced by the chemicals. Further agar plating confirmed that no viable parent cells were left after 48 hrs (Appendix Figure D-4 B). Using the previously established formula in [206], the optimised protocol can obtain a final yield of 5×10^9 mini SimCells/ml with high purity (>99.99%) and no parent cell contamination.

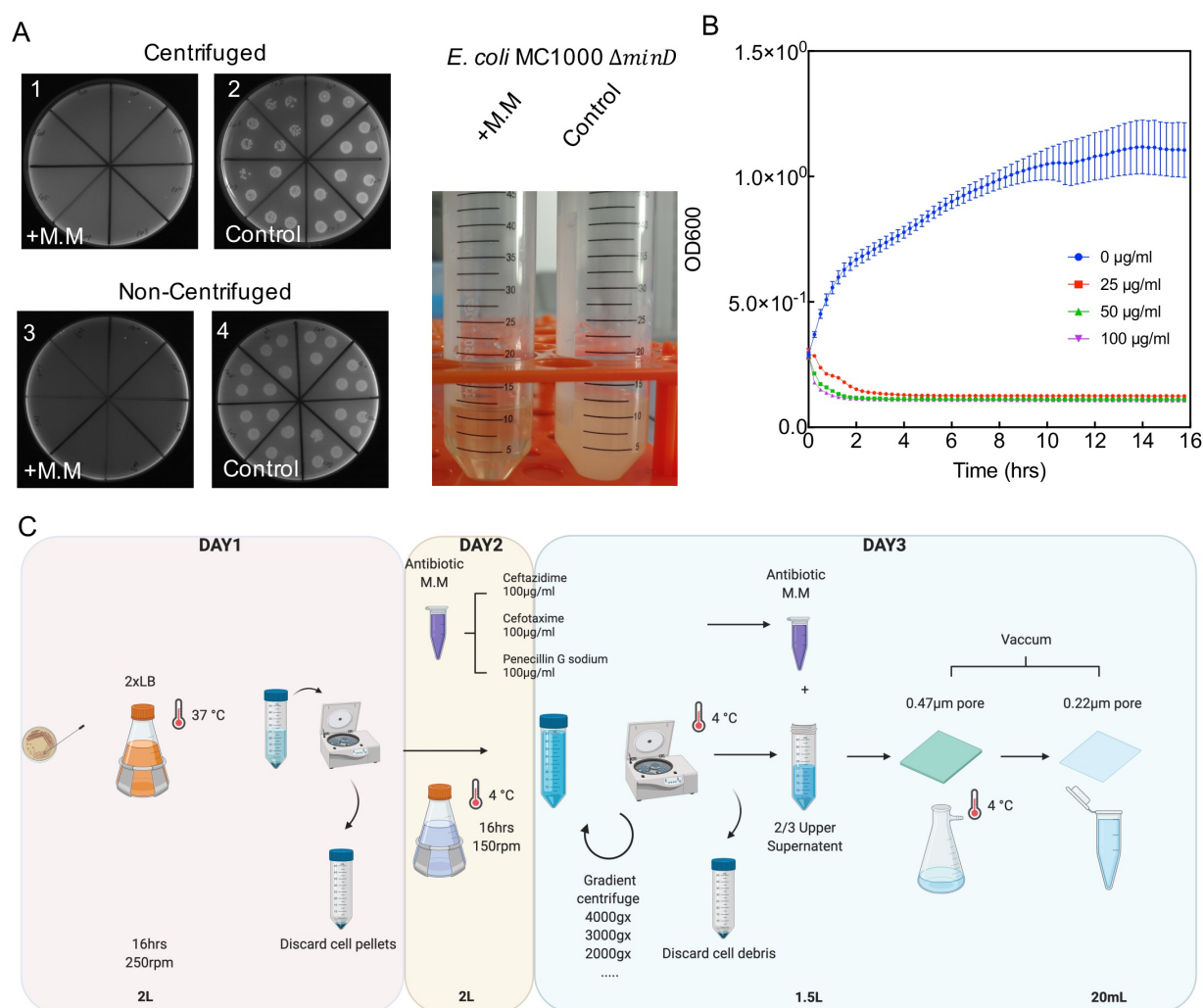


Figure 6.7. Mix cocktail with high parent-cell elimination efficiency.

Parent cell removal using master mixed (MM) cocktail (100 $\mu\text{g/ml}$ of Cefotaxime sodium, Cefpirome sulphate and Penicillin G sodium each). A) Centrifuged suspension of 1) treated vs 2) non-treated control. 3) and 4) indicate cell supernatant treated without the centrifuge step. The MM

can eliminate cells at high density (overnight culture) and without the requirement of the centrifuge step. B) OD growth of cells under a MM dilution gradient. N=3. C) A schematic diagram of the workflow is provided at the bottom.

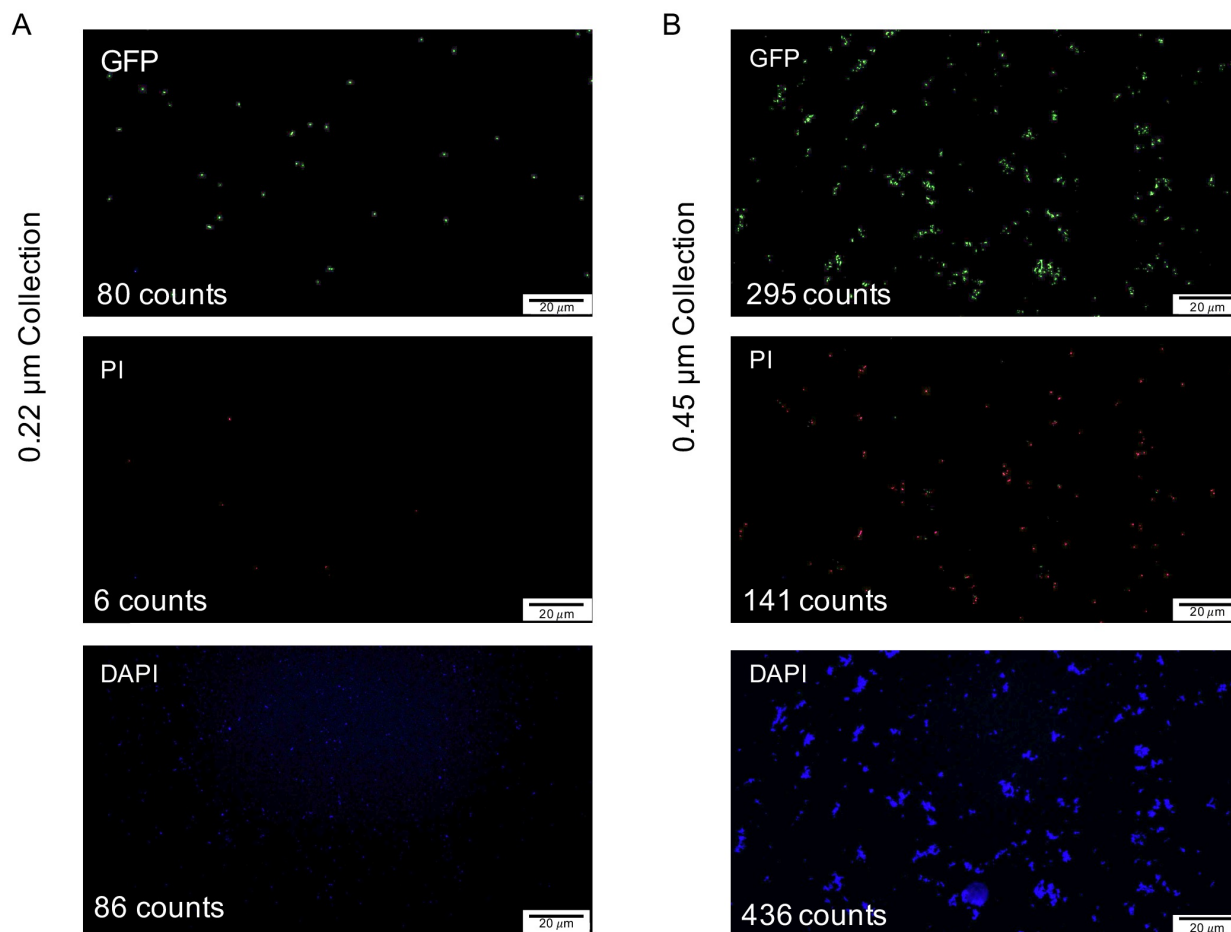


Figure 6.8. Mini SimCell homogenisation with an additional 0.45 µm filter step.

Microscope images of mini SimCells and cell debris after the antibiotic treatment. Mini SimCells collected using an A) 0.22 µm MCE filter. Prior to this, mini SimCells were homogenised using a B) 0.45 µm MCE filter. Both membranes were washed with 5 ml of PBS to dissolve the collected cells and stained with DAPI (Total/Blue) and PI (Dead/red) stains. Magnification $\times 400$. Scale bar = 20 µm.

6.3.4 SalR systems and NorR systems can function in mini SimCells

After establishing a working protocol to extract and purify mini SimCells, the next step was to test the optimised candidate from each control system (SalR, vRecA and NorR) in mini SimCells. As demonstrated in Figure 6.9 A, I implemented the positive feedback format SalR

construct ($P_{Sal_gfp_salR}$) into mini SimCells it could function in both parent cells and purified mini SimCells. In parent cells, the relative fluorescence is significantly higher than that of mini SimCells. In the case of the SalR circuit implementation, under the induction of 50 μ M aspirin, relative fluorescence reads of 86687 ± 1097 RFU were observed, while for mini SimCells, a level of 1467 ± 36 RFU was recorded. SalR mini SimCells also exhibited a decrease in the limit of detection (LOD) of aspirin. $LOD_{mini\ SimCells}$ is 2.5 μ M in contrast to a LOD_{parent} of 0.05 μ M. However, as shown in Figure 6.12 C, mini SimCells exhibited a faster response time at the saturated level (Aspirin = 50 μ M), compared to the 1.2-hrs response time observed in parent cells. Mini SimCells were induced without any delay (0.25 hrs) (Figure 6.9 C). This increase in response time might be due to the large relative surface–volume ratio. The induction kinetics in mini SimCells also displayed a linear pattern different from the sigmoid kinetics in parent cells. This difference in kinetic pattern suggests the SalR system could be susceptible to cellular context-dependence or other unknown regulators missing in mini SimCells. Fluorescent images confirmed the dosage response and no contamination was carried over for mini SimCells loaded with aspirin construct (Appendix Figure D-4).

The NorR-based system was also tested functionally in mini SimCells. As shown previously in Figure 5.6 A, I implemented the optimised $P_{NorV\beta_sfGFP_norR}$ constructs into *E. coli* MC1000 $\Delta minD$. The relative fluorescence intensity is also tenfold higher in parent cells (164100 ± 8161 RFU) than in purified mini SimCells (19076.67 ± 519.3 RFU). Unlike the decrease in sensitivity observed in the SalR cases, NorR mini SimCells displayed a lower detection limit (0.75 μ M) than parent cells (3 μ M). The increase in response time was conserved. As demonstrated in Figure 5.6 D, compared to a delayed response time of 1.25 hrs displayed by the parent cells, mini SimCells showed a faster induction (0.5 hrs) under 500 μ M DETA/NO. In summary, both

σ^{70} - and σ^{54} -based sensory constructs can function in mini SimCells without supplementing additional transcriptional and translational factors (RNAP, sigma factor). These findings ensured that mini SimCells could be used as a modular chassis for most of the genetic tools developed based on *E. coli*.

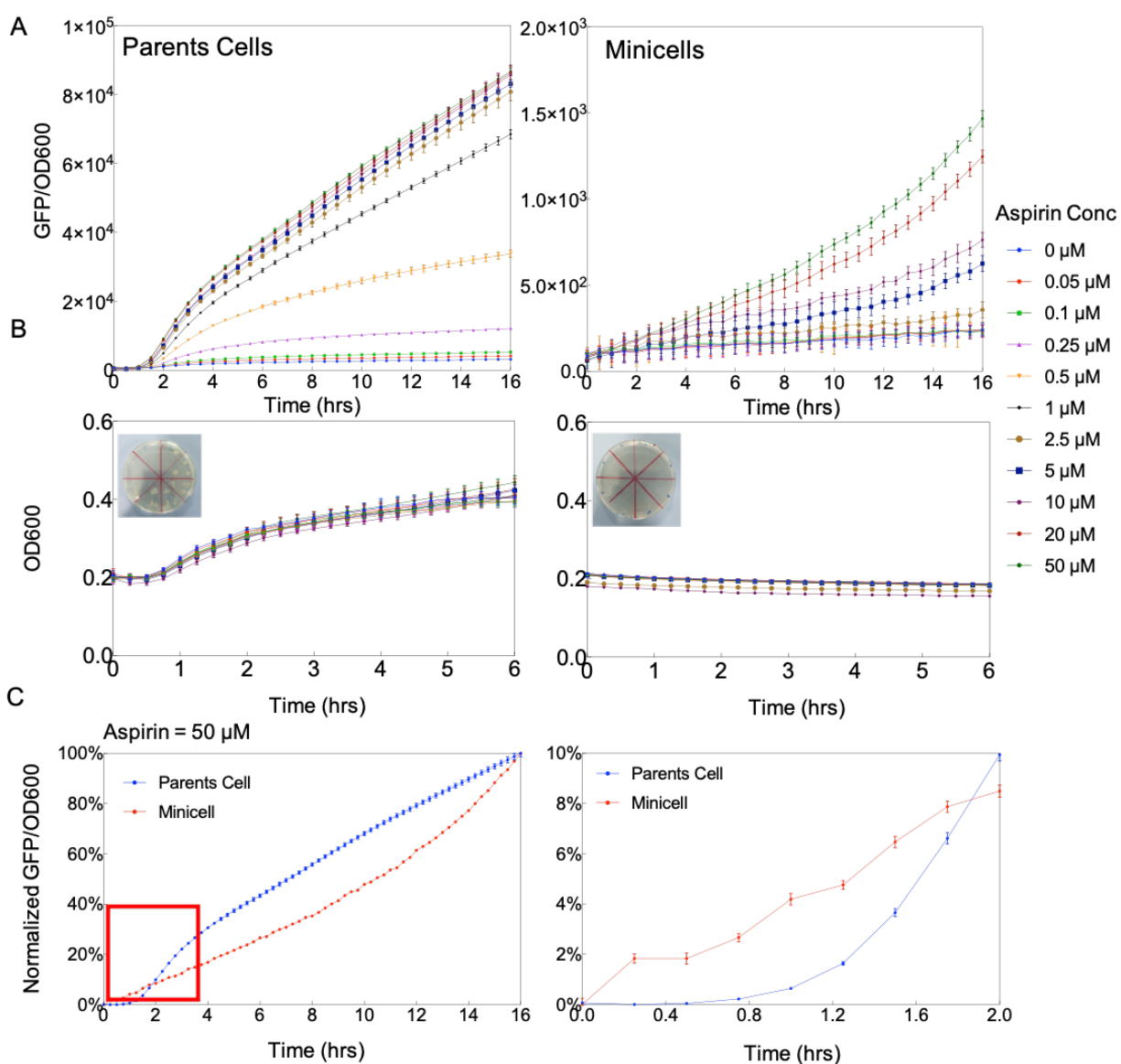


Figure 6.9. SalR circuits implementation in mini SimCells.

A) SalR system induction kinetics of parent cells and mini SimCells under aspirin gradient (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50 μ M). B) OD600 was measured to indicate potential parent cell contamination. Plate assays were done over 16 hrs. No viable parent-cell contamination found in mini SimCells filtrate. N=3. C) Normalised induction kinetics for parent cell and mini SimCells

under 50 μM aspirin (full induced concentration). Relative fluorescence reads were normalised against the highest reads to determine the response time and lagging time. The first 2 hrs were enlarged and shown on the right.

6.3.5 Antisense RNAP collision logic gate tested in mini SimCells

After validating the transcriptional and translational function of mini SimCells, I further investigated the potential of mini SimCells to execute an antisense RNAP collision-based logic gate to validate whether mini SimCells contain sufficient transcriptional machinery to perform complex logic functions similar to that of the wild-type cells. As shown in Figure 6.10 A, the optimised TetR-based antisense logic is fully functional in *E. coli* Mc1000 $\Delta minD$. As the concentration of tetracycline increased, the RNAP complex travelling from the reverse P_{TetO} promoter collided and abolished the forward expression of *salR* transcripts, subsequently leading to the repression of *gfp* transcription. Using this strategy, the leakiness can be suppressed from 3265 ± 217 RFU to 1819 ± 202 RFU. Circuits could also respond to a salicylate gradient (15 μM to 1mM) and displayed a sixteen-fold dynamical range from 3265 ± 217 RFU to 52289 ± 2508 RFU.

In the transformed and purified mini SimCells, the induction pattern of tetracycline and salicylate are retained (Figure 6.10 B). Circuits can respond to both salicylate induction and tetracycline suppression. However, the mini SimCells display a much lower relative intensity than that of the parent cells. As shown in Figure 6.14 C, with the absence of tetracycline and 500 μM salicylate, the circuits expressed a relative fluorescent signal of $(18643 \pm 268 \text{ RFU})$, compared to the control level (absence of both tetracycline and salicylate) of $10698 \pm 324 \text{ RFU}$. Such narrowing of dynamic range could be due to both a lack of energy regeneration mechanisms and building-block recycling within the mini SimCells. In Figure 6.10 D, OD measurement and plate

assay confirmed that each well contained the same number of mini SimCells and no parent-cell contamination.

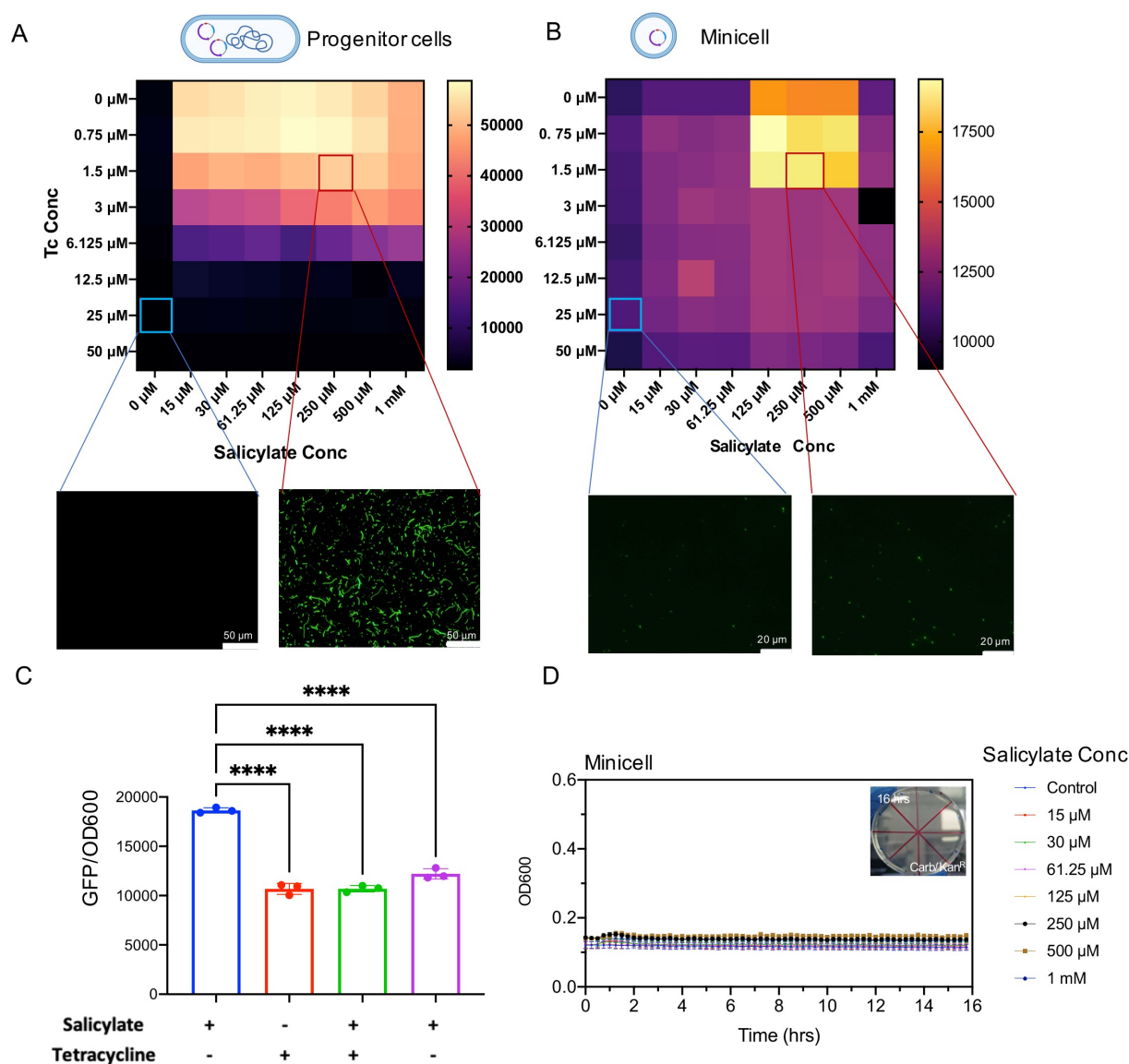


Figure 6.10. Testing RNap collision logic gate in mini SimCells.

A) Heatmap of the induction dynamics in parent cells (*E. coli* MC1000 $\Delta minD$) and B) induction pattern in mini SimCells. Two fluorescent microscope images were shown in the corresponding area of (salicylate 0 μ M, tetracycline=25 μ M) and (salicylate 250 μ M, tetracycline=1.5 μ M). Scale bar = 20 μ m. C) Steady-state relative fluorescent level (T = 16 hrs) of each condition was compared using paired t-tests. **** P<0.0001. D) OD600 measurements and plate assay were shown to indicate no parent cell growth over 16 hrs and each well contained the same number of cells.

6.3.6 Debug of vRecA system in mini SimCells

Unlike the NorR system and SalR system, the vRecA system did not function in mini SimCells. As shown in Figure 6.11 B, the optimised construct (P_{vRecA_AT} -sfGFP) was tested in *E. coli* MC1000 $\Delta minD$ and EcN was used as a positive control. Circuits displayed a strong host dependence and exhibited high basal line leakiness (63245 ± 1899 RFU) in the absence of an inducer (Mitomycin C), compared to its fully induced state (99678 ± 2981 RFU), only a marginal 57.6% increase. To further investigate whether genome *minD* gene knocks out contributes towards the high basal expression, the same circuit was tested in wild type *E. coli* MC1000. As shown in Figure 6.11 A, $\Delta minD$ strains exhibited an almost two-times higher basal expression (19788 ± 154 RFU) than the wild-type *E. coli* MC1000 (11089 ± 118 RFU), thus, it was suspected that upon differentiation from the parent cell, there would be an insufficient amount of LexA in the cytosol of mini SimCells to repress the P_{vRecA_AT} promoter and so it became constitutively expressed. As shown in Figure 6.11 C, there was no significant increase of fluorescence level in mini SimCells upon their induction with 250 nM of Mitomycin C. Conversely, circuits tested in EcN showed a clear induction profile with a much lower basal line. No apparent difference was found in cellular growth between parent cells and EcN.

To compensate for the missing regulator system, the LexA and RecA under their native promoter from *E. coli* Nissle 1917 were cloned and fused with the vRecA constructs into one master plasmid. As shown in Figure 6.12 A, the basal line was suppressed to a near background level (compared to LexA and RecA construct with no sfGFP), which indicated binding of LexA with the P_{vRecA_AT} promoter. However, induction behaviour was also lost when testing with the gradient of Mitomycin C. This suggests the cloned *E. coli* LexA failed to release the P_{vRecA_AT} promoter upon DNA damage or RecA activation, which could be further explained with the

supposition that as the LexA recycling protease (ClpX and Lon) was absent from the system, it in turn failed to facilitate the total release of LexA upon autolysis [404]. There was no significant difference in the final OD reads (Figure 6.12 B), which suggests that the insertion of an extra copy of LexA or RecA does not pose any additional cellular burden.

To our surprise, upon testing the same unified constructs ($P_{V_{\text{RecA_AT_repeats}}}\text{-sfGFP}$ with an additional copy of LexA and RecA) in EcN, the sensor dynamics exhibited both a broadening in dynamic range and a reduction in basal line. As shown in Figure 6.12 A, circuits with the LexA and RecA addition displayed a lower basal line of 1403 ± 84 RFU, compared to the case without (1934 ± 27 RFU). Higher fluorescent output was also achieved under $0.75 \mu\text{M}$ Mitomycin C induction. Unified constructs exhibited the highest relative fluorescence intensity of 6788 ± 382 RFU, a ~ 4.8 -fold change in full induction compared to the ~ 1.2 -fold change of the circuits without an additional regulator copy. Such amplification in sensitivity and reduction in baseline could be explained as both the DNA damage sensor module (RecA) and promoter repressing module (LexA) were increased. However, further experiments are required in LexA-deleted mutants to quantitatively capture the effect of RecA protein dynamics on overall SOS response dynamics.

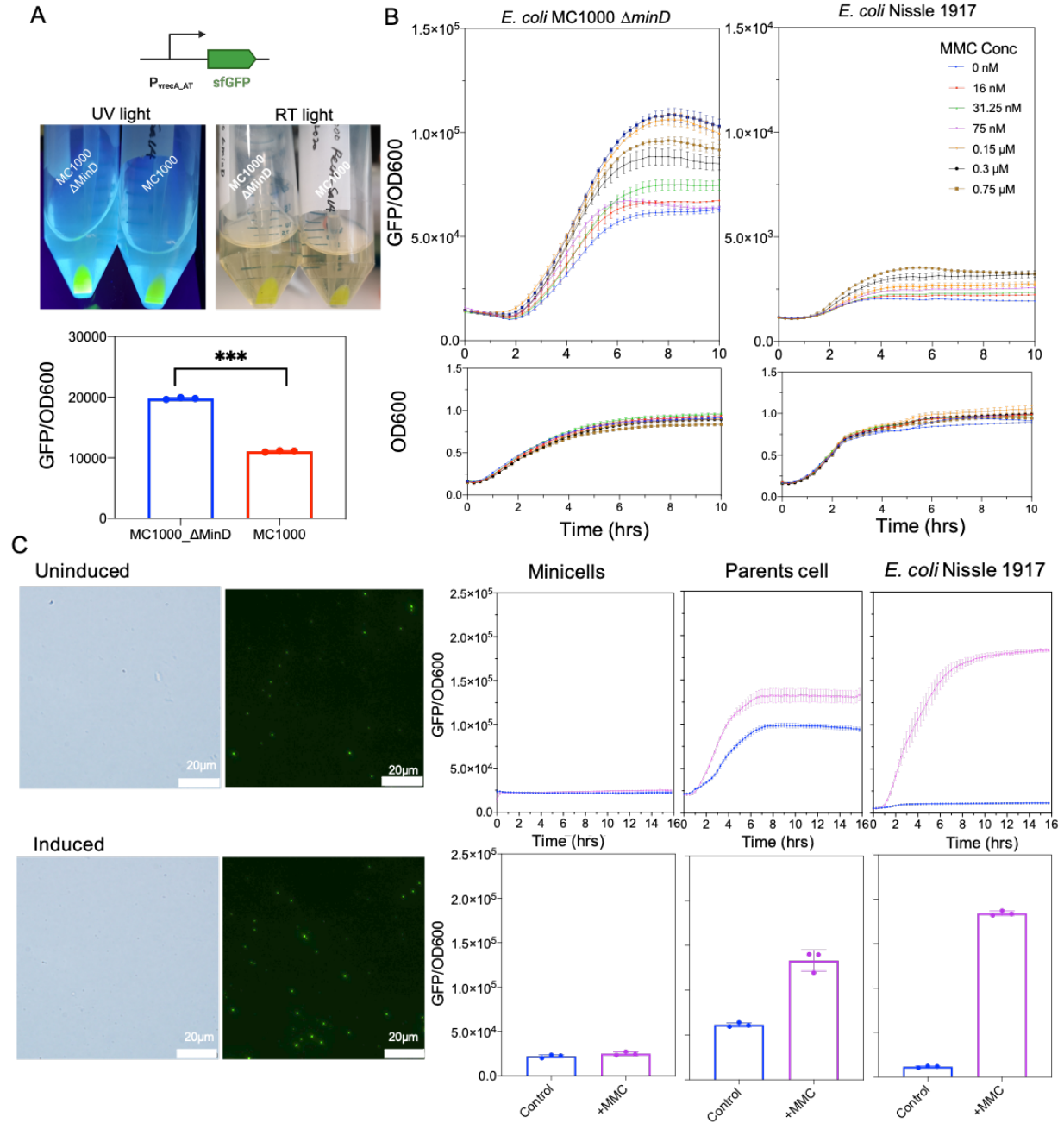


Figure 6.11. Constitutive expression of sfGFP under P_{VRecA_AT} promoter in mini SimCells.

A) Schematic diagram of the optimised constructs (P_{VRecA_AT} -sfGFP) and their background leakiness in *E. coli* MC1000 wild type and *E. coli* MC1000 $\Delta minD$. 302 nm UV light was used as an arbitrary way of indicating the fluorescence intensity of the centrifuged cell pellets. B) Induction kinetics of the same circuits tested in *E. coli* MC1000 $\Delta minD$ and EcN (*E. coli* Nissle 1917) with a gradient of Mitomycin C (0, 16, 31.25, 75, 150, 300, 750 nM). C) Induction kinetics of isolated mini SimCells, parent cells and EcN with 250 nM of Mitomycin C. Steady-state relative fluorescence level is shown (T = 16 hrs). N=3. Microscope images of mini SimCells are shown in both bright field and GFP channel. Scale bar = 20 μm .

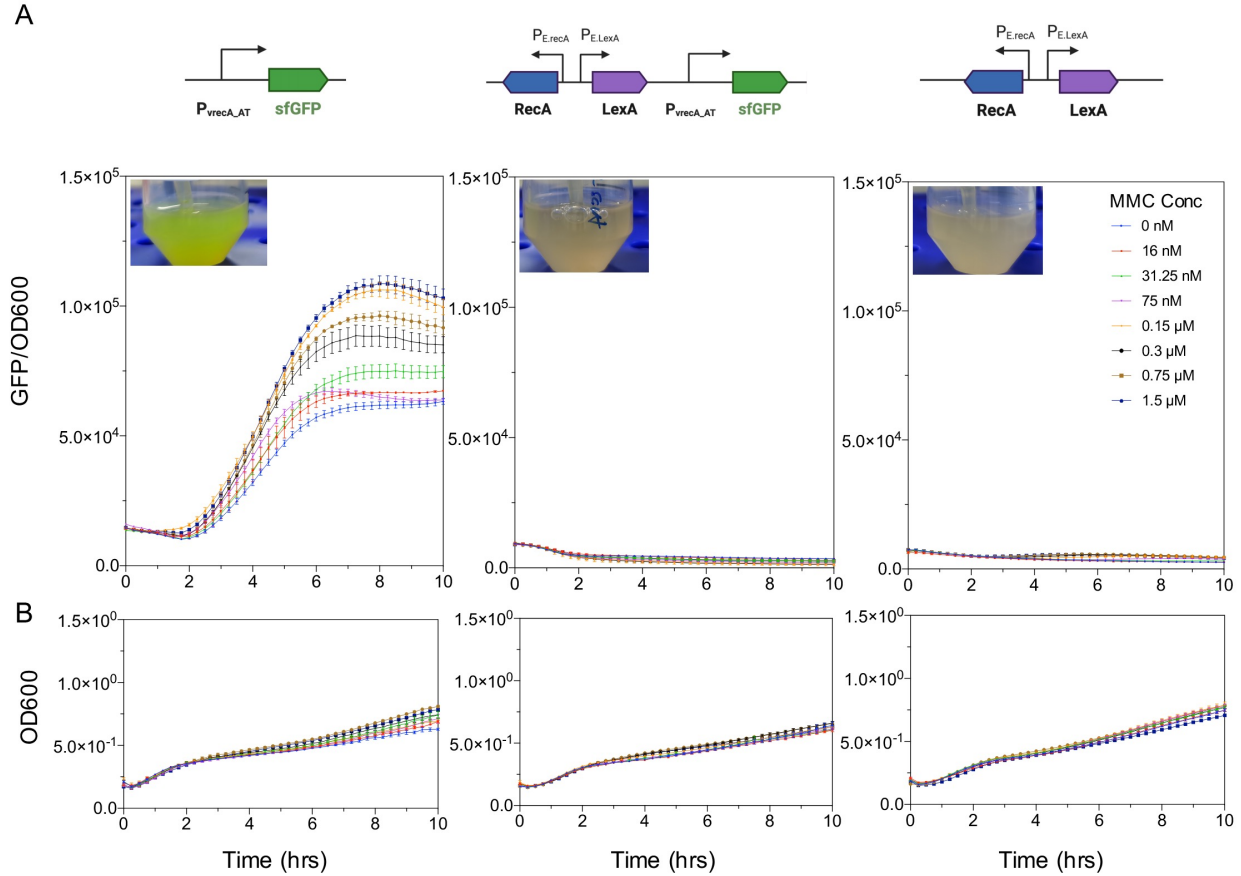


Figure 6.12. Circuits function with the addition of regulator RecA and LexA.

A) Induction kinetics of the original P_{vrecA} -sfGFP circuits, P_{vrecA} -sfGFP circuits with an additional copy of regulator (LexA and RecA), under its native promoter. P_{RecA} $recA$ P_{LexA} $lexA$ with no sfGFP was used as control. The assembled construct was tested in *E. coli* MC1000 $\Delta minD$. Mitomycin C gradient used was (0, 16, 31.25, 75, 150, 300, 750, 1500 nM). B) OD600 measurements for cell growth. N=3.

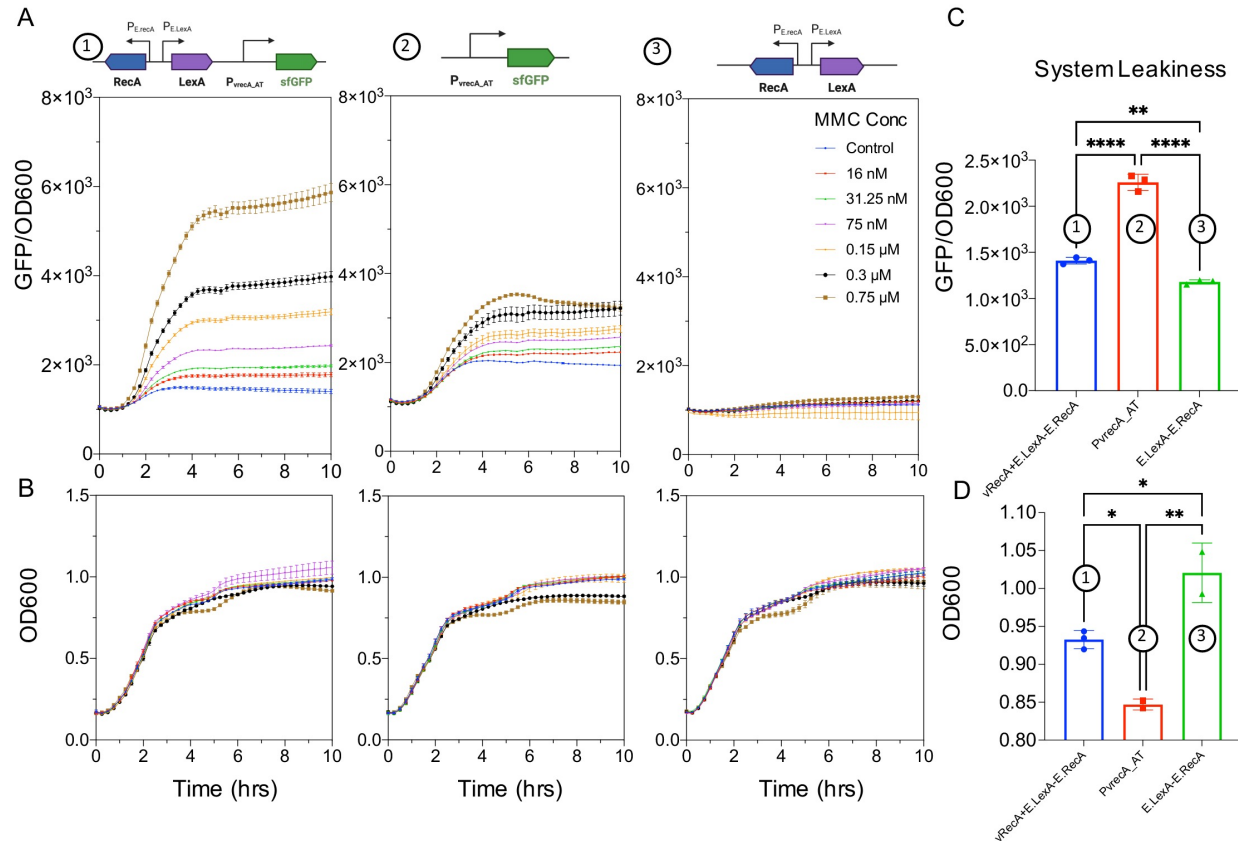


Figure 6.13. Performance improvement of the unified construct in EcN.

A) Induction kinetics of the unified constructs (P_{vrecA} -sfGFP with an additional copy of regulator), original P_{vrecA} -sfGFP and regulator only tested in EcN. Mitomycin C gradient used was (0, 16, 31.25, 75, 150, 300, 750 nM). B) OD600 measurements for cell growth. N=3. C) A paired t-test was carried out to determine the significance of the basal line difference and final OD. (T = 16 hrs) **** $P < 0.0001$ ** $P < 0.05$ * $P < 0.05$

6.3.7 Therapeutic cargo design: converting aspirin into catechol

After obtaining an optimised construct based on the vRecA system in EcN, this inspired us to further extend its potential medical usage with a therapeutic cargo module, *salA*, which was isolated originally from the *salAR* catabolic cluster that catalyses the conversion of salicylate into catechol. Its mechanism is shown in Figure 6.14 A. Aspirin was first converted to salicylate under non-specific esterase by *E. coli* and self-hydrolysis (Appendix Figure A-6). The non-specific esterase activity was quantified using an FDA assay and shown to be common to most of the *E. coli* subtypes (BL21DE3, DH5 α , S17 and EcN), but couples to the growth phase of the cells (Appendix Figure A-7). The salicylate was then oxidised by SalA and converted into catechol, which is a dark brown colour with an absorbance peak at 275 nm light (Figure 6.14). Catechol displays a strong anti-inflammatory effect and has been associated with cancer-specific suppression [393][394]. I added the codon-optimised *salA* gene (codon optimised based on EcN codon usage) into P_{VRecA_AT}_sfGFP and the modified P_{VRecA_AT}_sfGFP with additional regulator copy (LexA and RecA). As shown in Figure 6.14 B, the circuits demonstrated tight control (OFF) under the absence of an inducer (Mitomycin C) and no production of catechol was noted with supplementation of 1 mM of salicylate. Both systems can only be turned on with the supplement of both salicylate and Mitomycin C. In Figure 6.14 C, it was shown that under 37 °C, within 16 hrs, using 250 nM of Mitomycin C and 1 mM of salicylate, 93.2% of the salicylate can be converted into catechol by the unified construct (pUnified_LexA_RecA_salA) based on a standardised curve of catechol concentration (Appendix Figure D-5), while only 78.1% can be converted in the original constructs (P_{vrecA}_sfGFP_salA). This boost in SalA expression quantity is consistent with the previous amplification in GFP signal shown in Figure 6.13. This provides a functional therapeutic module to combine with other tightly regulated systems constructed in this work, making it useful for anti-inflammatory treatment or cancer-killing.

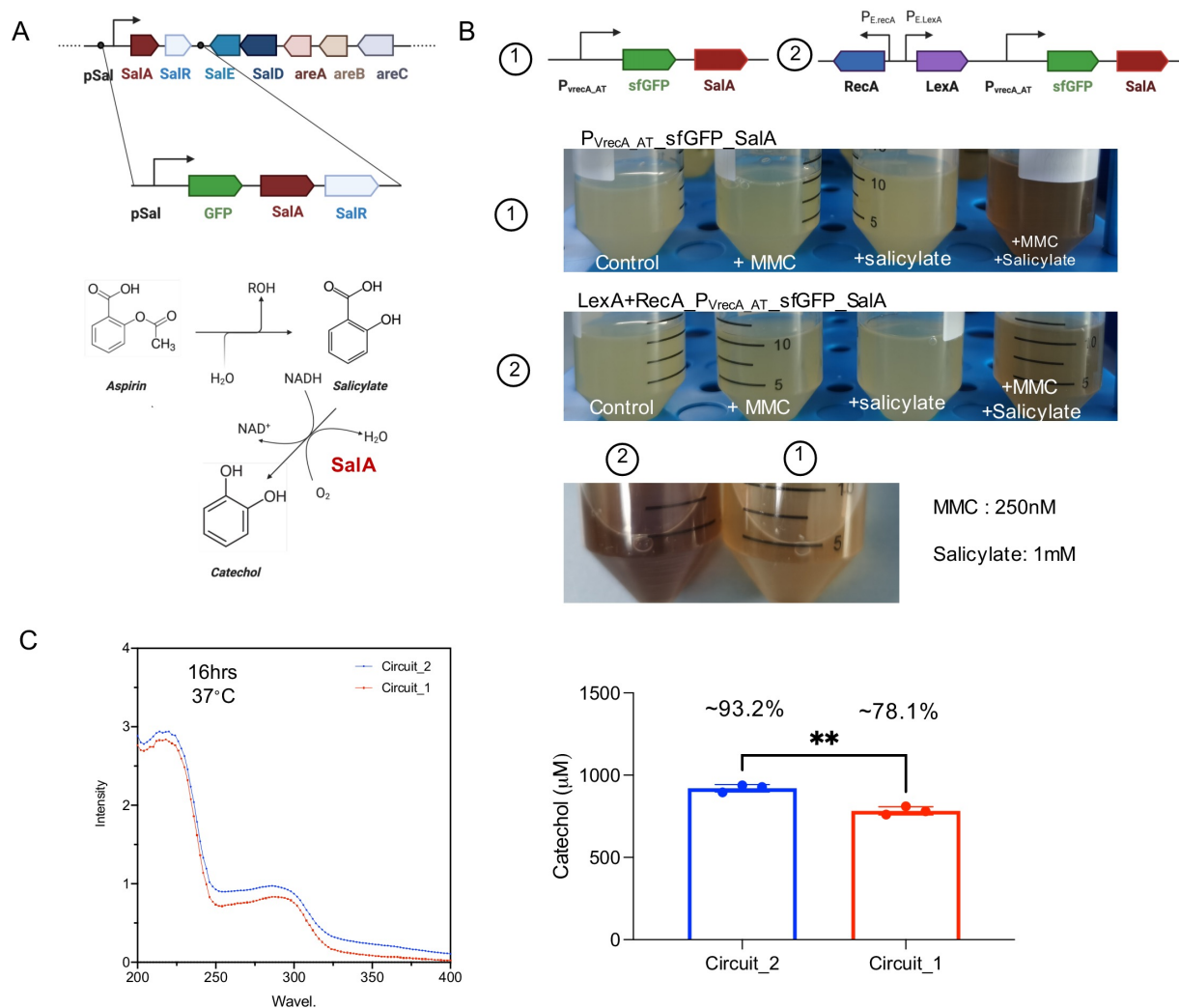


Figure 6.14 Conversion of aspirin into therapeutic catechol in EcN.

A) Schematic map of *salAR* catabolic operon and original P_{Sal} -gfp-*salA*-*salR*-based aspirin sensors construct. *SalA* can catalyse the oxidation of salicylate into catechol. B) Addition of codon-optimised *salA* orf to $P_{VRecA_AT_sfGFP}$ and unified $P_{VRecA_AT_sfGFP_LexA_RecA}$, both circuits were tested in EcN and induced under Mitomycin C (250 nM) and salicylate (1 mM) C) The final converted catechol was quantified using peak absorbance at 275 nm and standard curve. The results indicate the majority of salicylate can be converted by the end of 16 hrs at 37 °C. Paired t-tests were carried out for the final catechol concentration between each circuit. ** $P < 0.005$, $N = 3$.

6.4 Discussion

In this chapter, the *E. coli* mini SimCells were thoroughly characterised from various angles, including their size distribution, motility profile and the Raman spectrum. It was evident that *E. coli* mini SimCells lie in the range of 100–400 nm in diameter, the majority of the mini SimCells contain sufficient energy to migrate over 40 times their body length ($< 20 \mu\text{m}$) and that a small population, 11.5%, can migrate 80~150 times ($35 \mu\text{m} \sim 60 \mu\text{m}$). This provides potential clinical implications for penetrating the blood–tumour barrier (BTB) (300 nm~4700 nm) into a tumour’s necrotic core [405][397]. Furthermore, the fast movement speed could be useful for tumour colonisation with lower density and a faster time, as evident in previous studies indicating that overexpression of *flhCD* in *Salmonella* increased the tumour colonisation five times more than control strains [165]. However, while most of the whole-cell chassis display chemotaxis towards the tumour core, it is still unclear whether mini SimCells could retain such function, thus, further microfluidics-based chemotaxis experiments could be derived as a natural follow-up to address this point. Next, the chromosome loss in mini SimCells was confirmed using the single-cell Raman spectrum, which provides a potential label-free chemical marker for high-throughput Raman active cell sorting [401].

To obtain mini SimCells with high purity, I built a combinatorial approach to isolating mini SimCells based on [206] and [6]. A final yield of 5×10^9 mini SimCells/ml can be achieved without any parent cell contamination (total cell removal in overnight culture). In contrast to previous methods [198], this offers the highest efficiency in host cell clearance and practicability. To validate whether the mini SimCells can be a universal chassis system for the designed circuits, two transcriptional controls (*SalR* and *NorR*) from family members of σ^{54} and σ^{70} were tested for functionality in mini SimCells. The results showed that mini SimCells could execute biosensor function with an increased response time but lower relative intensity. This makes sense since mini

SimCells only contain a fraction of the cellular component from their parents ($\sim 0.45 \mu\text{m}^3$ cellular volume, which is fifty times smaller than the volume of parent cells) and lack energy regeneration modules. Still, they contain sufficient energy to consistently express sfGFP under aspirin induction for 16 hrs, as shown in Chapter 3 [141]. This provides an adequate time window for most medical applications. To further investigate the maximum working time of mini SimCells, inducible circuits with either *ssrA*-tagged sfGFP or *LuxCDABE* could be used and induced repeatedly to measure the total duration of functional induction cycles. Instead, I further tested mini SimCells with an RNAP collision-based transcriptional logic gate to validate whether two negatively coupled exogenous promoters would function similarly to the wild-type strain. The mini SimCells can indeed respond to both tetracycline and salicylate control to reproduce the quasi-XOR gate function. This paves the ways to many higher-ordered logic functions in decision making, such as building cellular memory and bandwidth filter.

To our surprise, the $P_{v\text{RecA}}$ promoter did not function as predicted in mini SimCells. I initially reasoned that this might be caused by an insufficient amount of regulators (LexA and RecA) present in the mini SimCells cytosol. However, with an additional copy of the regulator (LexA and RecA), the sensor exhibited a much lower basal fluorescence intensity but still failed to respond. This led us to suspect that protease (ClpXp and Lon) might also be also required for mini SimCells to execute the full release of LexA [306]. As previous literature indicated, insufficient autolysis of LexA could leave its N-terminal domain to bind and repress the promoter [306]. Upon implementing the unified constructs into a probiotics chassis (EcN), unexpected signal amplification and basal line reduction can be achieved. To build upon this finding, I linked and characterised a salicylate catechol-converting enzyme, SalA, with the unified construct in EcN. After coupling the codon-optimised SalA to the unified vRecA constructs, the results demonstrated

a tight control in the OFF state, while in its ON state, an efficiency of ~93.2% for conversion of salicylate into catechol was achieved. These advantages in terms of tight control (low baseline expression) and safety (probiotics chassis) would be useful for the future *in-situ* application. To conclude, mini SimCells could offer several unique advantages over the whole-cell chassis such as increased response time, higher induction fold and intact motility. However, certain design constraints might need to be considered for systems dependent on multiple cytosolic regulatory proteins.

Chapter 7 Conclusion and future works

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7.1 Key findings summary

Bacteria therapy offers several unique advantages over traditional cancer therapy and conventional treatment for chronic inflammatory diseases, making engineered synthetic living microbes one of the most promising applications of synthetic biology. However, due to the lack of accurate control mechanisms and concerns over biosafety, the clinical translation and commercialisation of bacteria therapy lag behind compared to other synthetic biology applications such as the optimisation of large-scale synthesis of valuable pharmaceutical products or the bioremediation of environmental pollutants.

In this work, I investigated and constructed three different kinds of transcriptional control systems. Each provides a unique control route that can be seamlessly combined with existing clinical practice (Figure 7.1). The end goal is to provide an integrated toolbox tailored for bacteria therapy that allows both external and internal regulation of the expression timing, dosage control and spatial activation. The constructed biosensors system allows three different control routes via (1) an orally ingestible drug (Chapter 3), (2) spatial control using UV or DNA-damaging

chemotherapeutic drug Mitomycin C (Chapter 4), and (3) internal disease biomarker nitric oxide (Chapter 5). The dynamical properties of the optimised toolbox are summarised in Table 7.1, Table 7.2 and Table 7.3, respectively.

A guideline for the ideal system characteristics was followed to fine-tune our transcriptional control systems. These requirements were: 1) Fast response time. The gene circuits need to be fully activated before the inducer chemicals are cleared by the human system. i.e., $T_{\text{response}} < T_{1/2}$ of the inducer. 2) Tight control. The system needs to demonstrate near-baseline leakiness that ensures low systematic toxicity to the patient and avoids any burden-derived mutation in genetic circuits during long term colonisation. 3) Medically relevant sensitivity and operational range. The activation threshold of the transcriptional circuits needs to be fine-tuned to a level that is sufficient to distinguish health/disease states or is lower than the toxicity level used in clinical practice. 4) Robustness. In the complex environments found within the human gut and heterogeneous tumour tissue, the designed circuits need to sustain their induction characteristics against external noises such as changes in pH, oxygen, or nutrient condition.

Nevertheless, during the actual optimisation process, there is often a trade-off between the different characteristics of the system. These requirements can take different orders of priority when considering the application niche. For instance, for an inducer chemical with a short half-life such as aspirin, the system needs to be fully induced before the inducer cue is degraded. This makes system response time critically essential and should be the first parameter to optimise. A different case is the hypersensitive SOS DNA damage sensor, where a transcriptional amplifier was added to increase its sensitivity, but this would also lead to unwanted amplification of the noise and make it more vulnerable against a constantly fluctuating environment. Therefore, robustness becomes the top priority for using a transcriptional switch operating in the nanomolar

range of inducers. Next, for a sensor that is used as a pathological disease detector, the priority is not only to distinguish between healthy state and disease state but also provides a semi-quantitative measurement of the pathological cue. This would require the activation threshold and operational range to be carefully balanced. Lastly, for cargo gene expression that is either highly toxic or metabolic intense for the cells, a tight control would be preferred for safety and efficacy purposes.

7.1.1 Integrated transcriptional toolbox tailored for bacteria therapy

To construct an orally ingestible control using a safe pharmaceutical drug (Chapter 3), a novel aspirin-responsive regulatory system was developed by employing the SalR system originally from *Acinetobacter baylyi* ADP1 and placing it into *E. coli* Nissle 1917 for characterisation of induction dynamics. The different regulatory design was induced in a dose-dependent manner in the presence of aspirin with a LOD level of 0.05 μM and response time within 50 minutes. By building an RNAP-based antisense NOT logic gate with antisense promoter ($P_{\text{tetO-TetR}}$), the expression level of the SalR could be fine-tuned, and the circuit output (GFP) displayed a near-background-level of tight control, making it a suitable system for delivering highly toxic cargo. In contrast with the previous existing case using a *P. putida*-isolated XylS regulator and NahR system [79], the SalR system displayed a much higher sensitivity ($\times 4000$) in its response to aspirin. Considering that the safe clinical dosage of aspirin for an adult is 75–300 mg per day, and therapeutic blood concentration for adults is between 111 and 555 μM , whereas toxic concentration is between 832 and 1665 μM [406], the optimised SalR system offers a much safer alternative using EcN as a chassis system for aspirin as a triggering mechanism.

Beyond this, constructing a spatial trigger that bypassed limitations relied on chemical diffusion. A highly sensitive DNA-damage responsive *recA* promoter was constructed in Chapter 4. P_{VRecA} from *Vibrio natriegens* was characterised, cloned and expressed into the probiotic strain

E. coli Nissle 1917. This was then rationally engineered according to predicted interactions between the LexA repressor and P_{VRecA}. The redesigned P_{VRecA-AT} promoter demonstrated a sensitive and robust response to DNA damage induced by UV exposure and genotoxic compounds. Further rational design of the P_{VRecA} coupled to a signal-amplification gene circuit increased the circuit output amplitude by 4.3-fold in response to a DNA-damaging compound mitomycin C. A TetR-based negative feedback loop was added to the P_{VRecA-AT} amplifier to achieve a robust SOS system that was resistant to environmental fluctuations in parameters including pH, temperature, oxygen and nutrient conditions. I also showed the potential of this P_{VRecA-AT} system as an optogenetic actuator, which can be controlled spatially through UV radiation. The final optimised hypersensitive circuits can be triggered via 6.125 nM Mitomycin C or 1 second of UV 302nm exposure; this provides a sufficient activation threshold when used in combination with conventional chemo/radio therapy, minimal dosage of radio/chemotherapy cues are required to turn on synthetic circuits without damaging the healthy tissue or causing other side effects, thus lowering the overall systematic toxicity.

Finally, through deciphering the probiotic sensory system of nitric oxide, which is an important disease biomarker found in many chronic inflammatory diseases and cancer, in Chapter 5, I developed a series of inducible systems based on the *norVW* operon isolated from *E. coli* Nissle 1917; the resulting sensor constructs can detect 15 μ M of DETA/NO within 25 minutes. Using a similar fine-tuning approach developed in Chapter 3, by tuning the constitutive promoter strength, overexpression of NorR results in the suppression of the output signal strength but not to the baseline level. The host endogenous copy of *norR* regulon also interfered with the synthetic constructs and suppressed its overall output intensity. The induction behaviour was further characterised in a positive feedback format to fine-tune the level of NorR. I examined the impact

of promoter variation through deletion/addition of an integration host factor binding site, providing that IHF2 was not essential for the induction behaviour. Instead, the deletion amplified the signal output by 25.3%, with no significant increase to the baseline. A substantial host interference caused by nitrogen depletion was noted when altering the induction medium. This can be successfully bypassed by using chromosome-free mini SimCells, thus eliminating the global regulator and potential competition of the σ^{54} pool. These findings fill the previous gap of identifying the relative contribution of each promoter component towards the overall dynamics of the NorR system.

7.1.2 Competitive binding of the regulator as a universal tuning strategy

Due to the different molecular background mechanisms involved in each case, a combinatorial tuning strategy was used in the fine-tuning process. (Figure 7.2) Nevertheless, I mainly focused on the interaction between regulator and promoter. For the SalR and NorR systems, I noticed that both regulators exhibited competitive binding mechanisms despite their intrinsically different activation mechanisms. The regulators could act as a dual repressor/activator towards the overall system's dynamics depending on the inducer presence. This could be used as a convenient universal tuning strategy for both σ^{54} - and σ^{70} -derived systems, simply through overexpression of the regulator to reduce the system leakiness or to probe the optimal expression level for amplifying the output as showcased in the NOT logic RNAP construct. In contrast with the classical mathematical expression of activator or repressor, the results indicated here that a finite equilibrium between two forms of regulator needs to be considered for future model construction. In the case of the P_{vrecA} system, the addition of an extra copy of the regulator boosted both the sensitivity and the dynamical ranges. However, due to the different mechanisms involved in SOS activation, we cannot conclude that the RecA/RecA* also exhibited a similar effect on LexA. This does emphasise the intracellular regulator level as the critical factor towards overall system

dynamics, thus should be a priority over other components (BRS, terminator and backbone ori) during the fine-tuning process. A detailed understanding of the regulator's interaction with the DNA needs to be defined before the DBTL cycle begins.

Another key strategy adopted in tuning the vRecA and NorR systems is via the rational design of a chimeric promoter. In vRecA, by modifying the GC content within the key regulator binding site (LexA), one can predict the binding affinity and relative dynamic performance from a thermodynamical angle. Furthermore, in the case of NorR, by deleting one non-essential IHF site (DNA twisting machinery), the system's induction output can be increased. This suggests that DNA architecture plays a vital role in transcription regulation and can potentially be used as a tuning knob for the future design of tightly controlled genetic circuits. The studies performed on the vRecA and NorR provides a well characterised and annotated foundation for the future design of signal integration chimeric promoters.

7.1.3 Mini SimCells as a versatile bacteria-bot for a functional biosensor

Beyond the aspect of genetic software, the emphasis on biosafety was enhanced by using probiotics chassis EcN and mini SimCells. All circuits were characterised and optimised in EcN instead of a more traditional *E. coli* lab strain. This ensured little discrepancy caused by host difference in later *in vivo* application. To ensure its genetic stability and to further strengthen the biosafety, I implemented the abovementioned circuits into *E. coli*-derived mini SimCells. The results demonstrated that both SalR and NorR could indeed function in mini SimCells with faster response time and higher induction fold. This could potentially be due to the high surface–volume ratio. Conversely, the P_{vrecA} system lost its induction function and exhibited constitutive expression upon differentiation into mini SimCells. An additional copy of its regulator LexA-RecA did suppress the leakiness; however, the circuits become unresponsive towards the inducer. This

indicates potential design constraints for mini SimCells implementation. Despite the fact that mini SimCells contain sufficient sigma factors and RNAP complex for transcription and translation, the regulator itself needs to be encoded onto the plasmids to fire the initial burst, the releasing and recycling of the repressor may also be needed to make a functioning biosensor. As shown in Table 7.4, all the working regulators that have been implemented in mini SimCells display either dual activator/repressor activity (SalR, NorR, AraC and CdaR), or in the case of TetR & AcuR system, the regulator simultaneously releases itself upon inducer binding (a high degree of cooperativity). Nevertheless, the sum of all working regulators validated the potential of using the mini SimCells as a functional biosensor platform. In this work, another key challenge I addressed was the purification and extraction of mini SimCells. The optimised protocol using both two-step filtration and an antibiotics cocktail demonstrated a high purity and yield (5×10^9 mini SimCells/ml) for mini SimCells production. This allowed us to have sufficient samples for the systematical characterisation of its motility, Raman fingerprint and size distribution. The small size (100 nm~400 nm) and high motility could make it an ideal candidate for building a bacteria-bot since most nanoparticle-derived swimmer bots face challenges in overcoming the Brownian effect and sustaining movement using limited energy. Furthermore, I addressed and validated the question regarding long-term storage. A freeze-dried mini SimCells with preloaded sensor constructs can be stored at room temperature and maintain its functionality over two months. This result demonstrated promising commercial potential for mini SimCells without the need for cold-chain transportation, thus lowering the overall cost while enhancing safety.

Transcriptional control for bacteria therapy

Different external/internal control system developed in this study

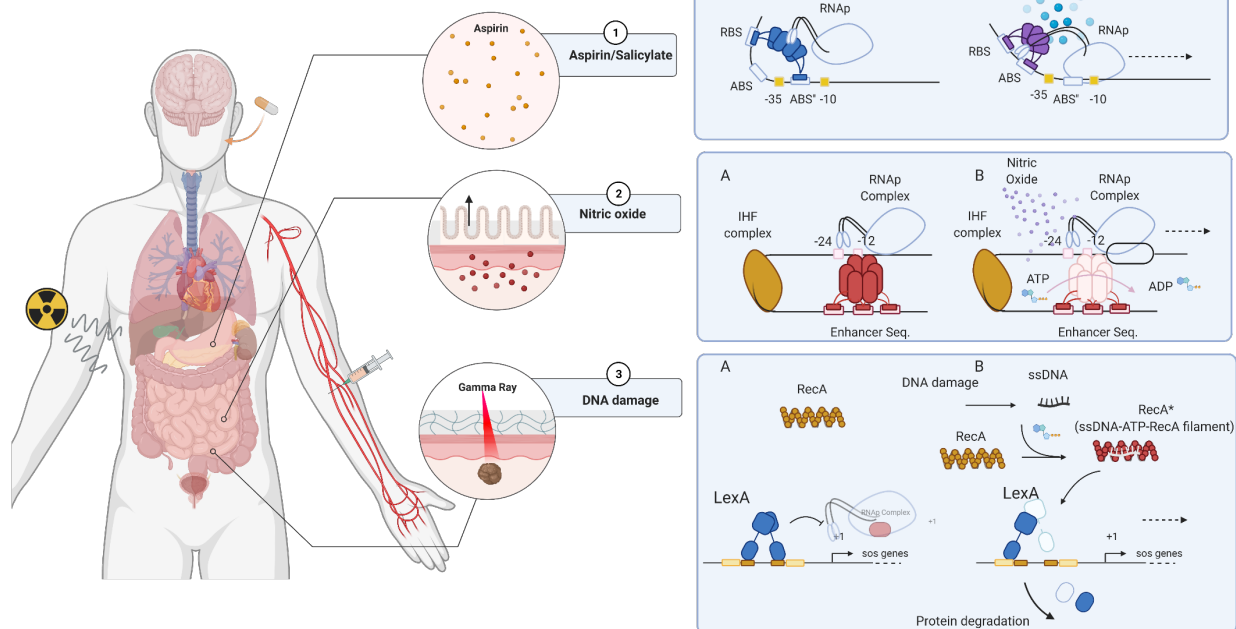


Figure 7.1 Overview of transcriptional control systems developed in this study

The molecular detail is summarised in the box with inducer absence (A) and system activation in the presence of inducer (B). Detail can be found in each related chapter.

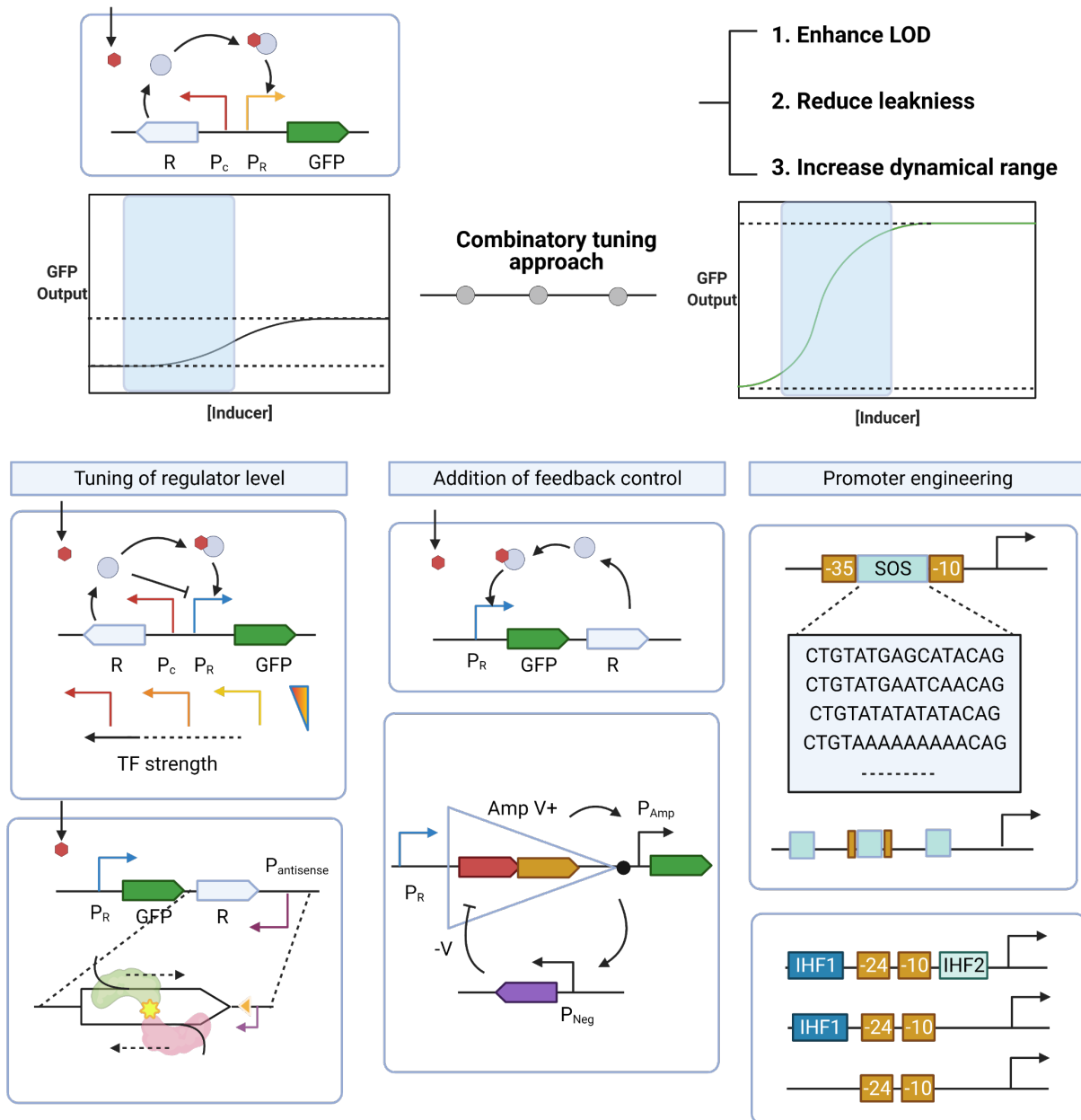


Figure 7.2 Tuning strategy adopted for fine-tuning transcriptional system

The transcriptional control system in the whole-cell biosensor can be fine-tuned following a combinatorial approach. The aim is to enhance the limit of detection (LOD) to a medically relevant range and expand its dynamical range (both reducing its leakiness and increasing the output). The tuning strategy mainly focuses on 1) methods to control the regulator level itself, 2) feedback control to achieve either signal amplification or increased robustness and 3) adjusting the interaction affinity between the regulator and promoter through rational promoter design

Table 7.1 Dynamical profiles for aspirin sensor toolbox

Circuits Name	Dynamical range (RFU)	Operational range (Aspirin/ μM)	Limitation of detection (Aspirin/ μM)	Strains tested	Response time (mins)
pP _{sal} -gfp-salR	5325 $\pm$ 362--32908 $\pm$ 939	0.05~5	0.05	DH5 α /EcN	~40
pP _{roD} -salR-P _{sal} -gfp	738 $\pm$ 99--8470 $\pm$ 893	0.05~5	0.05	DH5 α /EcN	~40
pP _{J109} -salR-P _{sal} -gfp	3201 $\pm$ 46--18119 $\pm$ 794	0.05~5	0.05	DH5 α /EcN	~40
pP _{sal} -gfp-salR-P _{bad} -araC_RNAp	7052 $\pm$ 58--47542 $\pm$ 648	0.25~5	0.25	EcN	~30
pP _{sal} -gfp-salR-P _{tetO} -tetR_RNAp	758 $\pm$ 7--16598 $\pm$ 301	3~200	3	EcN	~30

Table 7.2 Dynamical profiles for DNA damage sensor toolbox

Circuits Name	Dynamical range (RFU)	Operational range (UV/MMC)	Limitation of detection (UV/MMC)	Strains tested	Response time (mins)
pP _{ERecA} -sfGFP	2292 $\pm$ 40 -- 6738 $\pm$ 47	1 sec~ 10sec	1 sec	EcN	~30
pP _{vrecA-AT} -sfGFP	3588 $\pm$ 127--18844 $\pm$ 501/ 2193 $\pm$ 75 -- 13284 $\pm$ 307	1 sec~ 10sec/ 0.1 μM ~ 10 μM	1 sec/0.1 μM	EcN	~30
pP _{vrecA-AA} -sfGFP	53527 $\pm$ 2214 - 10068 $\pm$ 1965	1 sec~ 10sec	1 sec	EcN	~30
pP _{vrecA} -sfGFP	10941 $\pm$ 767-- 42957 $\pm$ 1075	1 sec~ 10sec	1 sec	EcN	~30
pP _{vrecA_Jungle_Down} -sfGFP	2089 $\pm$ 74 -- 7088 $\pm$ 121	1 sec~ 10sec	1 sec	EcN	~30
pP _{vrecA_Jungle_Both} -sfGFP	1271 $\pm$ 39 -- 3428 $\pm$ 281	1 sec~ 10sec	1 sec	EcN	~30
pP _{vrecA_Jungle_Up} -sfGFP	1464 $\pm$ 132 -- 6130 $\pm$ 162	1 sec~ 10sec	1 sec	EcN	~30
pP _{vrecA-AT} -Amplifier*	1361 $\pm$ 186 -- 12032 $\pm$ 257	6.125 nM ~ 200 nM	6.125 nM	EcN	~120
pP _{vrecA-AT} -Amplifier + Feedback ⁺	12864 $\pm$ 261-- 26100 $\pm$ 1105	6.125 nM ~ 100 nM	6.125 nM	EcN	~120
pP _{lexA_lexA_P_{recA}_recA_P_{vrecA}_AT} -sfGFP	1268 $\pm$ 47 -- 7579 $\pm$ 124	16 nM~ 750nM	16 nM	EcN	~40

: pP_{vrecA-AT} promoter connected with HrpRS transcriptional amplifiers. pP_{vrecA-AT}-hrpRS-P_{hrpL}-sfGFP

⁺: pP_{vrecA-AT} promoter connected with HrpRS transcriptional amplifiers and negative feedback constructs. pP_{vrecA-AT}-hrpRS-P_{hrpL}-GFP-TetR + P_{TetO}-tdtomato-hrpV⁺

Table 7.3 Dynamical profiles for nitric oxide sensor toolbox

Circuits Name	Dynamical range (RFU)	Operational range (DETA/NO μ M)	Limitation of detection (DETA/NO μ M)	Strains tested	Response time (mins)
pP _{norVnull} -sfGFP-norR	2989 $\pm$ 128—5540 $\pm$ 172	1000~2000	1000	EcN	~60
pP _{norV} -sfGFP-norR	2847 $\pm$ 36--13931 $\pm$ 193	1.5~500	1.5	EcN	~25
pP _{norVβ} -sfGFP-norR	3118 $\pm$ 35--20762 $\pm$ 2326	1.5~500	1.5	EcN/ DH5 α	~25
pP _{roD} -norR-P _{norV} -sfGFP	1847 $\pm$ 8--2257 $\pm$ 79	1.5~500	1.5	EcN	~25
pP _{roC} -norR-P _{norV} -sfGFP	1728 $\pm$ 35--3465 $\pm$ 136	1.5~500	1.5	EcN	~25
pP _{roA} -norR-P _{norV} -sfGFP	1614 $\pm$ 82--8220 $\pm$ 816	1.5~500	1.5	EcN	~25

Table 7.4 Existing transcriptional biosensor tested functionally in mini SimCells

Regulator name	Family	Binding mechanism	σ family	Inducer molecules	Ref
SalR	LysR	Activator/Repressor	σ^{70}	Aspirin/Salicylate acid	[34]
NorR	Ntrc/NifA	Activator/Repressor	σ^{54}	Nitric oxide	This work
TetR	TetR	Repressor	σ^{70}	Tetracycline	[113]
AraC*	AraC	Activator/Repressor	σ^{70}	L-arabinose	[407]
CdaR*	CdaR	Activator/Repressor	σ^{70}	Glucarate	[408]
AcuR*	TetR	Repressor	σ^{70}	Acrylate	[87]

* Represents study done earlier within the group [198].

7.2 Future outlook

7.2.1 Design constraints of compatible software used in Mini SimCells

A number of research directions can emerge as natural follow-ups from the foundation work this thesis has outlined. The first is to outline any design constraints and further troubleshoot for the malfunctioning DNA damage sensor in the mini SimCells (Chapter 6). It is reasonable to assess other SOS response steps: these can be split into 1) the generation of ssDNA and 2) RecA-mediated LexA self-cleavage and protein recycling. The former can be potentially assessed using a decoy plasmid with a high copy number to mimic the ssDNA production in the mini SimCells system. However, based on existing data shown in Chapter 6, the sensor system with an extra copy of LexA and RecA exhibiting total silencing suggest the failed autocleavage of repressor LexA dimer is more likely the case. This hypothesis can be assessed through decoupling the RecA–ssDNA-mediated LexA autocleavage using a chimeric LexA. For instance, Jeffery et al. developed a small-molecule responsive SOS gene network through the rational insertion of a TEV linker within the LexA dimer. Upon triggering P_{tetO} with aTc and expression of TEV protease, the SOS system can be switched on without DNA damage [409]. By adopting and validating such a system in mini SimCells, we can narrow down our scope to troubleshoot any specific step caused for the failure in the mini SimCells. Furthermore, to assist the release of LexA dimer, mini SimCells plasmid should include the degradation and recycling enzymes such as ClpXP and Lon proteases. It was previously reported that the accumulation of the LexA DNA binding NTD could silence and suppress SOS activation [306].

7.2.2 Future development of Mini SimCells

The successful implementation of the dual input SalR quasi-XOR gate suggests that mini SimCells contain sufficient cellular machinery and energy to execute complex signal processing

functions. It is important to investigate further the total energy capacity of mini SimCells and strategies for creating an auxotrophic energy regeneration system. One strategy adopted previously in a different anucleate cell is to insert a plasmid-encoded modular glycolysis pathway [388]. Compared with any cell-free or liposome-based simplified system, the intact native cellular membrane provides a unique edge for building an artificial cell with preloaded machinery. The ATPase proton pump could be rewired to boost up the fluorescence output for biosensors (Figure 7.3C).

Similarly, given that the mini SimCells has been shown to preserve motility function and intact flagellum structure, the membrane-bound chemotaxis sensory array can be potentially rewired with synthetic plasmid to reconstitute a synthetic chemotaxis function tailored to tumour-related metabolites. (Figure 7.3A). There are 32 different classes of two-component chemosensory modules with 11700 copies per cell resident on a typical *E. coli* membrane [410][411]. To harness this complex yet highly precise sensory system, mini SimCells approach has the intrinsic advantage compared to the fully bottom-up approach. Furthermore, it is evident that these large chemosensory arrays preferentially localise to the polar regions of the cells [412], making mini SimCells more ideal for a model cell used in synthetic chemotaxis construction. Other aspects of improving the system, such as increasing protein mobility during segregation [413] and plasmids dipole movement are also worth investigating. It was shown that different origin of replication has location preference (polar end of the cells or centre) [414] and expression of some actin like flameouts structure can push plasmids to the dipole ends of the cells [41][415].

During the creation of the *minCD* deleted EcN strain, numerous difficulties were encountered using either SacB or lambda-red derived recombination system (data not shown). Compared to the well-characterised *E. coli* K12, point mutation of the SacB and non-specific

integration of the resistance cassette (Cm^{R}) were often found during the making process of gene knockout. Despite extending the homology arm (~1100bp) and alternatively using a more established temperature-controlled curating plasmids system, the issue persisted. It was previously speculated the two stably replicating cryptic plasmids in EcN, pMUT1 and pMUT2, might exhibit protection of the carrier bacterium from attacks by mobile genetic elements, thus influencing the genetic stability of the strain [416][3]. However, by deleting one or both cryptic plasmids, the cell exhibited little difference in its metabolic profile [417]. A further lack of precise functional annotation for these cryptic plasmids makes cryptic-free EcN an uncertain direction. In contrast, based on the established cases of CRISPR-Cas9 and dCas9 work implemented in EcN [418] [419], it is ideal for making a portable plasmid-based *minCD* repression system (Figure 7.3B) controlled via an external chemical cue. Cas9 with defective nuclease activity (dCas9) has been assessed and validated as a suitable repressor for transcription silencing. Given the flexibility of gRNA design, developing a dCas9 mediated *minCD* silencing platform system, it could potentially convert many other strains that are hard to culture or lack suitable genetic tools into mini SimCells-producing strains.

Finally, from the perspective of disease diagnosis and future clinical application, general medical practice often involves multiple biomarkers to be assessed at the same time to increase confidence in a diagnostic decision. This should be reflected in the synthetic circuits design. Therefore, the well-characterised nitric oxide sensor could be coupled with other inflammatory disease inputs such as tetrathionate and thiosulphate [85], where each of the individual signals can be fused to construct AND logic based on a highly fragmented T7 system [153]. This could be further tested in a mouse model or pre-clinical samples. Non-dividing mini SimCells might exhibit increased robustness and steady performance, as it has been demonstrated that 100% serum or

urine samples pose a strong growth inhibition effect on bacteria cells [143]. In summary, the mini SimCells can be regarded as a simplified system with many different advantages over the bottom-up and top-down chassis system. Through expanding suitable modules and regulatory circuits, we can transform the bacteria bot from a science fiction concept into a reality.

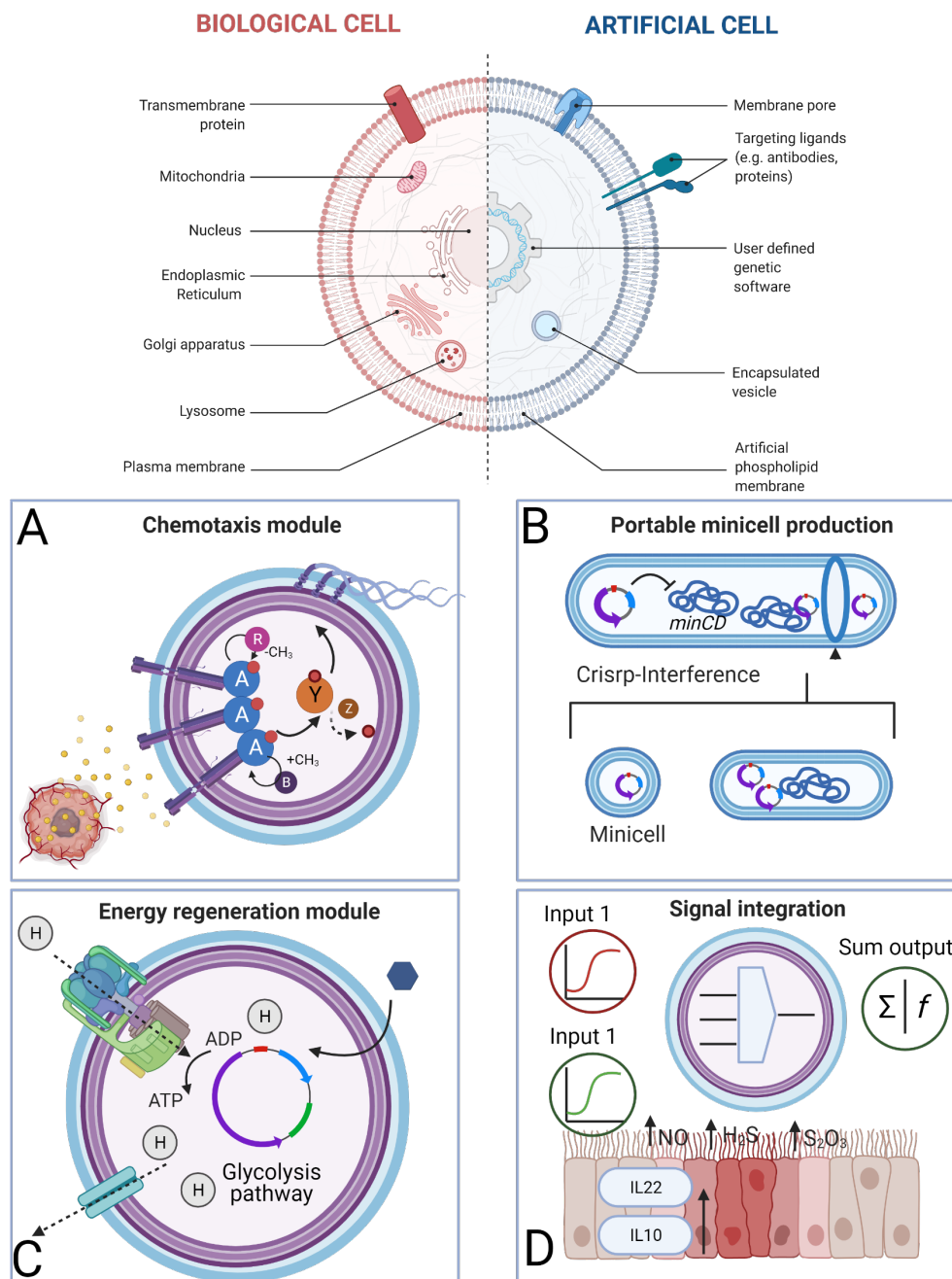


Figure 7.3. The future research directions of mini SimCells

Mini SimCells as a programmable chassis platform. Four future research directions can be built upon the foundation this thesis has outlined. A) synthetic chemotaxis modules that rewire the chemosensory array on the membrane to sense tumour-related metabolites. B) Portable mini SimCells production plasmid system via dCas9 interference of the host *minCD* operon. C) Insertion of additional glycolysis modules to boost ATP regeneration. D) Signal integration to further increase the confidence of diagnosis outcome.

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Appendix A. Chapter 3. SI Figures

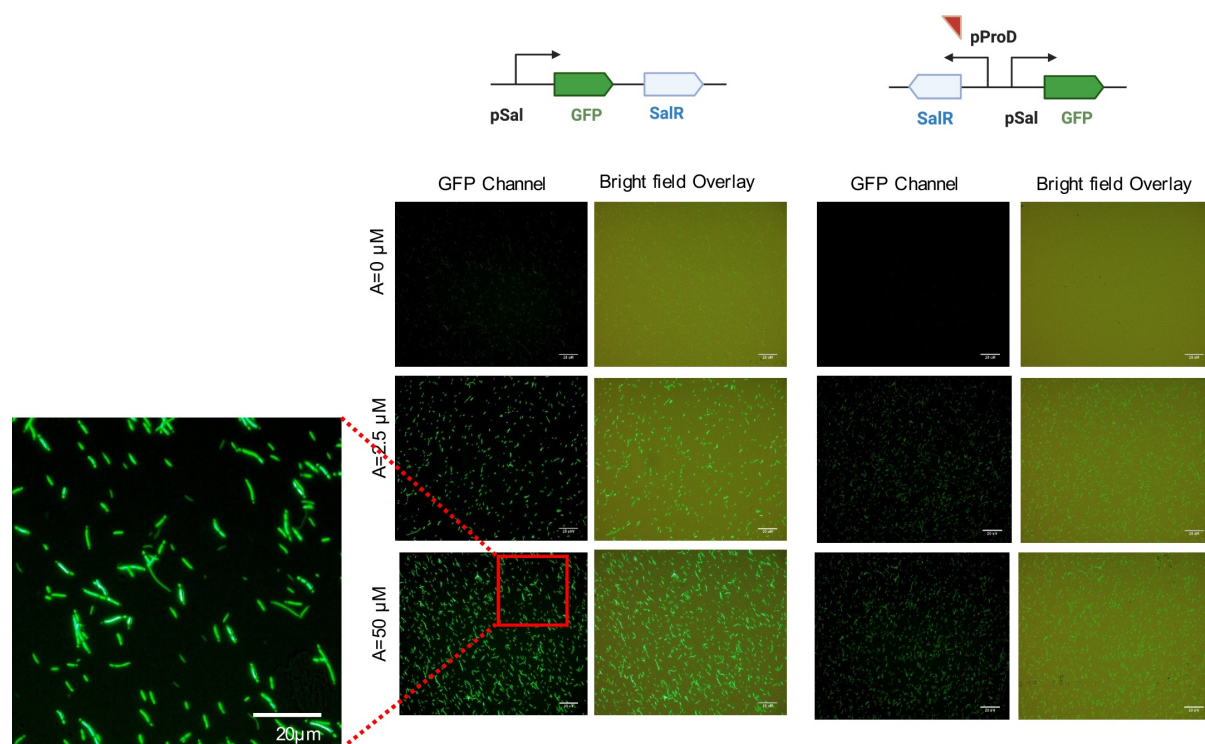


Figure A.1 Microscope images of SRS and PAR.

Images were taken at both GFP channel and overlaid with Bright field channel for indication of potential truncated circuits or non-transformed bacterial cells. The specific concentration of aspirin is indicated beside the plot. On the left, enlarged images of the PAR circuit when induced with aspirin (50 μM) clearly show two separate populations with different GFP intensity (High/Low) as reflected in result from flow cytometry.

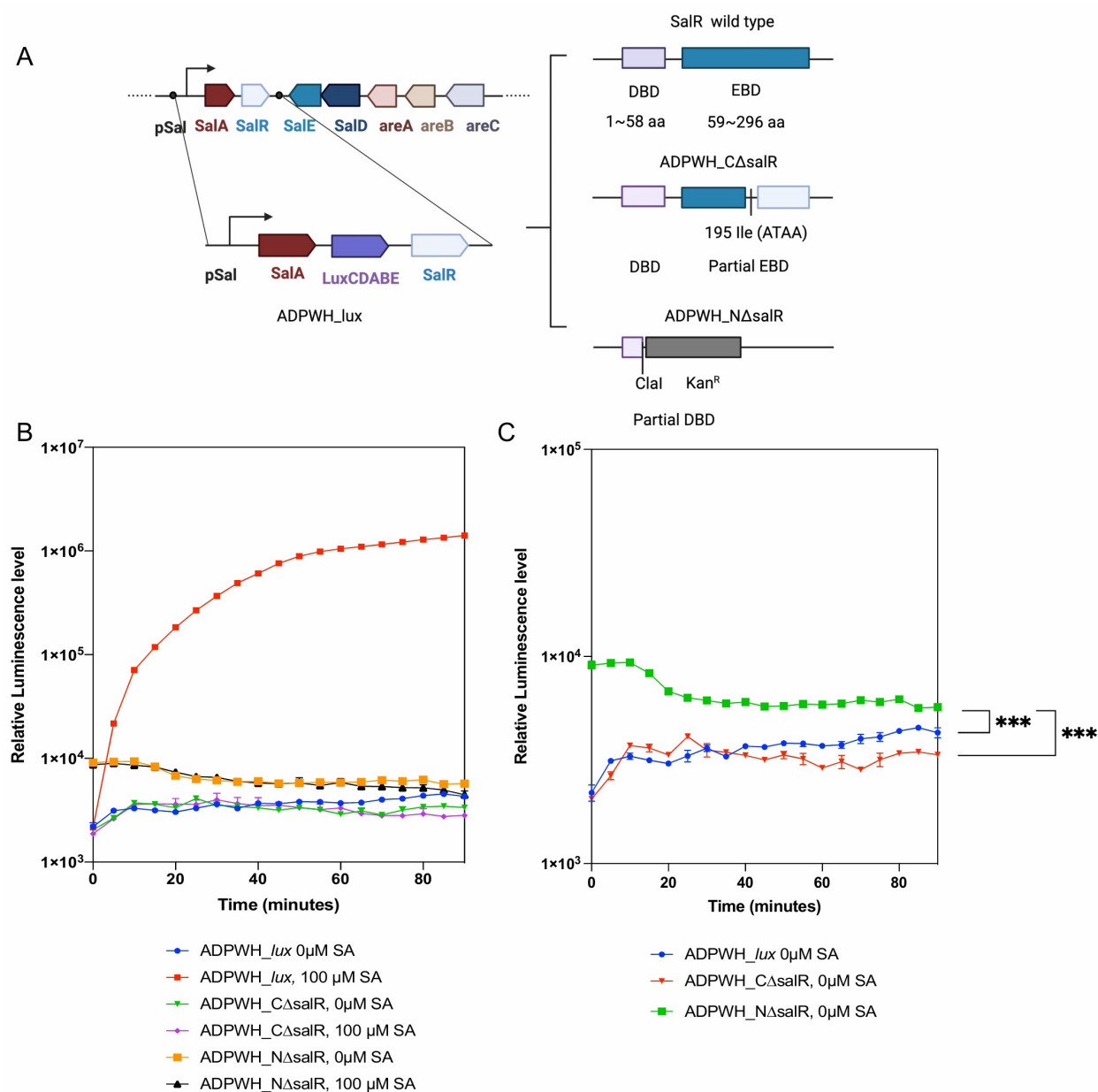


Figure A-2. Salicylate induction towards mutated ADPWH_salR.

A) Schematic diagram for creating N/C mutant of SalR. B) Bioluminescence response to salicylate (SA) of ADPWH_lux, ADPWH_CΔsalR and ADPWH_NΔsalR. ADPWH_lux has a strong, rapid, response to SA, while the two mutants with incomplete *salR* do not respond at all. Standard deviation was plotted for all data replicates (n=4), the enlarged log scale for non-induced ADPWH_CΔsalR, ADPWH_NΔsalR and ADPWH_lux was provided in C) as a clear indication of increased background level in the case of ADPWH_NΔsalR. $P < 0.001$ ***

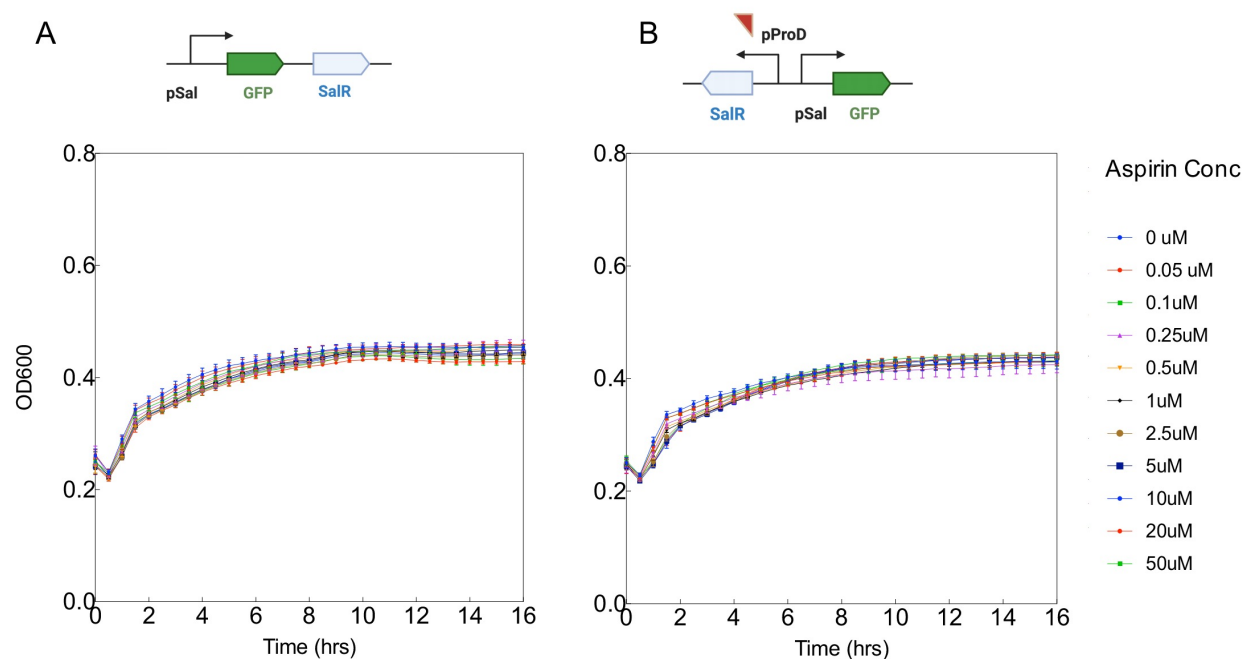


Figure A-3. OD growth of induced SRS and PAR circuits.

OD600 was monitored for 16hrs for both A) PAR (P_{Sal} -gfp-salR) and B) SRS (P_{ProD} -salR- P_{Sal} -gfp). No clear growth difference was found. Standard deviation and mean value were plot for all data replicates (n=3)

Table A-1. Parameters used for simulation of SalR competitive binding

Parameter	Description	Value	Units
δ	Degradation rate of proteins.	3.5×10^{-5}	1/s
α_1	Combined transcription/translation rate of salR in the PAR system.	6.02×10^{-3}	nM/s
α_2	Combined transcription/translation rate of salR in the SRS system.	0.153	nM/s
α_2^*	Combined transcription/translation rate of salR in the SRS* system.	0.0217	nM/s
α_3	Combined transcription/translation rate of GFP in all systems.	1.88	nM/s
K_R	Equilibrium binding constant of salR in its repressive (non-induced) form.	3.75×10^2	nM
K_A	Equilibrium binding constant of salR in its activating (induced) form.	10	nM
K_a	Equilibrium binding constant of binding between Aspirin and salR.	0.457	μ M
n	Exponent for hill function governing Aspirin-salR binding.	1	none
L	Transcriptional leakage rate from P _{Sal} as a proportion of maximal transcription output.	0.104	none

Appendix A 3.1 Simulation Approach

The model is implemented using MATLAB 2017b; numerical integration is performed a Runge-Kutta 4th method, and parameters are fit using the *fmincon* function. Simulation start-time corresponds to addition of $[\alpha]$. We therefore assume that initially $[\text{GFP}] \approx 0$ in all systems. The initial salR concentration, $[\text{salR}]_0$, is determined by solving (with $[\alpha] = 0$) at steady-state with the

parameters fit for the LB growth media. This is because all cells were grown in LB prior to commencement of each experiment.

Appendix A 3.2 Parameter Fitting

Parameter values (listed in Table A-1 along with definitions) were fit to experimental data as follows. Because there is insufficient experimental data to identify all parameters in our model, we set $n=1$ as which corresponds to non-cooperative binding of the inducer. Exponents of $[\text{salR}]$ are likewise unity throughout, though lack of cooperativity in binding P_{sal} does not imply that salR does not dimerise quickly (compared to other binding processes) once it is expressed. The value of K_A was set to an arbitrary value of 10 nM, meaning that the fit value of K_R governs the relative strength of repressive vs activating binding of salR , and expression rates of salR in each system ($\alpha_1, \alpha_2, \alpha_2^*$) govern the absolute level of salR -promoter interaction. The free parameters ($\delta, \alpha_1, \alpha_2, \alpha_2^*, \alpha_3, K_R, K_a, L$) were then least-squares fit to the experimental data for each circuit variant (Figures 3.4 and Figure 3.5 of the main text). The resulting parameters (Table A-1) demonstrate some underlying phenomena. It was observed that the fit value corresponds to a protein half-life of approximately 5.5hours, in the ballpark expected for relatively stable proteins. The ratio of transcription for the SRS/SRS* circuits is $\alpha_2 / \alpha_2^* \approx 7$, which is a similar factor of change to that observed in Figure 3.5 of the main text. As expected, it was found that $K_R \gg K_A$, supporting our operational hypothesis that binding of salR in its active (inducer bound) form to P_{sal} is favourable compared to binding of inactive salR . Furthermore, the equilibrium constant for salR -Inducer binding (K_a) falls into the middle of the range of inducer concentrations tested, where we anticipate salR action is becoming dominated by its active form.

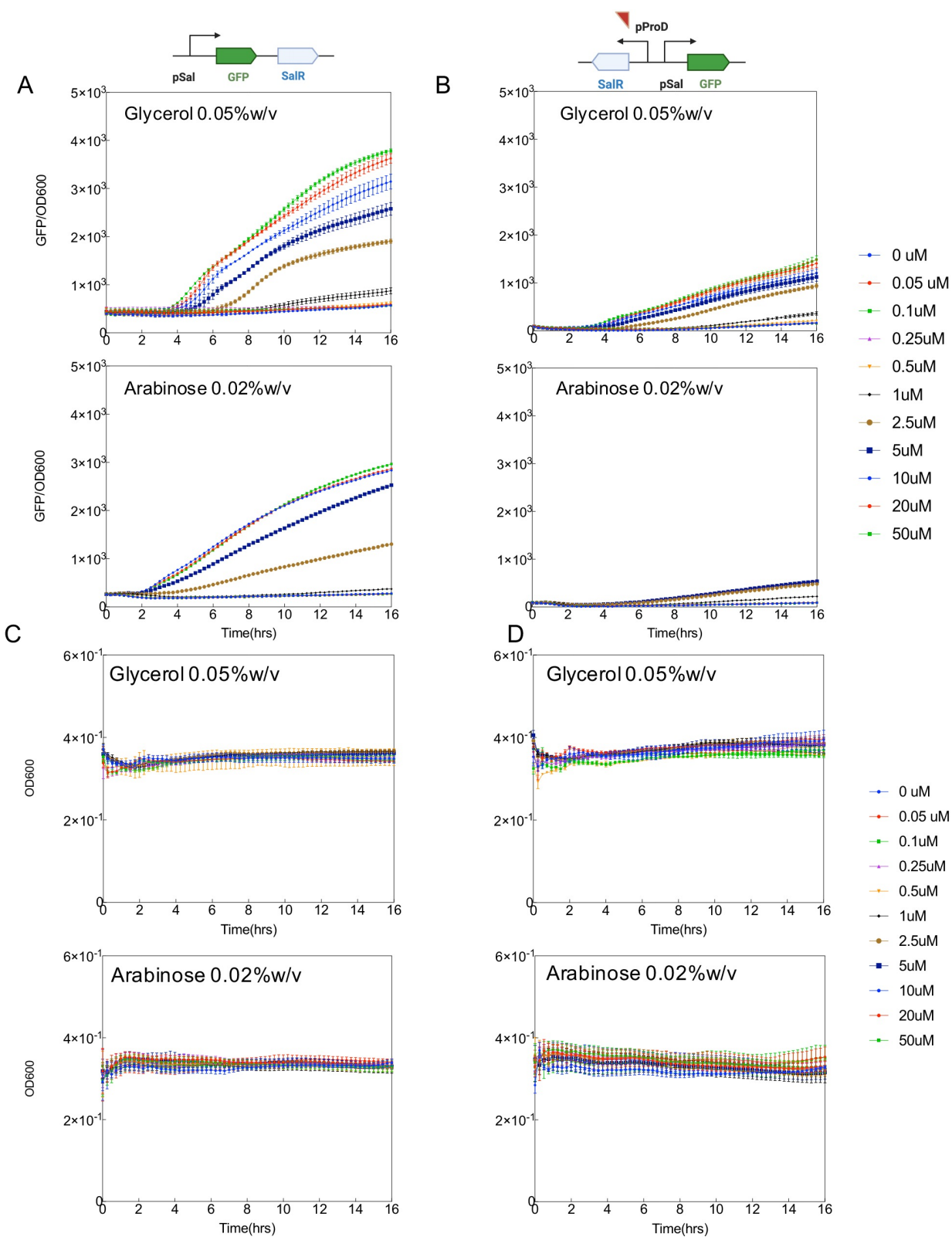


Figure A-4 The effect of carbon source and growth in designed systems.

Induction kinetics for the A) SRS and B) PAR construct transformed in DH5 α and tested PBS supplemented with Glycerol (0.05%v/v) and PBS supplemented with 0.02% v/v L-arabinose. Aspirin was added at time zero and fluorescence is observed for 16hrs. Color coded induction levels are indicated on the right legend. The induction gradient is 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50 μ M. Cell intrinsic fluorescence and background was removed. Standard deviation and mean value were plot for all data replicates (n=4) C) OD600 growth for cells transformed with SRS in corresponding induction kinetics supplemented with glycerol or arabinose and D) OD600 growth of cells transformed with PAR construct.

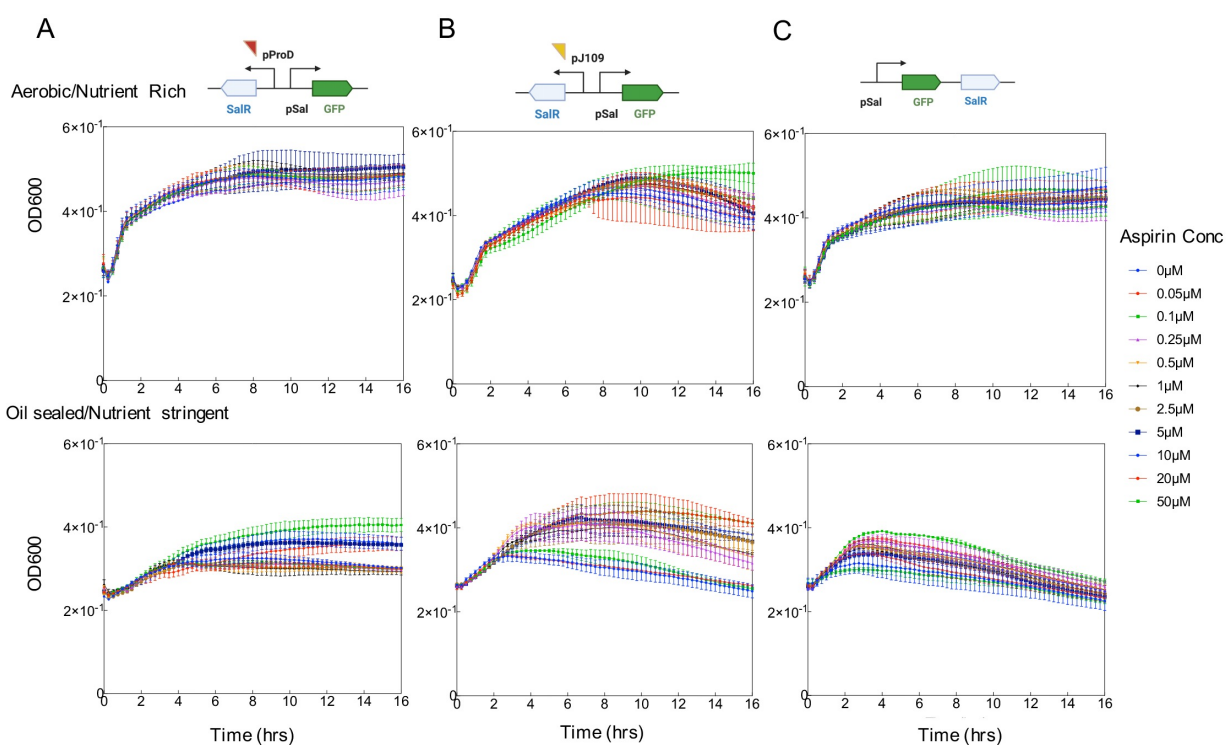


Figure A-5. Growth kinetics for the SRS SRS* and PAR constructs in EcN

OD600 measurement over 16hrs for SRS (P_{roD} -salR- P_{Sal} -GFP); SRS* (P_{J109} -salR- P_{Sal} -GFP) and PAR (P_{Sal} -GFP-salR) under aspirin gradient in both aerobic/nutrient rich (LB) and microaerobic/nutrient stringent condition (M9 medium supplemented with glucose 0.4% w/v). N=3 Standard deviation and mean value was plot for three biological replicates

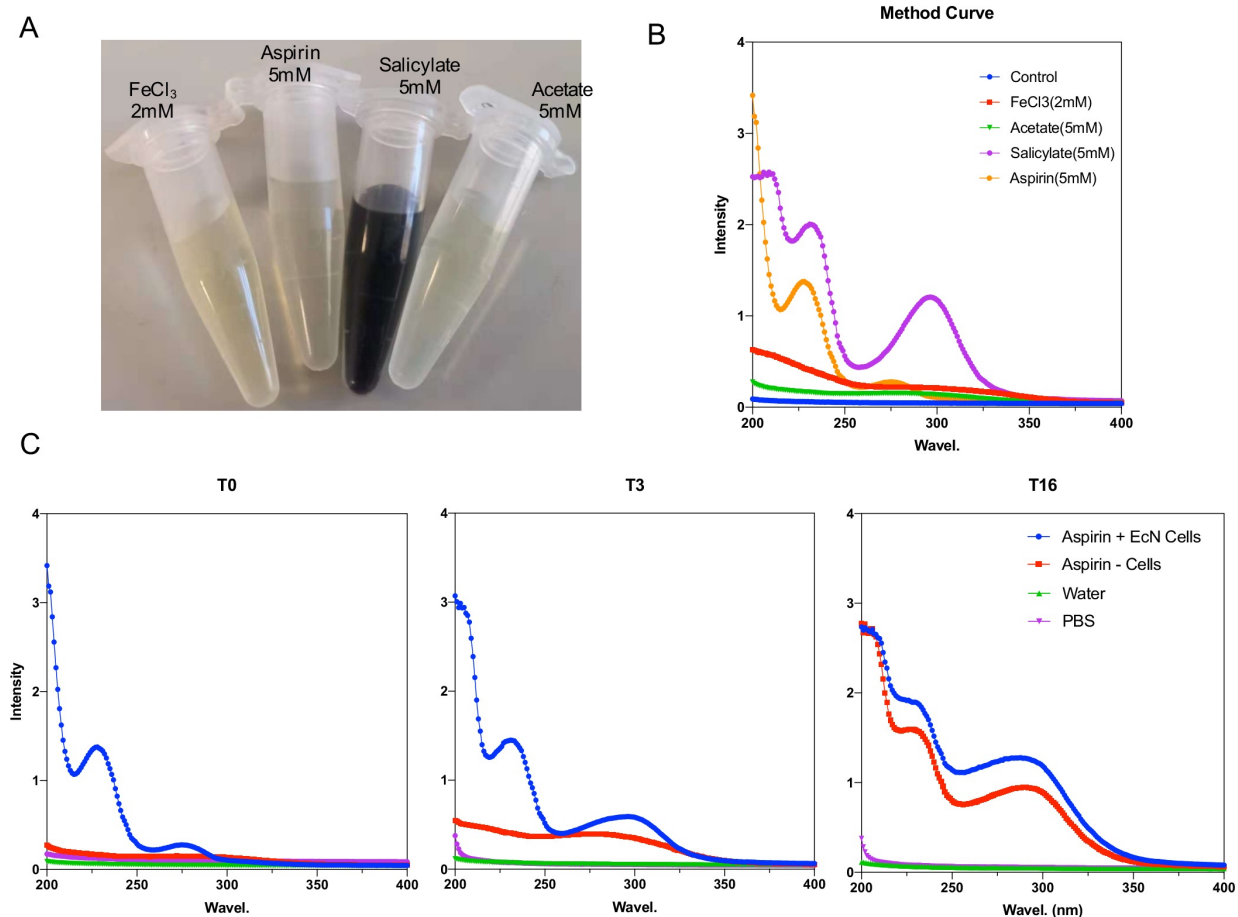


Figure A-6. EcN facilitate the aspirin conversion to salicylate.

Fe (III)Cl₃ assay for identification of salicylate. A) Titration of 2 mM FeCl₃ with 5 mM of aspirin, salicylate and acetate respectively, only salicylate react with FeCl₃ yielding a dark purple color. And a B) Unique absorbance peak at 300nm indicates the presence of salicylate) Cell supernatant was collected at time point T0, T3 and T16 hrs after the initial aspirin induction of cells. Aspirin added into PBS medium without cells is used as control. EcN facilitate higher titre of salicylate conversion.

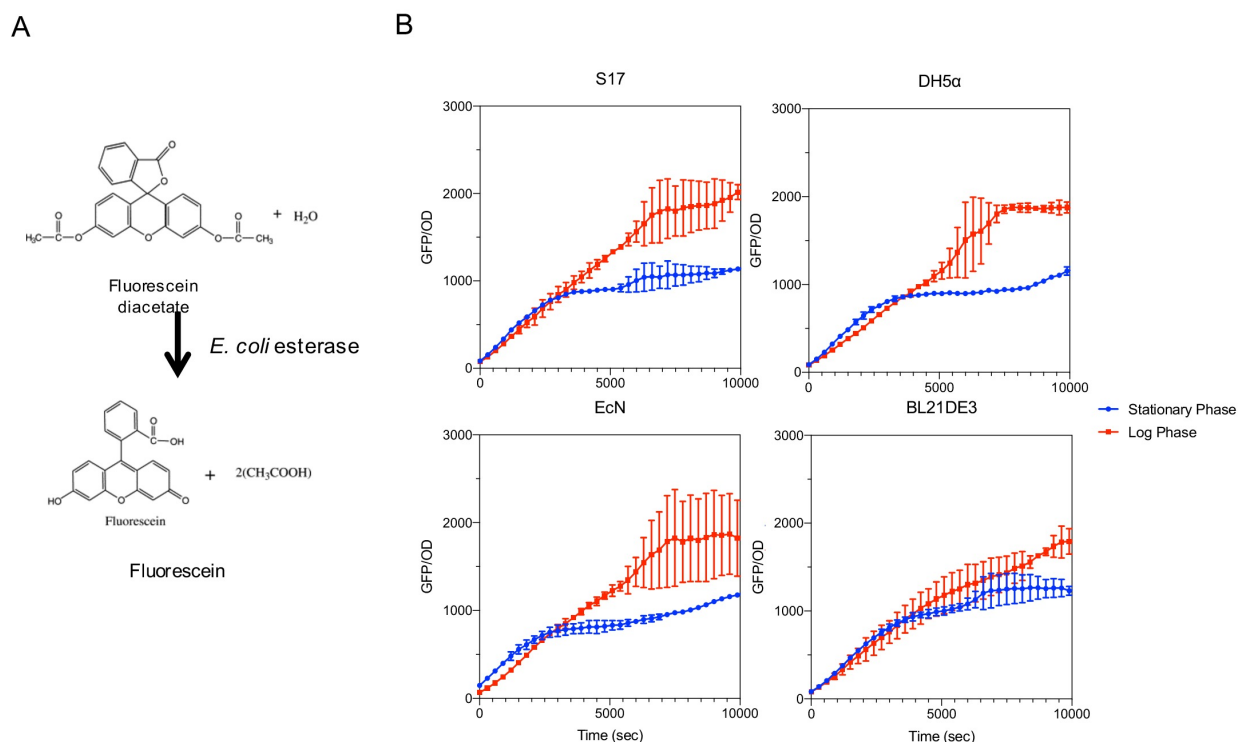


Figure A-7. Non-specific esterase activity displayed by different *E. coli* strains.

Standard Fluorescein diacetate (FDA) assay for quantification of esterase activity. A) Fluorescein diacetate (FDA) is converted into Fluorescein by *E. coli* esterase and yielded an increase reads in GFP signal. B) Non-specific esterase activity is strain independent but varied in different growth stage. Equal number of cells were diluted and seeded at the beginning. Standard deviation and mean value were plot for three biological replicates (N=3)

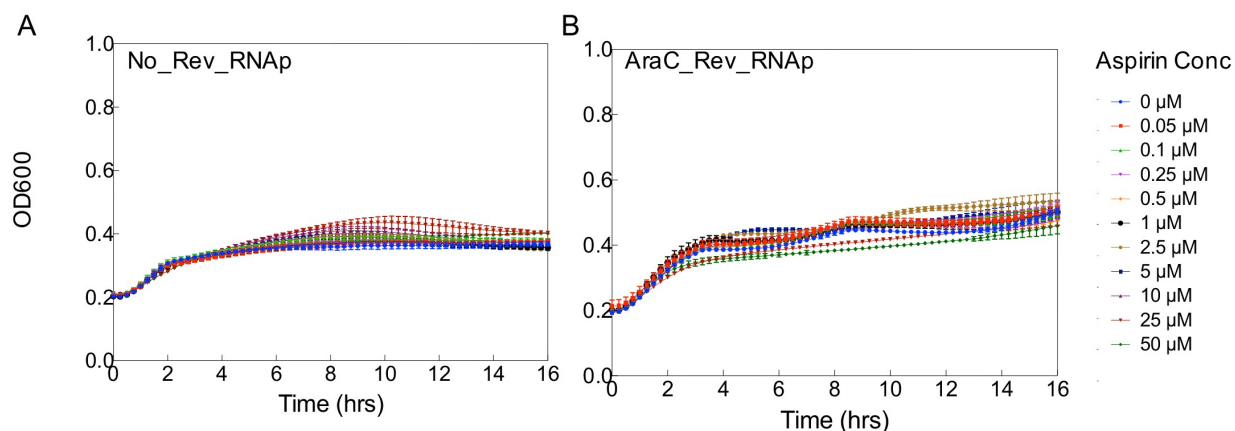


Figure A-8. OD growth with the addition of AraC_Rev_RNAP and without.

A) OD growth of EcN transformed with the original PAR construct and B) AraC_Rev_RNAP constructs. Both induced with an aspirin gradient. N=3. Standard deviation and mean value were plot for three biological replicates.



A) Schematic diagram of SOS DNA repair mechanism. In its native state, SOS machinery is dormant and repressed by LexA while low level of RecA is stored inside cell in its inactive form: upon DNA damage, single-stranded DNA will activate RecA (ssDNA-ATP-RecA filament) which subsequently trigger the self-cleavage of the LexA dimer and further degradation by the Lon protease and ClpXP, to fully release it from the SOS binding sites, resulting the activation of 40 different DNA repair genes. B) Overview of the SOS DNA damage system where LexA/RecA interaction regulates a cascade of 40+ DNA repair and proofreading genes. Upon DNA damage, each gene can be turned on in sequential manner dependent on the promoter affinity with LexA. C) LexA protein sequence alignment between *V. natriigene* and *E. coli* K12, a similarity score of 72% and amino acid residue responsible for DNA binding region is highlighted in yellow.

Table B-1. Alignment of SOS promoters and LexA binding motifs in different bacteria.

Species Name	LexA binding site	Ref
<i>Citrobacter youngae</i>	ATGACG <u>CCATTACTGT</u> ATAAAAAA <u>ACAGGTACAA</u> ATA	[420]
<i>Pseudomonas aeruginosa</i>	TAATCC <u>CAGTCACTGG</u> ATAAAAAC <u>ACAGAGCGAC</u> GAC	[421]
<i>Shewanella piezotolerans</i>	CTGATT <u>AAATTACTGT</u> ATATACTA <u>ACAGGTACTGT</u> TAT	[422]
<i>Escherichia coli</i> K-12.MG1655	AAACAC <u>TTGATACTGT</u> ATGAGCAT <u>ACAGTATAAT</u> TGC	This study
<i>Escherichia coli</i> Nissle 1917	AAACAC <u>TTGATACTGT</u> ATGAGCAT <u>ACAGTATAAT</u> TGC	This study
<i>Vibrio natrigene</i>	CAGGTA <u>TAGACACTGT</u> ATGAATCA <u>ACAGTATAAT</u> AAC	This study
<i>Salmonella entrica</i>	AATCAC <u>CTTTAACTGT</u> ATATACTC <u>ACAGCATGAT</u> TGT	[43]

Table B-2. Comparison of SOS promoters and LexA binding motifs in different *Vibrio* spp. with different doubling time.

Species Name	LexA binding site	Doubling time (mins)	Ref
<i>Vibrio cholerae</i>	CAGGGG <u>TGGACACTGT</u> ACAAATCA <u>ACAGTATAAT</u> GAA	~40	[423]
<i>Vibrio natriegens</i>	CAGGTA <u>TAGACACTGT</u> ATGAATCA <u>ACAGTATAAT</u> AAC	~10	[424]
<i>Vibrio fischeri</i>	AGCATA <u>GCACAGCTGT</u> ATATACTC <u>ACAGTGAAC</u> TGTA	~280	[425]
<i>Vibrio parahaemolyticus</i>	CAGGTA <u>TAGACACTGT</u> ATGAATCA <u>ACAGTATAAT</u> GAC	~10	[426]
<i>Vibrio vulnificus</i>	CAGGGG <u>TAGACACTGT</u> ACAAATCA <u>ACAGTATAAT</u> GAG	~20	[427]

Sigma factor -35 & -10 shown in underlined and bolded font

LexA binding (CTGTN₈ACAG)-motif shown in cyan font

Table B-3. Medical usage of mitomycin C in treating the different type of tumour.

Targeting diseases	Method	Dosage (μM)	Ref
Esophageal carcinoma	IV	12.1	[428]
Anal cancer	IV	20	[429]
Breast cancer	IV	22.1	[430]
Bladder tumours	IV	23.92	[431]

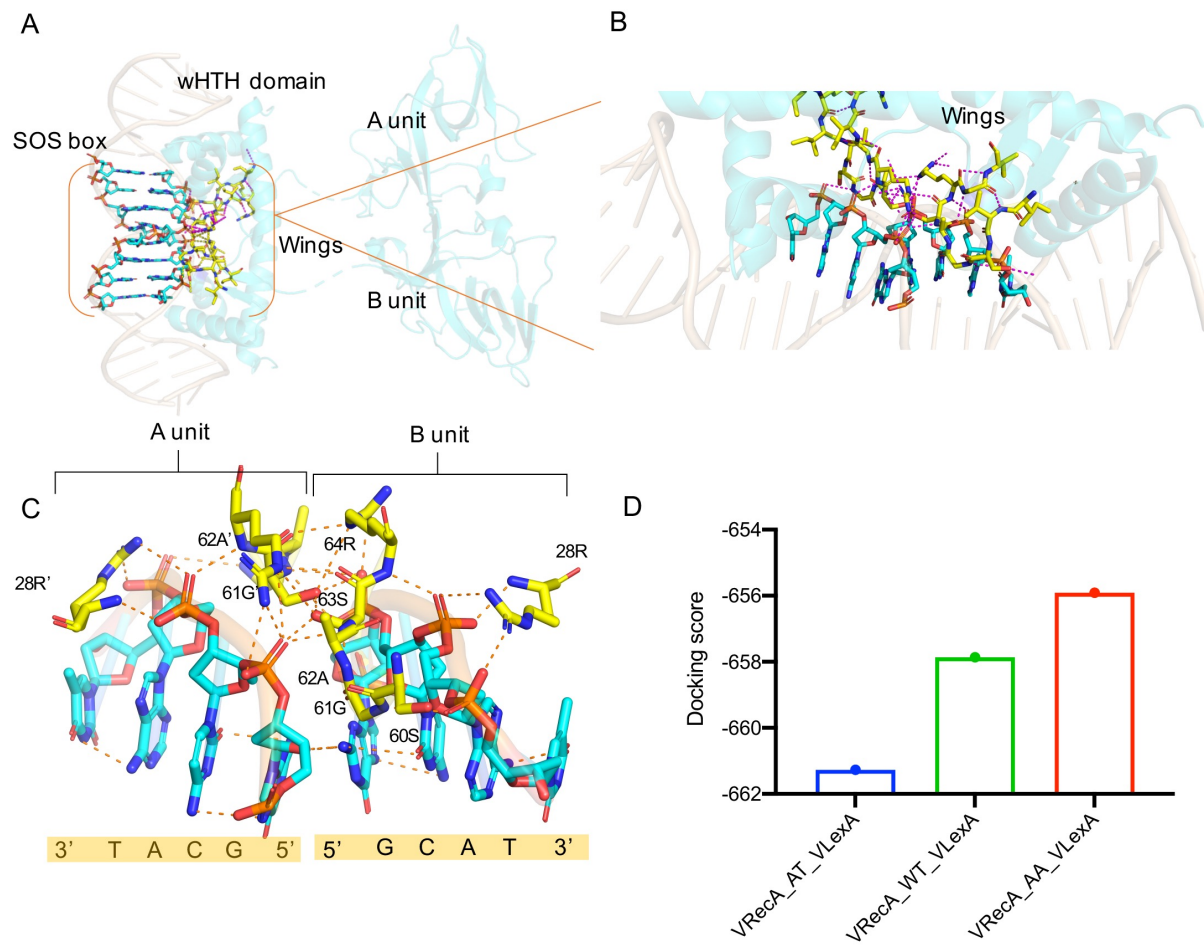


Figure B-2. LexA DNA interaction and docking simulation.

A) 3D structure of LexA interaction with SOS box. Crystal structure obtained from Uniprot ID P0A7C2. (B) Interval nucleotide flanked by LexA binding site & interacting amino acid residues highlighted. (C) H-bond between base pair and polar contact with B unit single chain (LexA) are highlighted. (D) Docking score for P_{VRecA}, P_{VRecA_AT} and P_{VRecA_AA}. The docking score marks the Gibbs energy required for complete docking. The higher the score, lower the affinity between DNA and LexA.

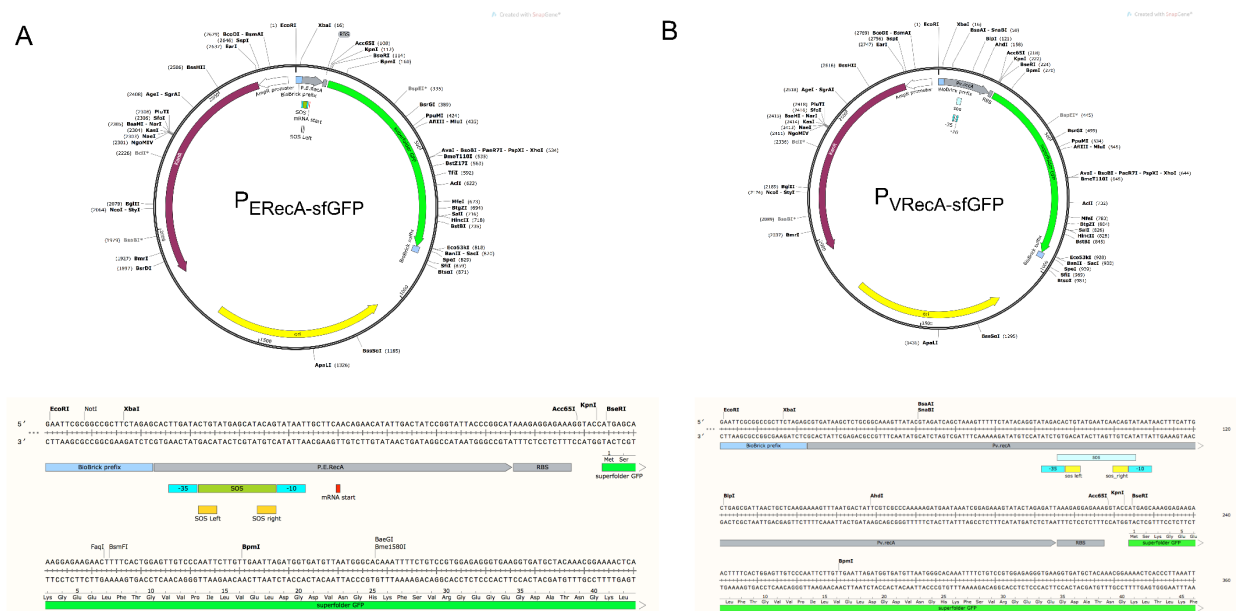


Figure B-3. Plasmids map for p_{PERecA} and p_{PVRecA_sfGFP} .

The $recA$ SOS binding region of A) the *E. coli* p_{PERecA} and B) *V. natrigenes* p_{VRecA} were annotated with LexA binding site (Yellow), Sigma factor binding site (Blue) and potential mRNA start site (Red).

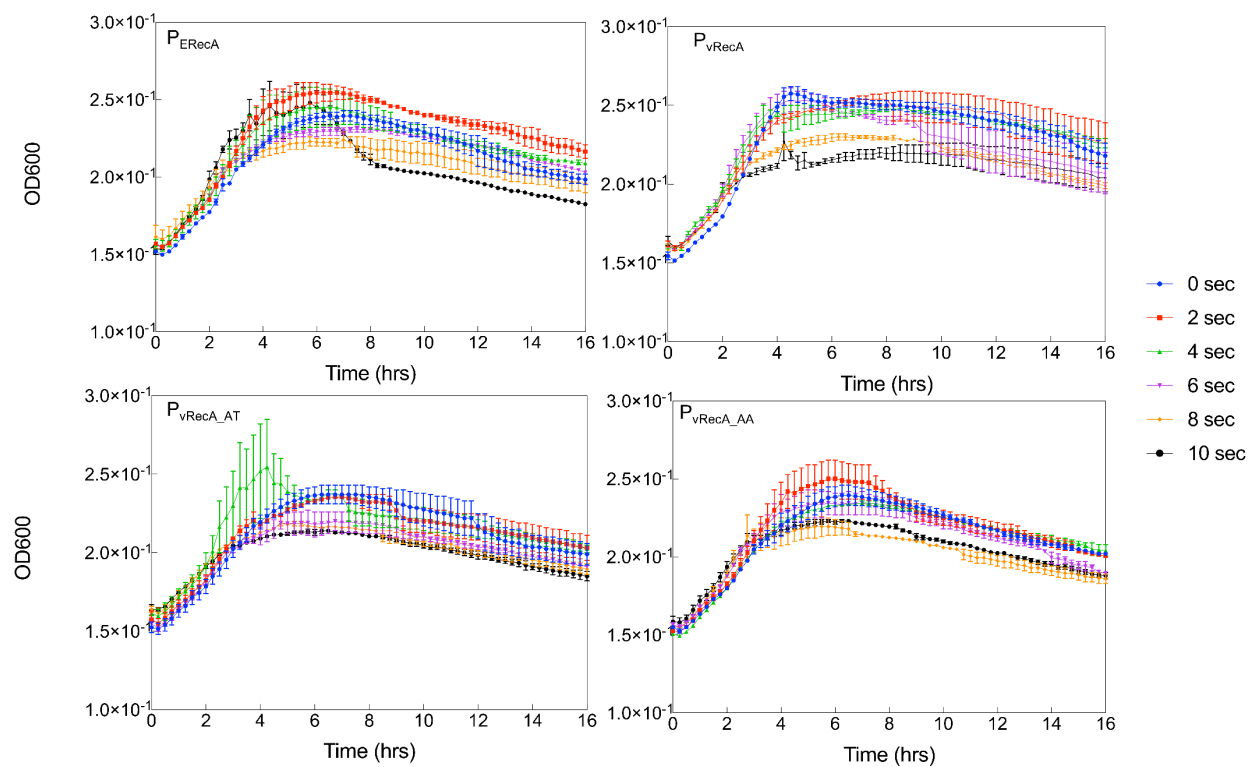


Figure B-4. OD growth of induced SOS system under UV.

OD growth curve for all three P_{VRecA} promoters with different SOS binding box and P_{ERecA} promoter tested with (0 ~ 10 sec) 302nm UV exposure. The promoters were transformed into *E. coli* Nissle 1917 supplemented in M9 medium with 0.4% glucose and Kanamycin 60 $\mu\text{g/ml}$. $N=3$. Standard deviation and mean value were plot for three biological replicates.

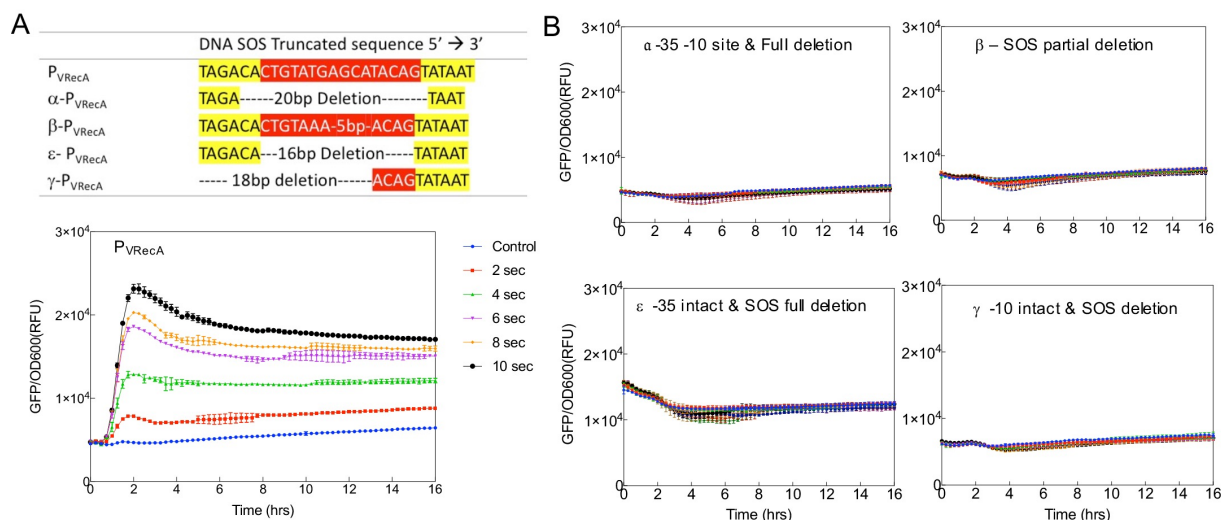


Figure B-5. Performance of P_{VRecA} promoter variants induced by 302 nm UV.

A) Table indicates different variants of P_{VRecA} promoter. Yellow box indicates the sigma binding site and Red box indicates the SOS box. B) Induction profile over 16hrs. Control (Wild type P_{VRecA} promoter) is shown on the left. N=3. Standard deviation and mean value were plot for three biological replicates.

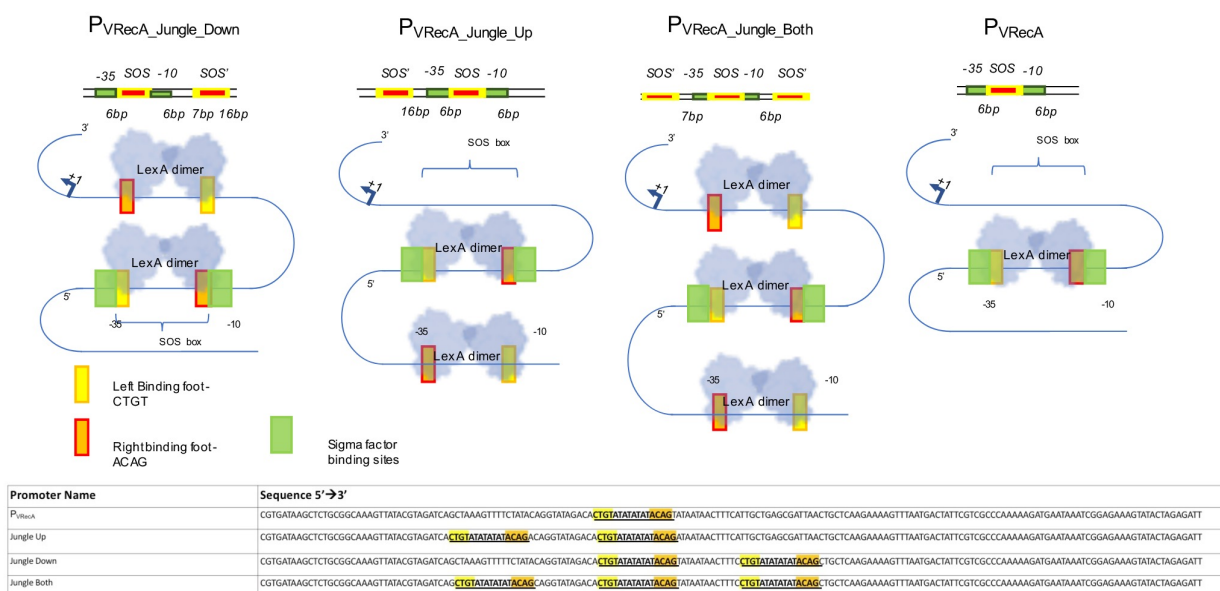


Figure B-6. Design of chimeric SOS promoters.

Promoter design and conceptual illustration of chimeric promoters binding with LexA dimer. Additional SOS box (AT repeat) was placed in different part of the P_{VRecA} promoter with the same 7bp gap. -35 and -10 remained unaltered.

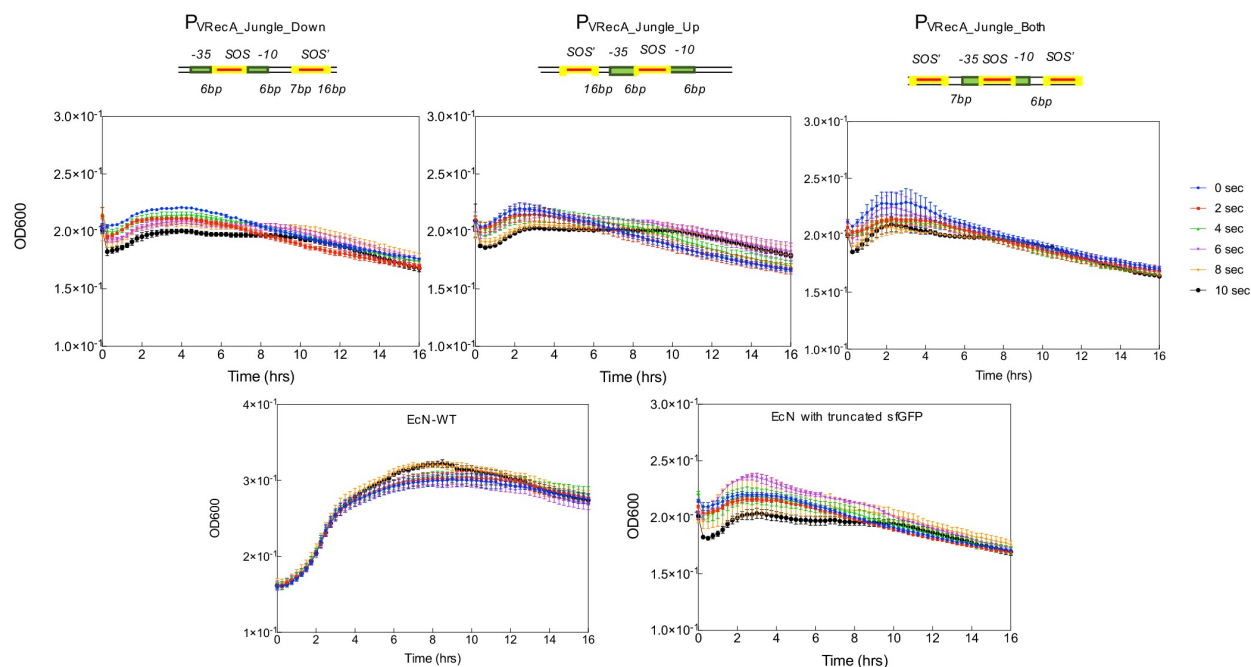


Figure B-7. OD growth of different chimeric promoters under UV exposure.

OD growth curve for all three P_{VRecA} promoters with different No of SOS binding box tested with (0 ~ 10 sec) 302nm UV exposure, transformed E. coli Nissle 1917 was supplemented in M9 medium with 0.4% glucose and Kanamycin 60 μ g/ml. OD growth curve for EcN wild type and EcN transformed with truncated sfGFP backbone were shown at the bottom. N=3. Standard deviation and mean value were plot for three biological replicates.

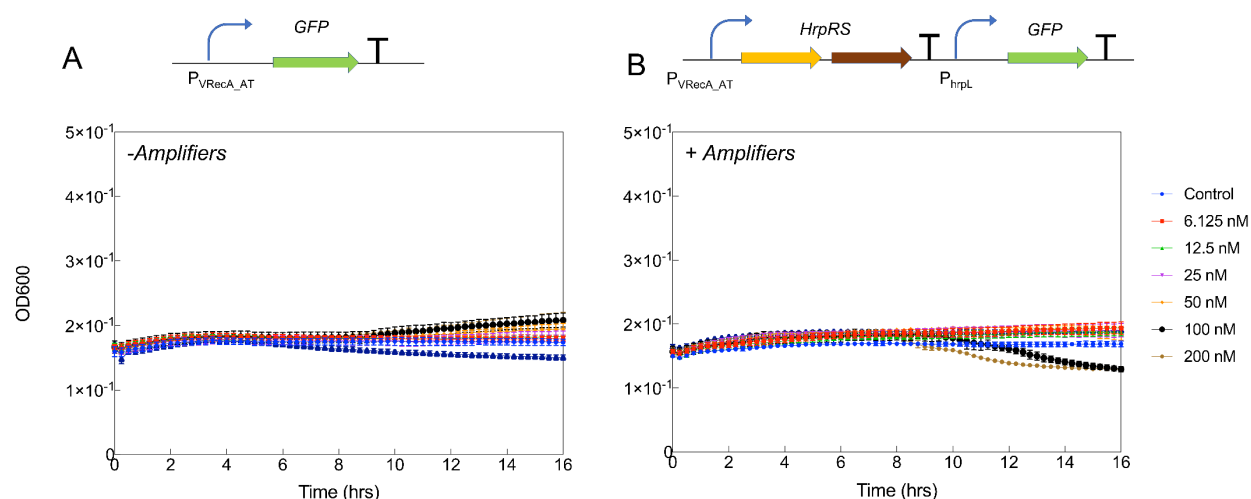


Figure B-8. Bacteria growth with and without amplifiers addition

OD600 of EcN transformed with the $P_{VRecA_AT_GFP}$ plasmid with and without the addition of HrpRS transcriptional amplifiers modules. A) P_{VRecA_AT} promoter fused with GFP. B) Optimised

P_{VRecA_AT} promoter fused with HrpRS subunits followed by a GFP output controlled by P_{HrpI} . Both systems were tested with a concentration gradient of Mitomycin C (0, 6.125, 12.5; 25, 50, 100; 200 nM) over 16hrs. N=3. Standard deviation and mean value were plot for three biological replicates.

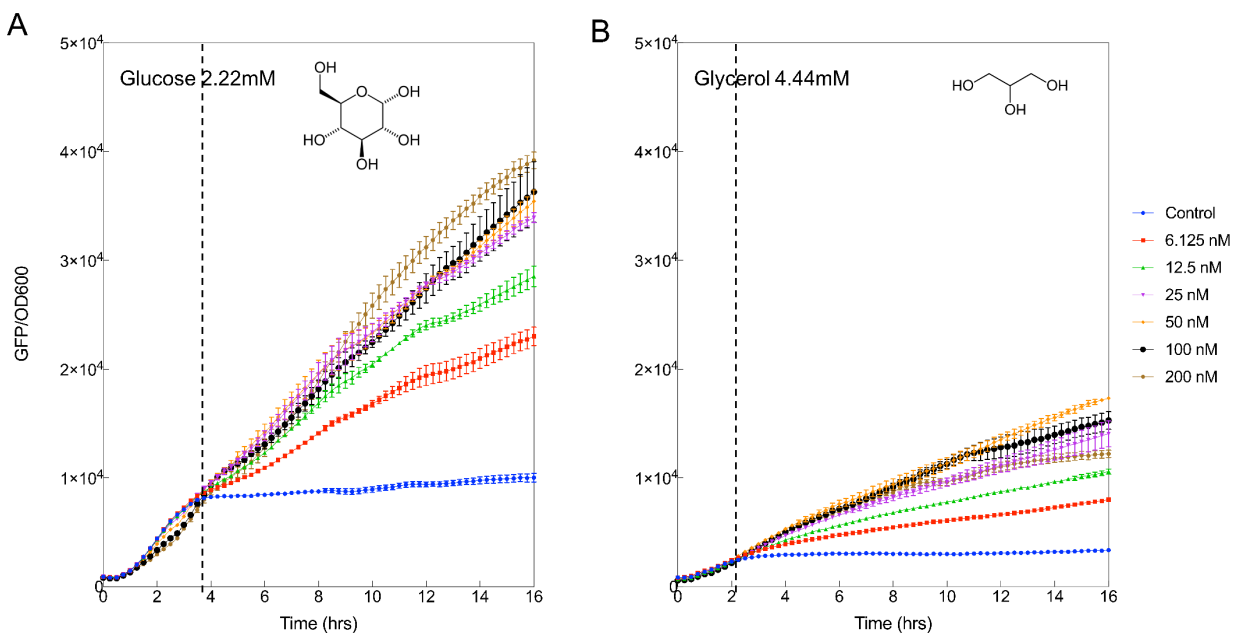


Figure B-9. Response time of amplifiers in different carbon source

Circuit performance of P_{VRecA_AT} amplifier system using different carbon source. The system is tested in M9 media supplemented with A) 2.22mM of glucose and B) 4.44mM of glycerol. Both experiments were done with a concentration gradient of Mitomycin C (0, 6.125, 12.5; 25, 50, 100; 200 nM) over 16hrs. The bottom row shows the OD600 profile of both experiments. N=3. Standard deviation and mean value were plot for three biological replicates.

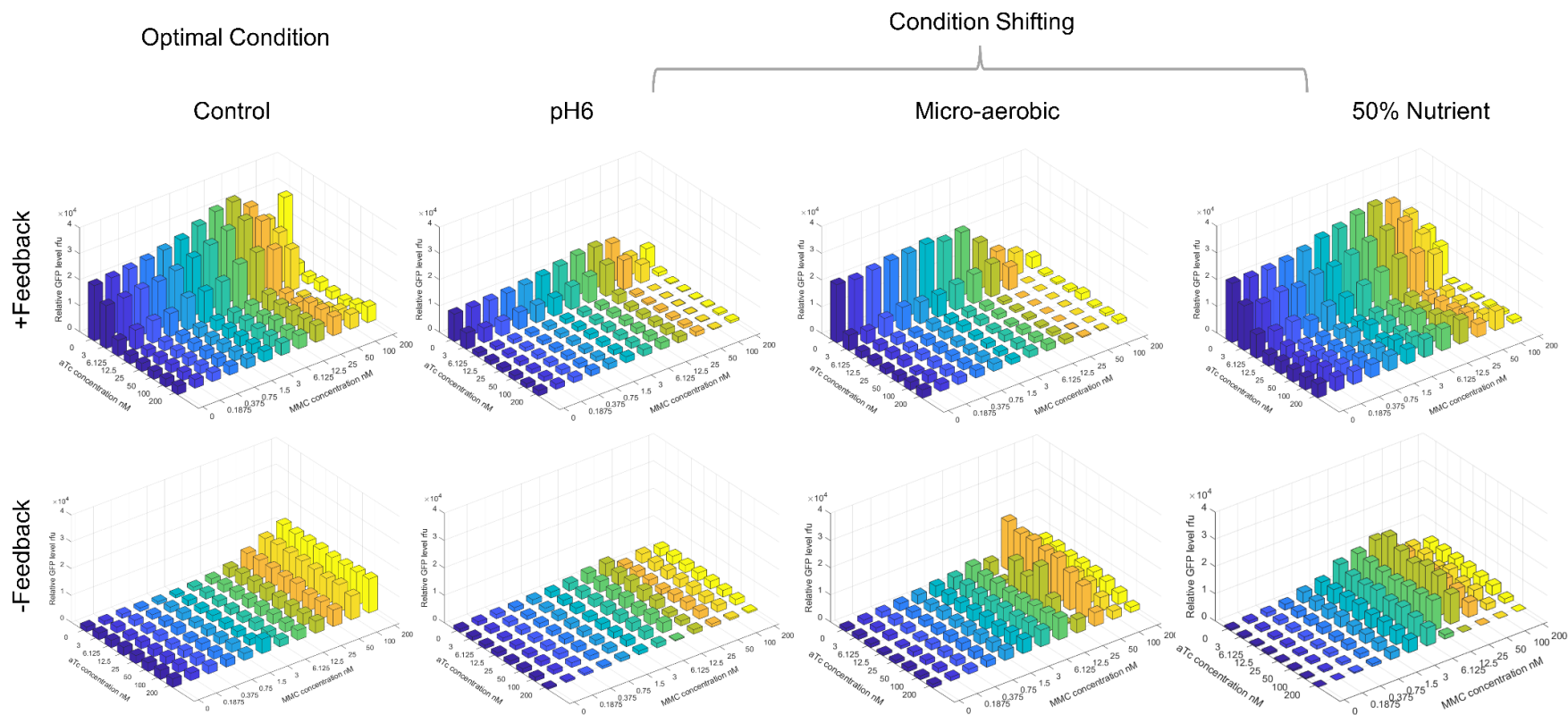


Figure B-10. System robustness against condition shift

3D bar chart representing the performance of P_{VRecA_AT} operational amplifier system subjected to different type of environmental disturbances (Acidic pH; Oxygen limitation and Nutrient limitation). Top row: P_{VRecA_AT} amplifier system containing the negative feedback modules tested with MMC and aTc from 0 to 200 nM under different conditions. Bottom row: P_{VRecA_AT} amplifier system without the negative feedback modules tested with MMC and aTc from 0 to 200 nM under different conditions. Microaerobic condition was achieved using 100 μ L mineral oil sealing the 96 well plates. pH was adjusted by addition of 1M HCl. Optimal condition was defined as pH 7.5 at 37 $^{\circ}$ C in a M9 media supplemented with 4% (w/v) glycerol without oil sealing.

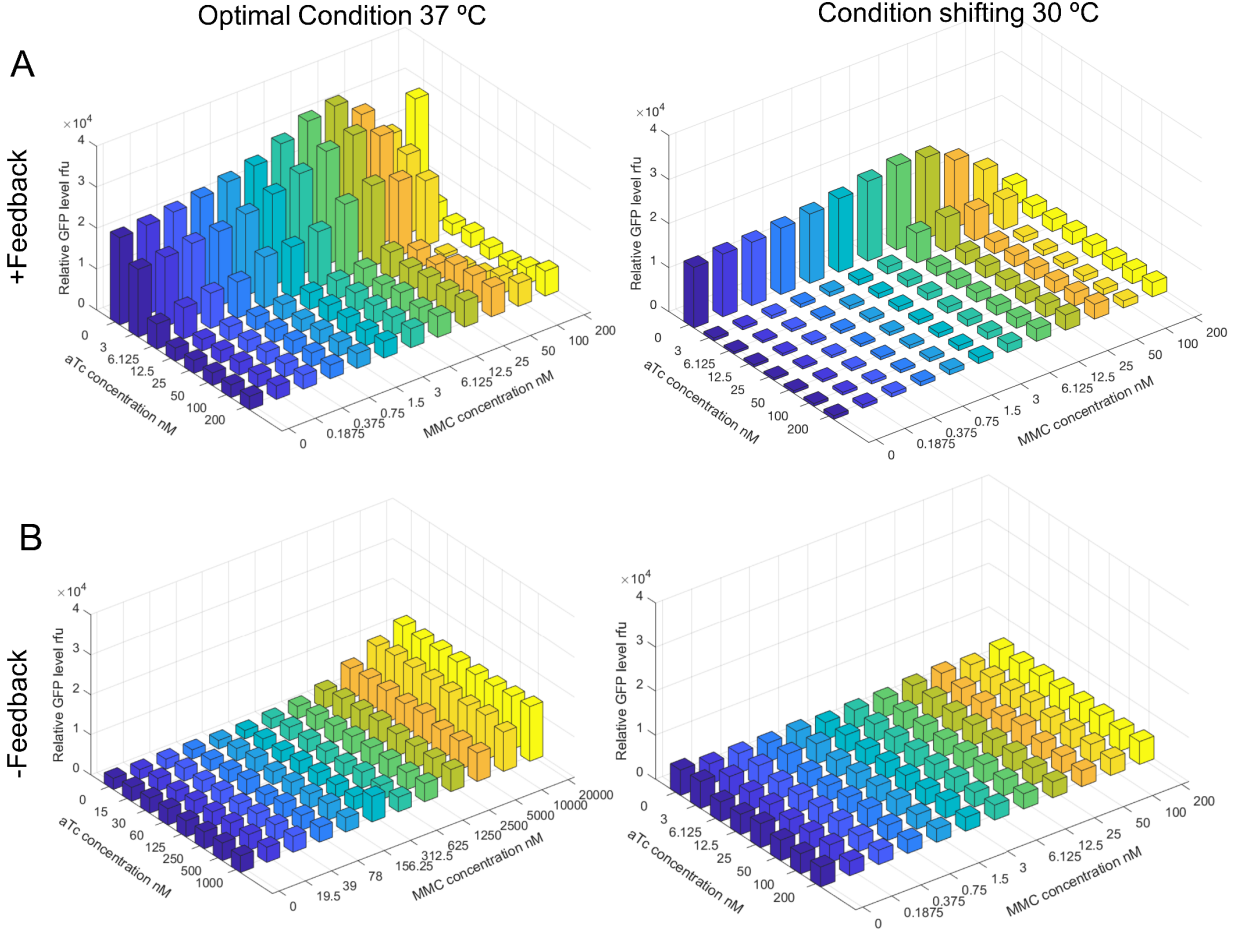


Figure B-11. System performance against temperature shift

3D bar chart representing the performance of P_{VRecA_AT} operational amplifier system subjected to temperature shift. A) P_{VRecA_AT} amplifier system containing the negative feedback modules tested with MMC and aTc from 0 to 200 nM. B) P_{VRecA_AT} amplifier system without the negative feedback modules tested with MMC and aTc from 0 to 200 nM.

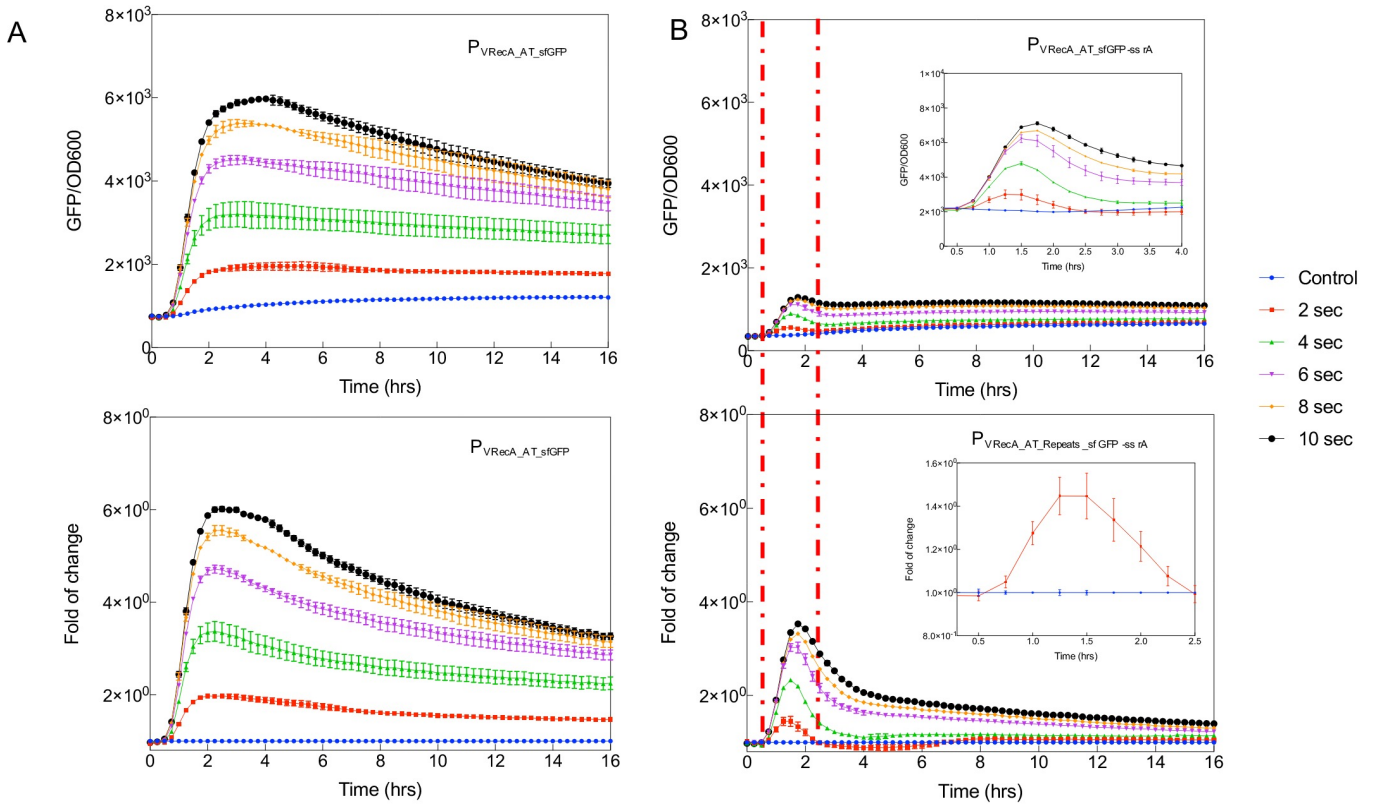
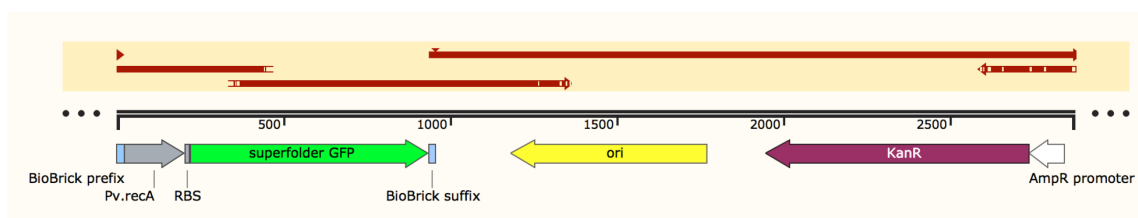


Figure B-12. System dynamics with ssrA tagged sfGFP

Induction performance of P_{VRecA_AT} (ATrepeat) with ssrA tagged sfGFP. P_{VRecA_AT} with A) normal sfGFP and B) ssrA tagged sfGFP. Fold of change upon induction was normalized to highlight the expression window upon 2sec induction. Region highlighted with dash line is shown in the top right window. System kinetics were measured for 16 hrs after the exposure of 320 nm UV at a duration gradient (0 sec; 2 sec; 4 sec; 6 sec; 8 sec and 10 sec). $N=3$. Standard deviation and mean value were plot for three biological replicates.

P_{VRecA_AT-sfGFP}



P_{VRecA_AT-sfGFP-ssrA}

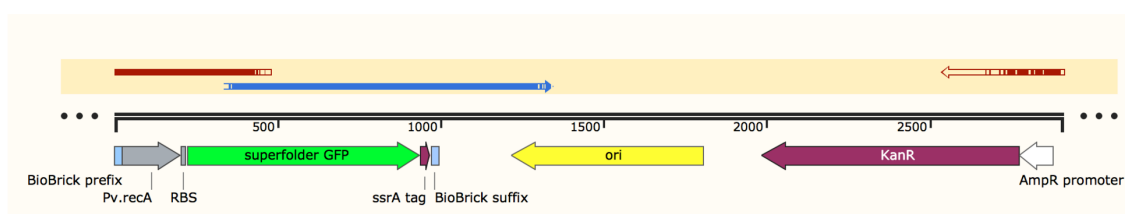


Figure B-13. Sequence check of transformed plasmids.

Cell supernatant was retrieved from turbidostats and sequenced to check plasmids integrity after 40hrs of UV exposure. Sequence alignment shown on top indicated sequence reads and plasmids map shown below. Mismatches are indicated as white space. No mutation was found in the core region of the sensor construct.

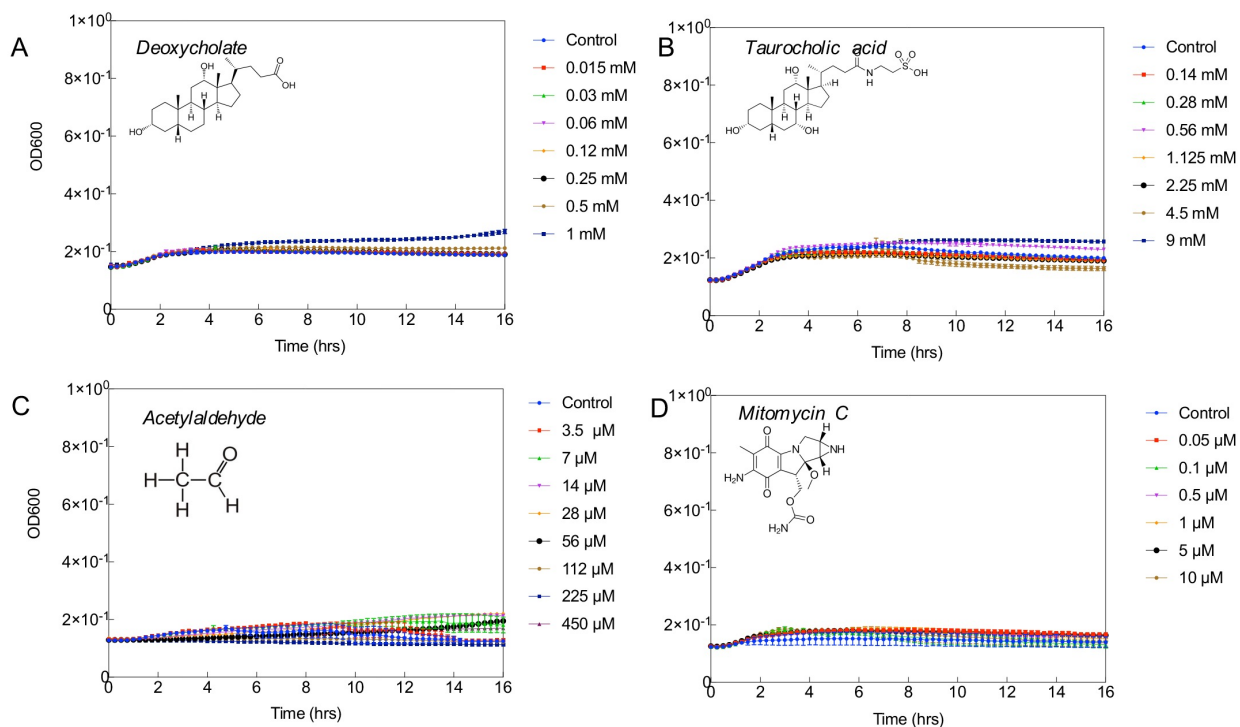


Figure B-14. OD Growth of bacteria tested with different secondary bile salts

OD600 of EcN transformed with optimized P_{VRecA_AT} promoter in detecting colon cancer related carcinogens. A) Deoxycholate B) Taurocholic acid and C) Acetylaldehyde. D) Mitomycin C N=3

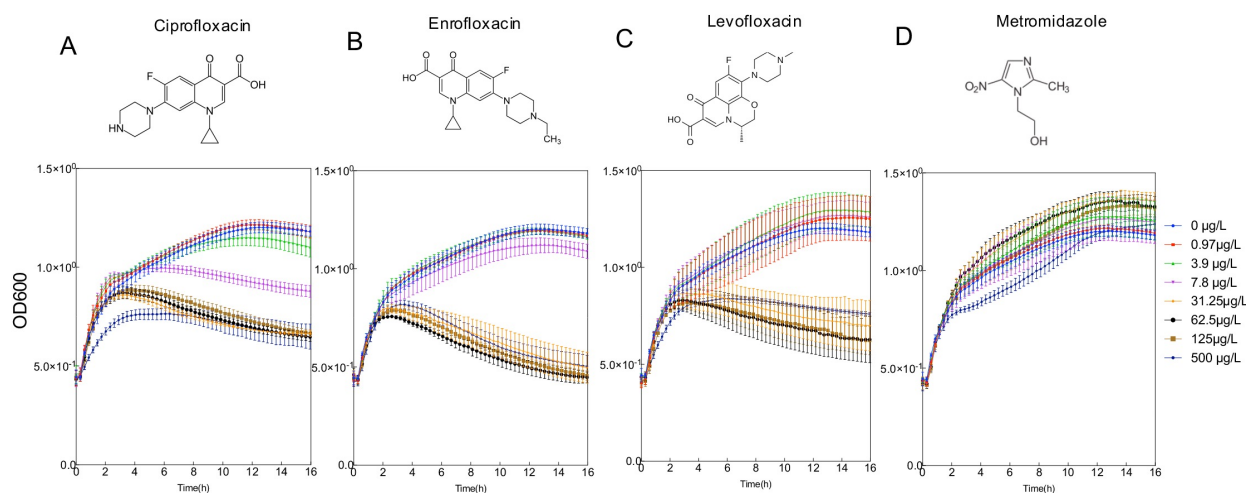


Figure B-15. OD Growth of bacteria tested with carcinogenic antibiotics

OD600 of EcN transformed with optimized P_{VRecA_AT} promoter in detecting commonly used carcinogenic antibiotics. A) Ciprofloxacin B) Enrofloxacin C) Levofloxacin and D). metronidazole N=3

Appendix C. Chapter 5. SI Figures.

Figure C-1. Sequence alignment between *E. coli* DH5 α and *E. coli* Nissle 1917

Alignment of NorR regulator.

Score	Expect	Method	Identities	Positives	Gaps
1017 bits(2630)	0.0	Compositional matrix adjust.	504/504(100%)	504/504(100%)	0/504(0%)
Query 1		MSFSVDVLANIAIELQRGIGHQDRFQRLITTLRQVLECDASALLRYDSRQFIPLAIDGLA			60
Sbjct 26		MSFSVDVLANIAIELQRGIGHQDRFQRLITTLRQVLECDASALLRYDSRQFIPLAIDGLA			85
Query 61		KDVLGRRFALEGHPRLEAIARAGDVVRFPADSELPDPYDGLIPGQESLKVHACVGLPLFA			120
Sbjct 86		KDVLGRRFALEGHPRLEAIARAGDVVRFPADSELPDPYDGLIPGQESLKVHACVGLPLFA			145
Query 121		GQNLIGALTLDGMQPDQFDVFSDEELRLIAALAAGALSNALLIEQLESQNMLPGDATPFE			180
Sbjct 146		GQNLIGALTLDGMQPDQFDVFSDEELRLIAALAAGALSNALLIEQLESQNMLPGDATPFE			205
Query 181		AVKQTQMIGLSPGMTQLKKEIEIVAASDLNVLISGETGTGKELVAKAIHEASPRAVNPLV			240
Sbjct 206		AVKQTQMIGLSPGMTQLKKEIEIVAASDLNVLISGETGTGKELVAKAIHEASPRAVNPLV			265
Query 241		YLNCAALPESVAESELF GHVKGAF TGAISNRSGKFEMADNGTLFLDEIGELSLALQAKLL			300
Sbjct 266		YLNCAALPESVAESELF GHVKGAF TGAISNRSGKFEMADNGTLFLDEIGELSLALQAKLL			325
Query 301		RVLQYGDIIQVRGDDRCLRDVVRVLAATNRDLREEVLAGRFRADLFHRLSVFPLSVPLRE			360
Sbjct 326		RVLQYGDIIQVRGDDRCLRDVVRVLAATNRDLREEVLAGRFRADLFHRLSVFPLSVPLRE			385
Query 361		RGDDVILLAGYFCEQCRLRQGLSRVVL SAGARNLLQHYSFPGNVRELEHAIHRAVVLARA			420
Sbjct 386		RGDDVILLAGYFCEQCRLRQGLSRVVL SAGARNLLQHYSFPGNVRELEHAIHRAVVLARA			445
Query 421		TRSGDEVILEAQHFAPFEVTLPTPEVAAPVVKQNLREATEAFQRETIRQALAQNHNNWA			480
Sbjct 446		TRSGDEVILEAQHFAPFEVTLPTPEVAAPVVKQNLREATEAFQRETIRQALAQNHNNWA			505
Query 481		ACARMLETDVANLHRLAKRLGLKD	504		
Sbjct 506		ACARMLETDVANLHRLAKRLGLKD	529		

Alignment of P_{NorV} between two strains.

Score	Expect	Identities	Gaps	Strand
327 bits(177)	6e-95	183/186(98%)	0/186(0%)	Plus/Plus
Query 1	CTTTGCCTCACTGTCAATTTGACTATAGATATTGTCATATCGACCATTGATTGATAGTC			60
Sbjct 1	CTTTGCCTCACTGTCAATTTGACTATAGATATTGTCATATCGACCATTGATTGATAGTC			60
Query 61	ATTTTGACTACTCATTAATGGGCATAATTTATTATATAGAGTAAAAACAATCAGATAAAA			120
Sbjct 61	ATTTTGACTACTCATTAATGAGCATAATTTATTATATAGAGTAAAAACAATTAGATAAAA			120
Query 121	AACTGGCACGCAATCTGCAATTAGCAAGACATCTTTTTAGAACACGCTGAATAAATTGAG			180
Sbjct 121	AACTGGCACGCAATCTGCAATCAGCAAGACATCTTTTTAGAACACGCTGAATAAATTGAG			180
Query 181	GTGCT	186		
Sbjct 181	GTGCT	186		

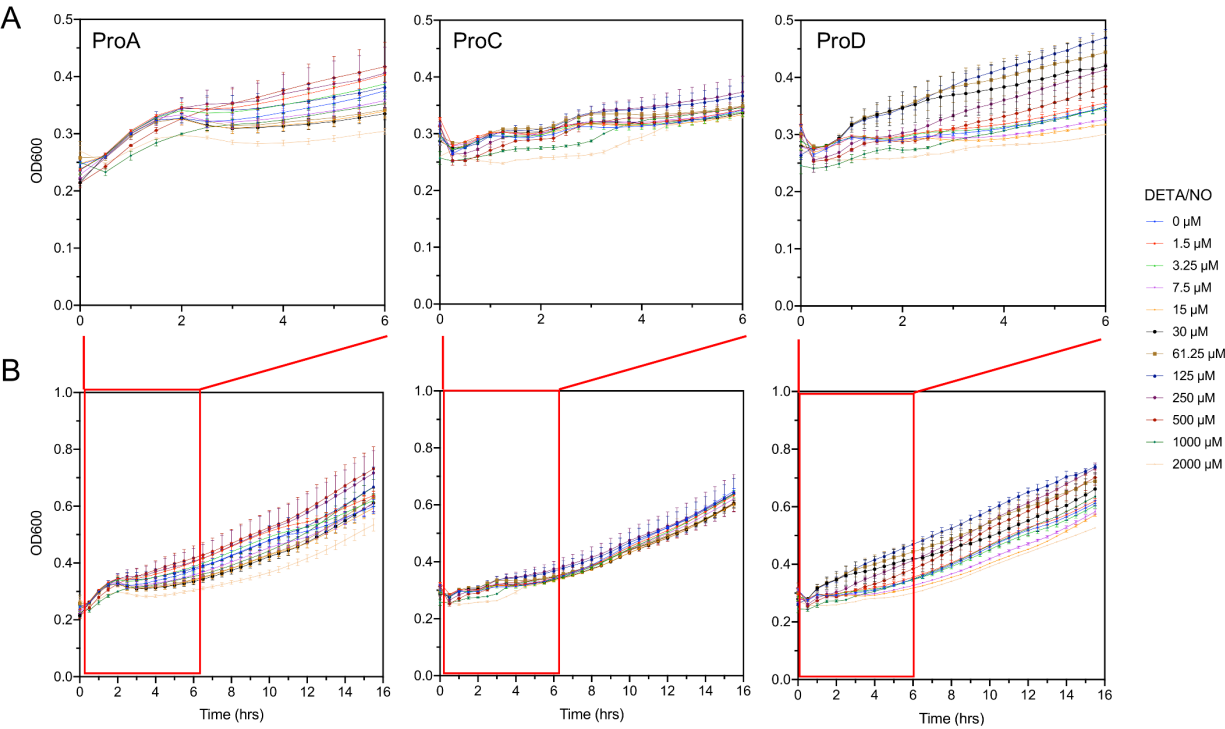


Figure C-2. Growth curve of induced circuits in open loop format.

OD growth of transformed EcN with different NorR construct (pProA,pProC and pProD). System induced with DETA/NO gradient of (0,1.5,3.25,7.5,15,30,61.25,125,250,500,1000,2000 μM) A) OD growth of the first 6hrs were enlarged and B) the total 16 hrs. Standard deviation and mean value of three bio-replicates were plotted in each graph.

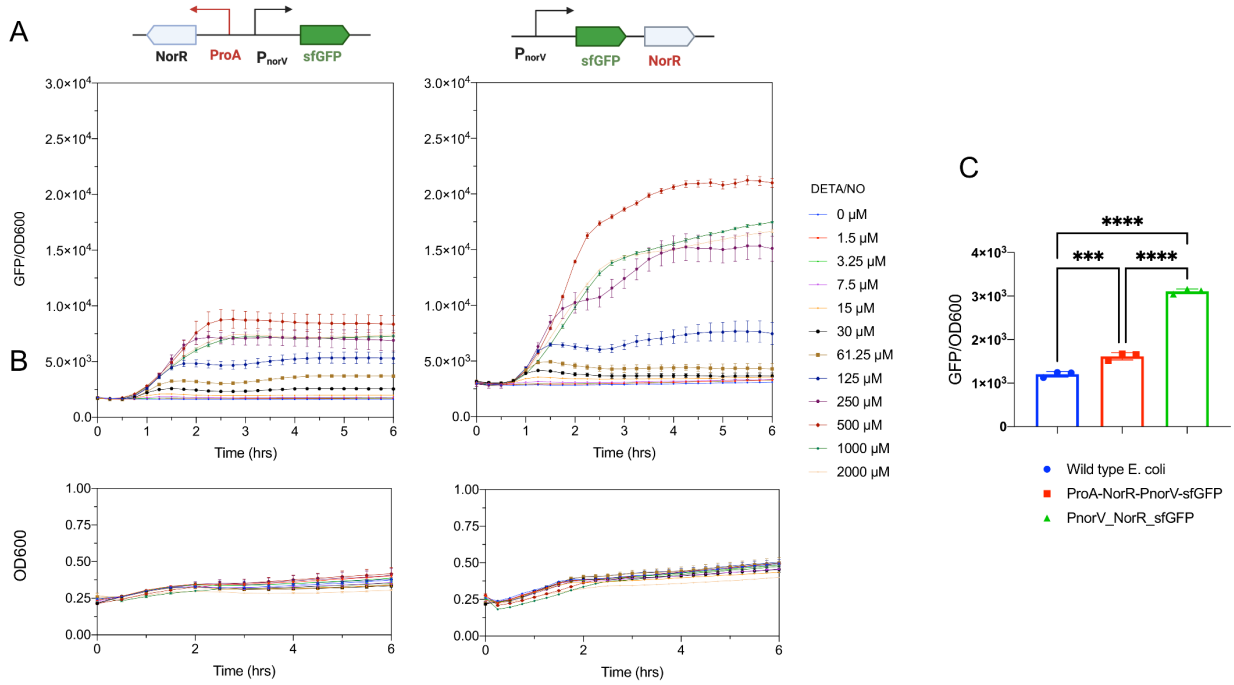


Figure C-3. Comparison of the system with and without the addition of positive feedback.

A) Induction kinetics of the open loop circuit ($\text{P}_{\text{roA}}\text{-norR-P}_{\text{NorV}}\text{-sfGFP}$) and positive regulated circuit ($\text{P}_{\text{NorV}}\text{-sfGFP-norR}$) shown in the same scale. B) The growth curve shown as OD600. C) Baseline difference compared between non-transformed *E. coli* Nissle 1917 and the two systems. Standard deviation and mean value of three bio-replicates were plotted in each graph.

Table C-1. Parameters used for simulation of NorR competitive binding

Parameter	Description	Value	Units
α_1	Constitutive expression strength of P_{roD}	100	nM/min
α_2	Constitutive expression strength of P_{roA}	27.8	nM/min
α_3	Constitutive expression strength of P_{roC}	3	nM/min
δ_1	Degradation rate of NorR protein	0.3	1/min
δ_2	Degradation rate of GFP protein	0.3	1/min
α_{norV}	Combined transcription/translation rate of NorR	200	nM/min
Kr	Binding affinity between non-induced, repressed NorRr and P_{NorV}	0.1	nM
Ka	Binding affinity between NO and NorR	10	nM
KA	Binding affinity between induced, activated NorRi and P_{NorV}	5	nM
N	Hill coefficient for NO-NorR binding	2	-

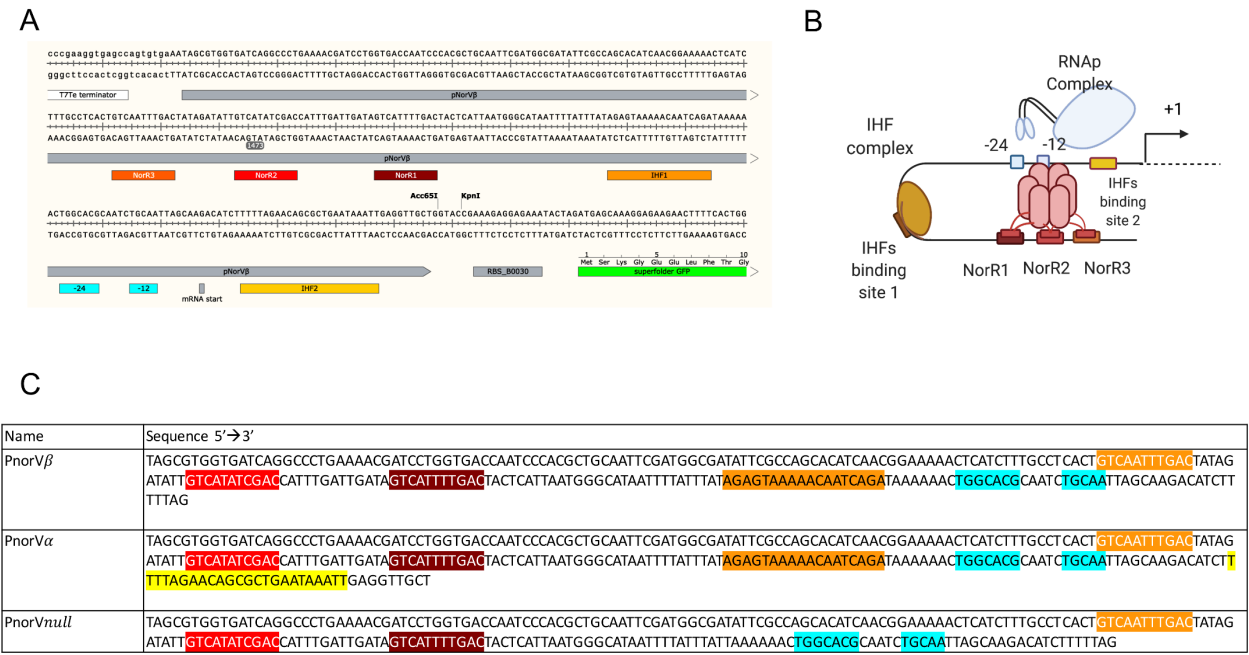


Figure C-4. Sequence annotation and alignment of *pnorV* & different design.

A) Annotated P_{NorV} promoter sequence. Three NorR binding site has been labelled with color. NorR binding site 1 and 3 both contain inverted repeat GTCA-(N3)-TGAC, while site 2 is GTCA-(N3)-CGAC. Sigma factor binding site has been shown in cyan and two of the IHFs shown in orange. B) Schematic diagram and the spatial layout of each component. C) Detailed sequence map for P_{NorV} with one IHF (P_{NorVβ}), two IHF (P_{NorVα}) and none (P_{NorVnull})

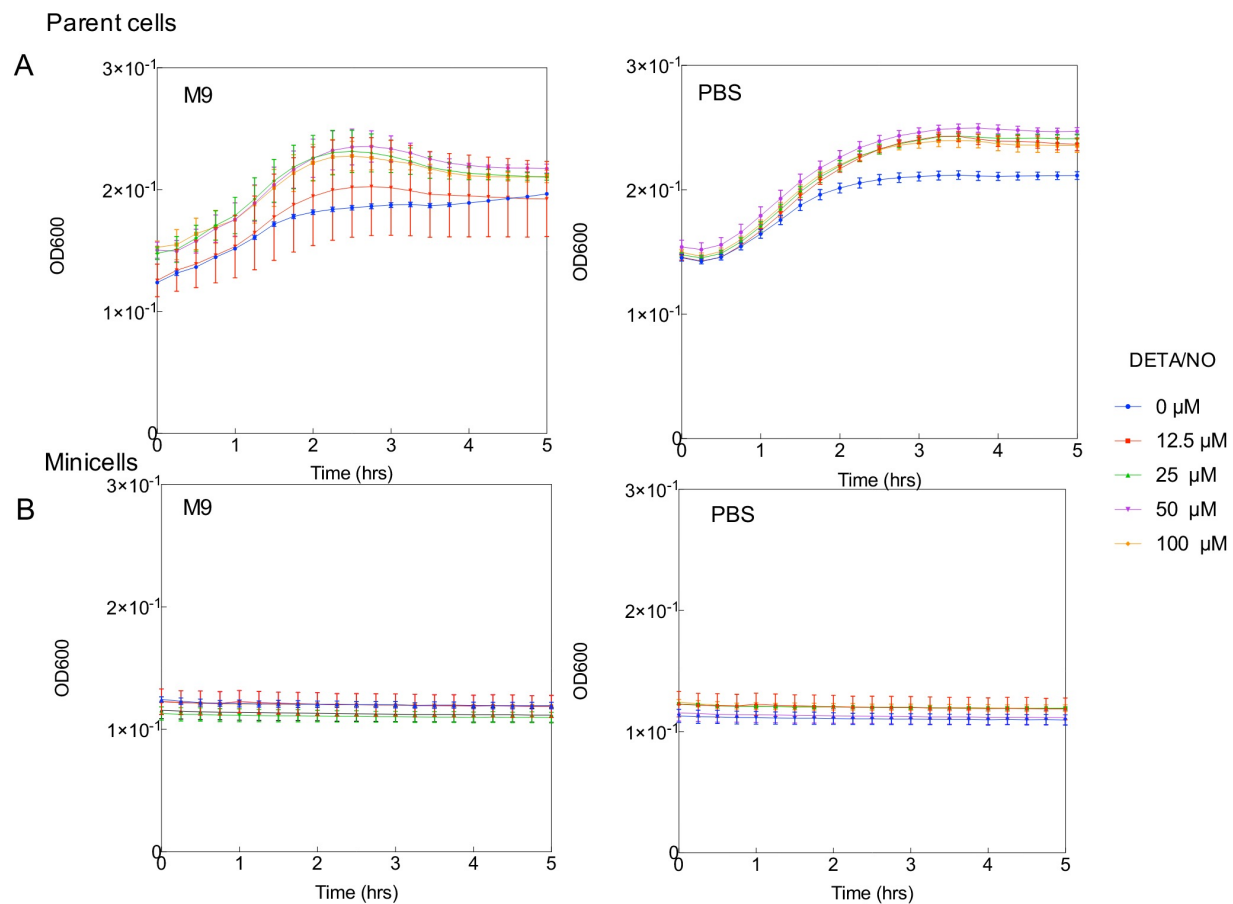


Figure C-5. OD growth curve of minicells and parents in different growth medium

OD600 of the transformed parent cell (*E. coli* MC1000 Δ *minD*) and mini SimCells induced in different medium. Standard deviation and mean value of three bio-replicates were plotted in each graph.

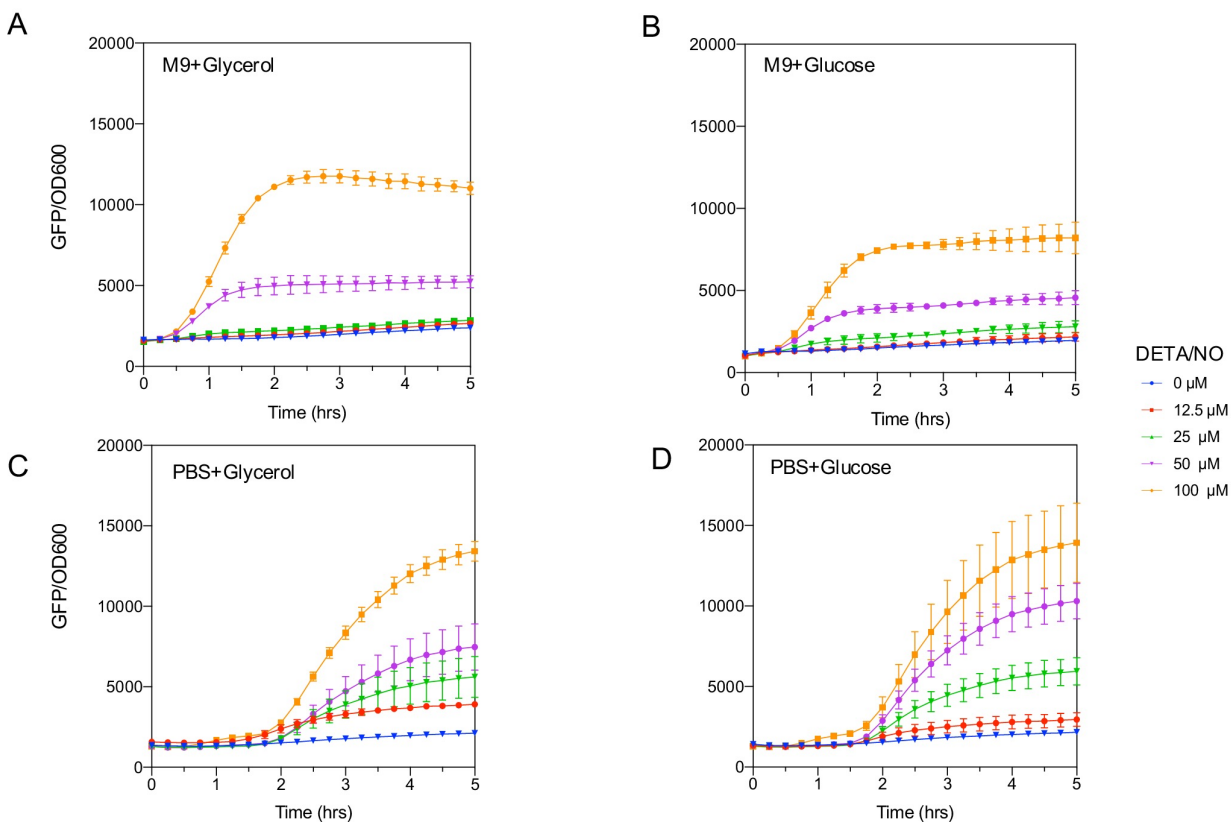


Figure C-6. Impact of carbon source and nitrogen source towards system dynamics.

A) Transformed EcN was induced under M9 supplemented with 4% w/v glycerol. B) transformed EcN induced at the same condition however, equal molar of glucose 2% w/v was supplemented instead. C) Nitrogen absent PBS medium was used instead of M9. Carbon source of 4% w/v glycerol unchanged D) PBS supplemented with glucose 2% w/v. Standard deviation and mean value of three bio-replicates were plotted in each graph.

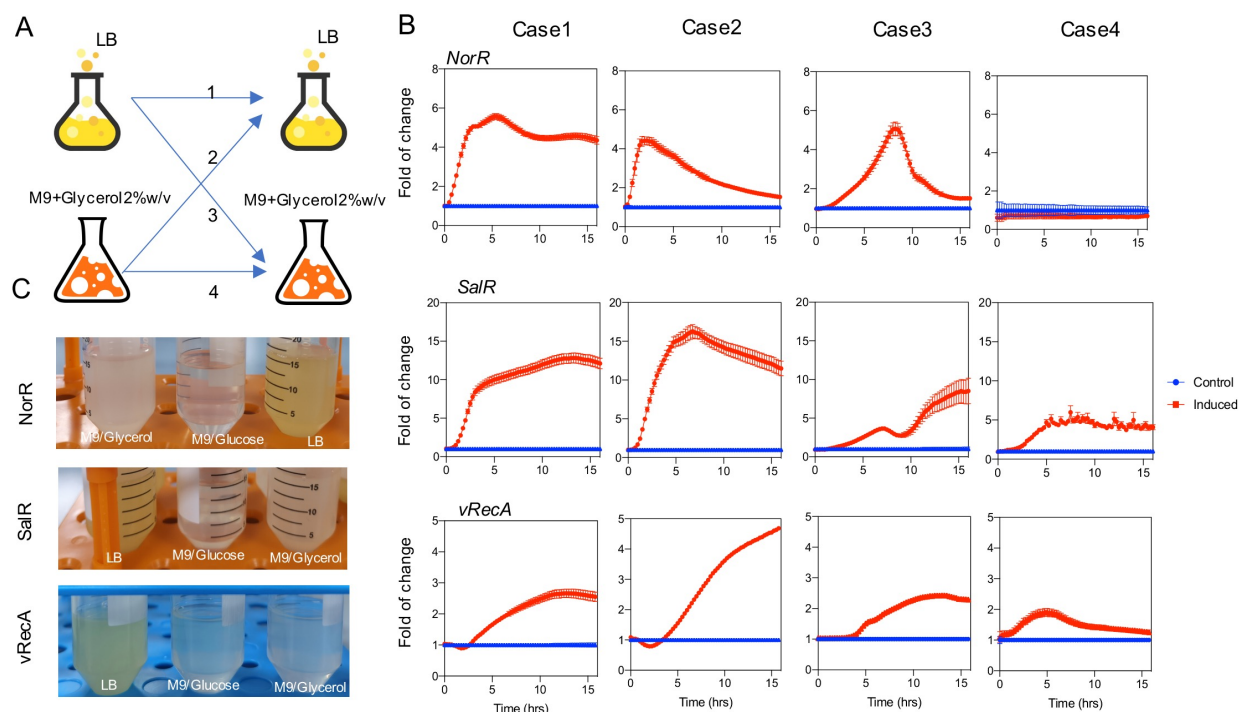


Figure C-7. NorR system is more vulnerable towards medium change.

NorR, *vRecA* and *SalR* system were challenged via medium shift to examine circuits memory dependent on the previous state. A) Graphical representation of the experiment procedure undergoes to test the cellular memory. In case one, transformed cells were inoculated in LB for 16 hrs and induced under the same LB condition in microplate; Case two, transformed cells were firstly inoculated in LB but induced under a more nutrient stringent condition: M9 supplemented with 2% w/v glycerol. Case three and Case four started in vice versa. B) Induction kinetics profiles shown as fold of change for *SalR* system ($P_{Sal_gfp_salR}$), *vRecA* ($P_{vRecA_AT_sfGFP}$) and *NorR* ($P_{NorV\beta_sfGFP_NorR}$); Fully saturated induction concentration was used for each system: aspirin (100 μM), Mitomycin C (500nM) and nitric oxide (1 mM). C) Transformed *EcN* failed to be inoculated in M9 supplemented with glucose 1%w/v after picking from fresh colony. N=3

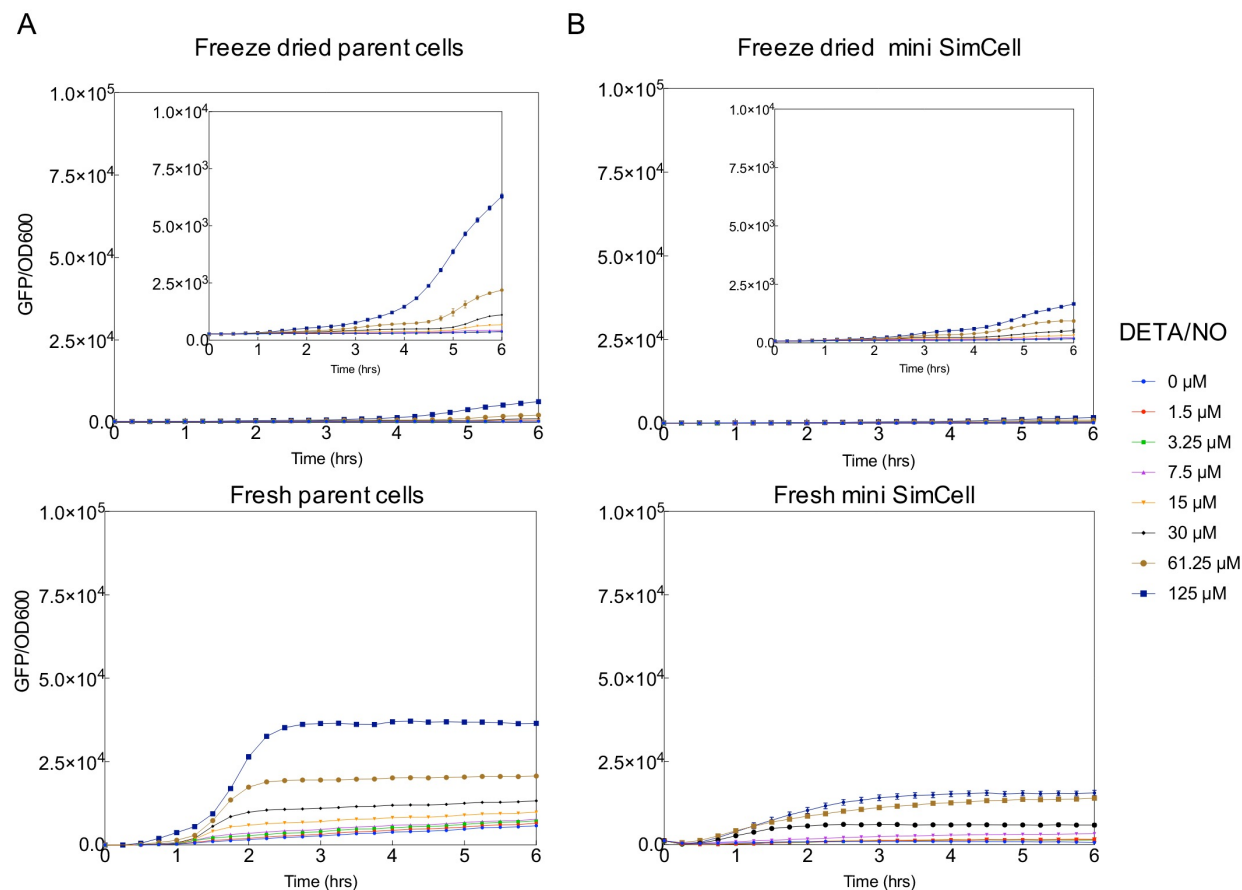


Figure C-8. Direct comparison of fresh and lyophilised whole cell biosensor versus mini SimCells.

A) Freeze dried parent cell (pPnorV β transformed into *E. coli* MC1000 Δ *minD*) and freshly transformed parent cell induced over a gradient of DETA/NO (0, 1.5, 3.25, 7.5, 15, 30, 61.25, 125 μ M). B) Freeze dried mini SimCells and freshly purified mini SimCells. For better visualisation, freeze dried data were shown in enlarged box. Standard deviation and mean value of three bio-replicates were plotted in each graph.

Appendix D. Chapter 6. SI Figures.

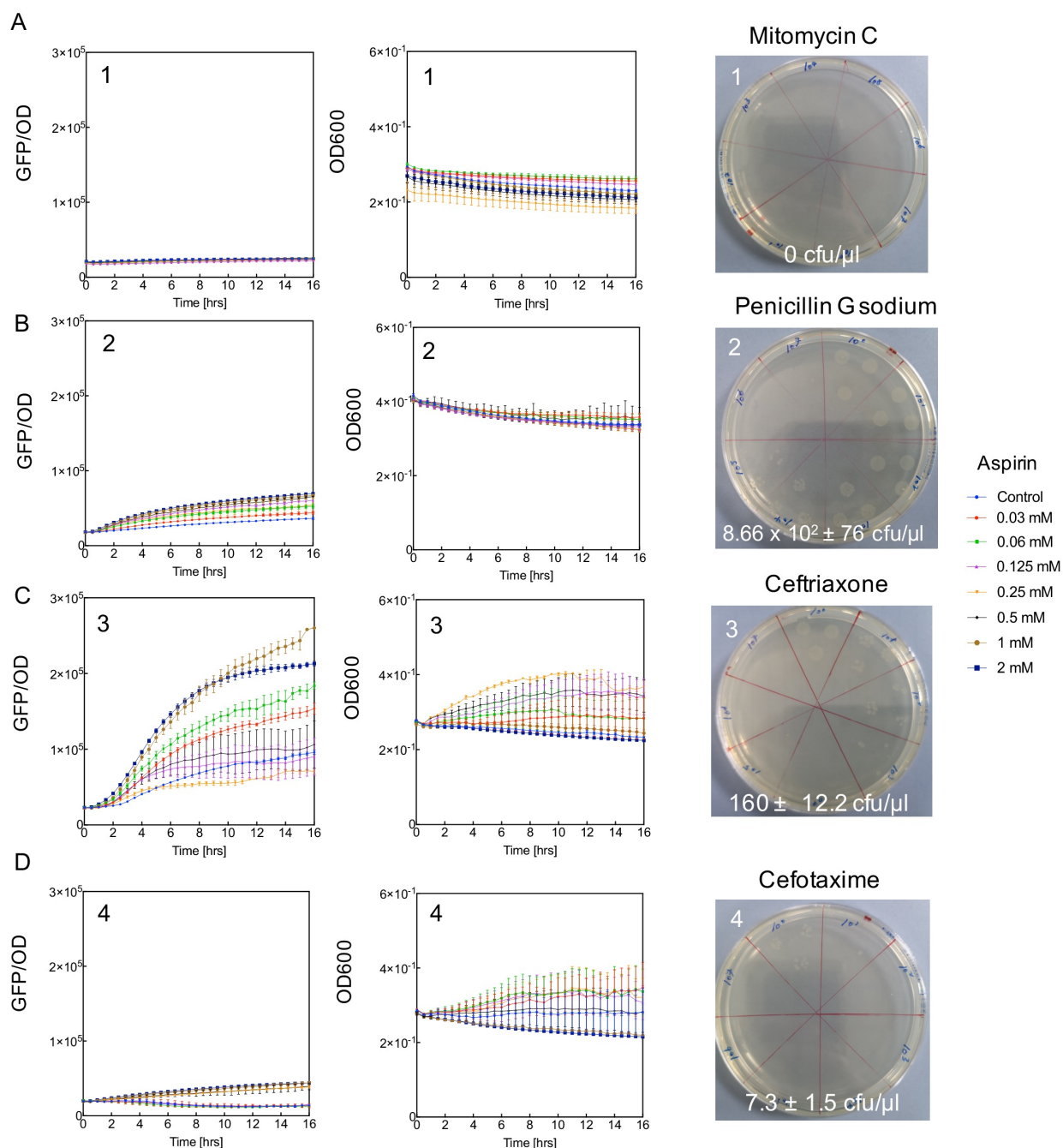


Figure D-1. Single antibiotics cannot eliminate parent cell contamination.

Purification of mini SimCells using four different antibiotics. A) Mitomycin C B) Penicillin G sodium C) Ceftriaxone and D) Cefotaxime. For function testing, *E. coli* MC1000 $\Delta minD$ was transformed with aspirin sensing construct ($P_{Sal_gfp_salR}$), purified mini SimCells were induced

with an aspirin gradient (0,0.03,0.06,0.125,0.25,0.5,1,2 mM). From left to right, the induction kinetics (GFP/OD600) and OD600 growth curve were shown. plate assay was done to quantify the parent cell contamination for 16hrs. Colony-forming unit (CFU) of the mini Simcell filtrate was indicated in each case. N=3

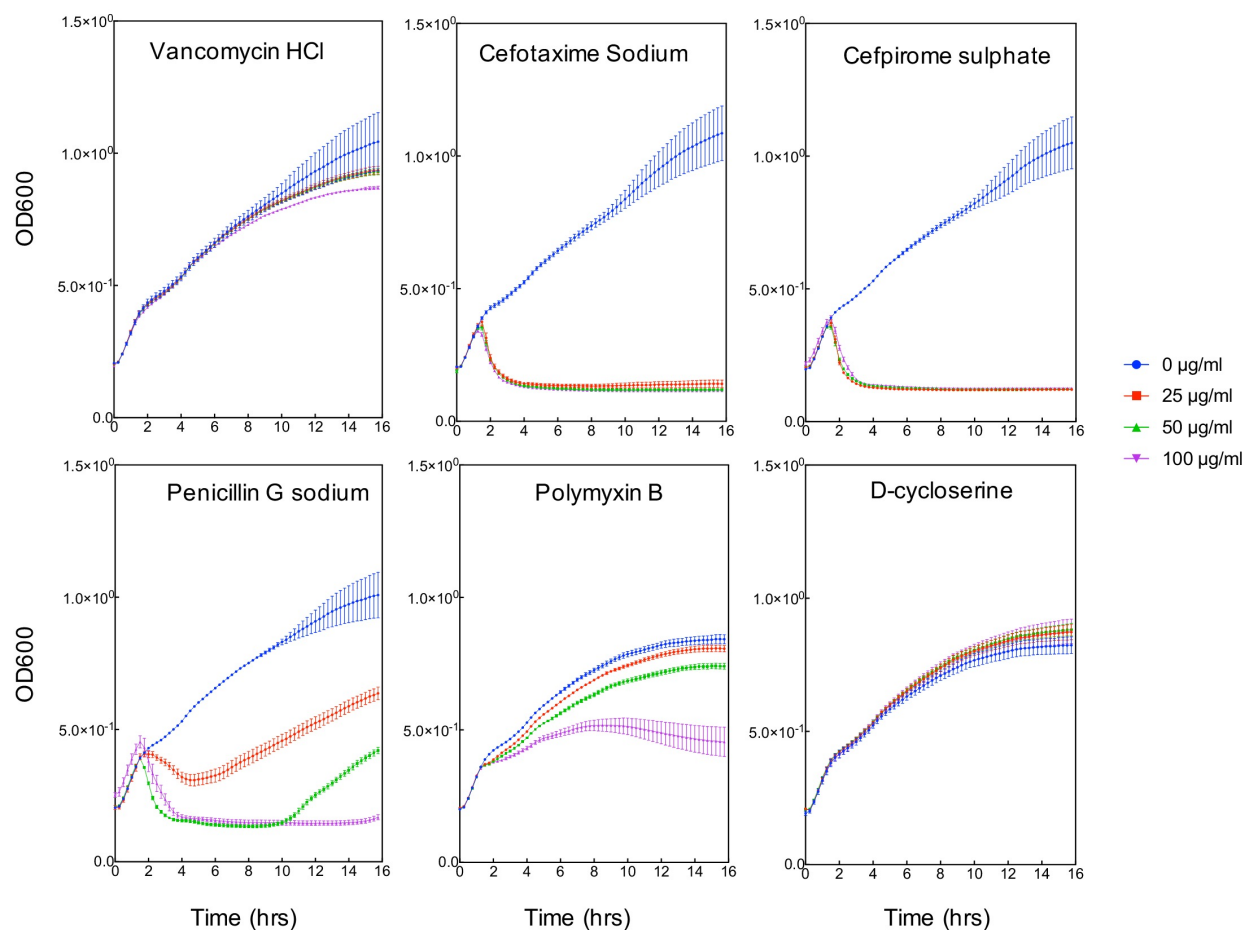


Figure D-2. Screen for better antibiotics candidate for cell wall lysis.

Six different cell wall lytic antibiotic candidates were screened to determine the minimal inhibition concentration (MIC) of each one. A gradient of 25 ug/ml, 50 ug/ml and 100ug/ml were tested for each compound. N=3.

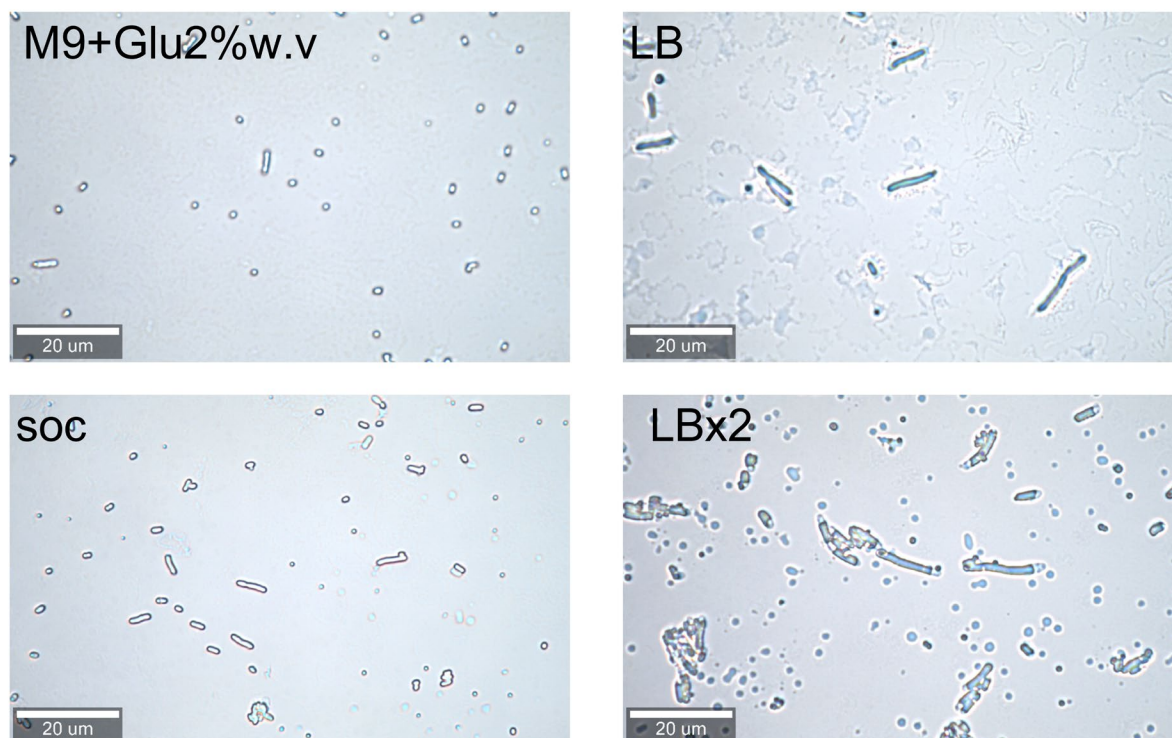


Figure D-3. Minicells culturing using different medium.

Culturing of minicells producing strains *E. coli* MC1000 $\Delta minD$ using different medium composition to further enlarge the parent cells. LB and 2-times concentrated LB successfully showed parent cells with increased cell length over 16 hrs culturing. Scale bar = 20 µm.

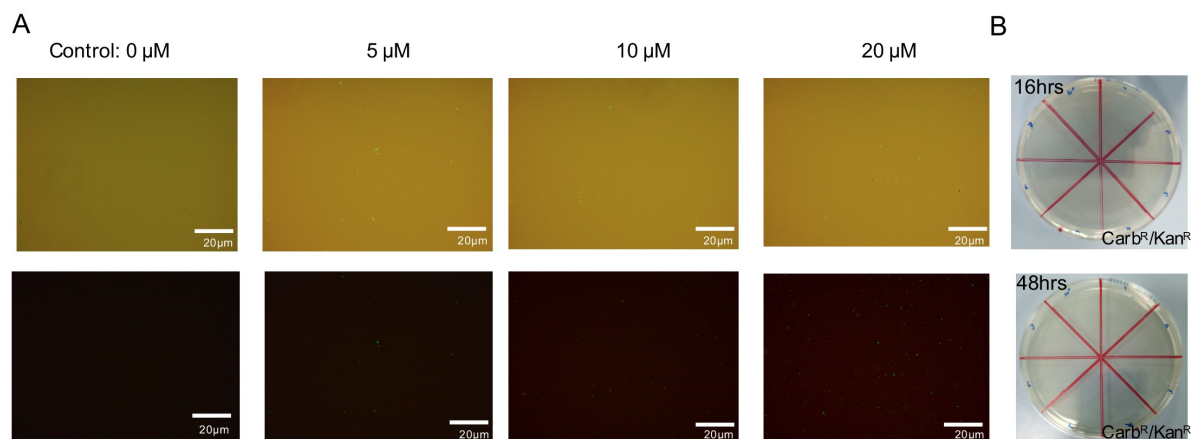


Figure D-4 Microscope images of minicell loaded with aspirin sensor constructs.

A) Images were taken at both GFP channel and overlaid channel for indication of potential mutated circuits or non-transformed minicells. The specific concentration of aspirin used is indicated above plot. Background was filtered using ImagJ. Scale bar = 20µm B) Plate assay for purified mini SimCells filtrate was done over 16hrs and 48hrs respectively.

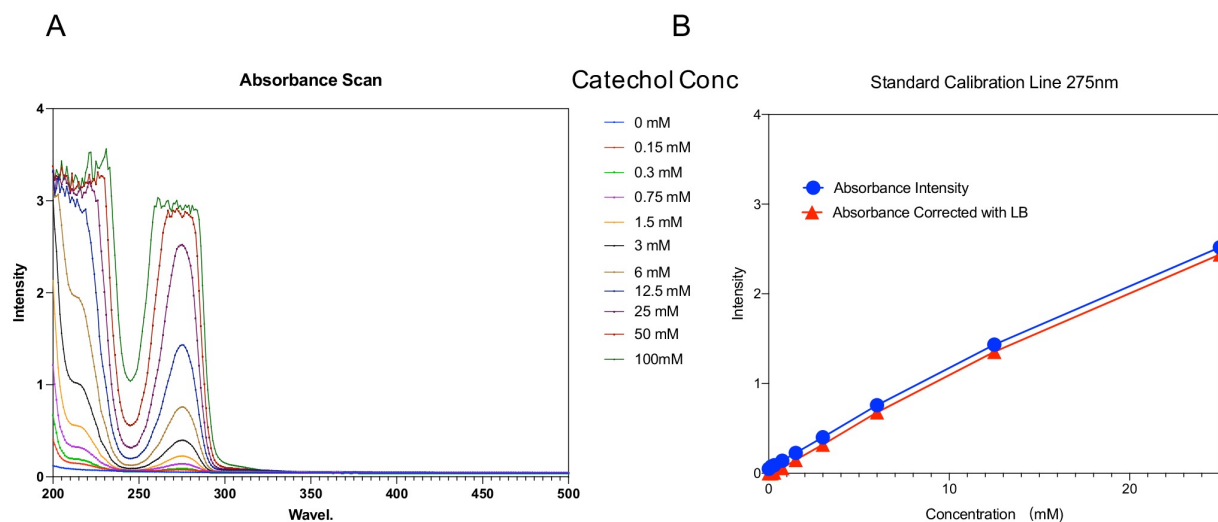


Figure D-5. Standardised curve for quantifying catechol production.

A) Absorbance scan of catechol ranges from 0, 0.15, 0.3, 0.75, 1.5, 3, 6, 12.5, 25, 50, 100 mM B) standardized curve plotted using peak value at 275 nm and background removed using LB.