Synthetic Temperature Inducible Lethal Genetic Circuits in *Escherichia coli*

by

Stephanie Pearce
B.Sc. Honours, University of Victoria, 2013

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

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Abstract

Temperature-sensitivity (TS) is often used as a way to attenuate microorganisms to convert them into live vaccines. Studies indicate that live vaccines are often necessary for the complete clearance of certain pathogenic organisms. In this work we explore the use of TS genetic circuits that express lethal genes for their potential utility as a widely applicable approach to TS attenuation. Here, we use restriction endonucleases as the lethal gene products. We tested different combinations of TS repressors and cognate promoters controlling the expression of genes encoding restriction endonucleases inserted at four different non-essential sites in the *Escherichia coli* chromosome. We found that the presence of the restriction endonuclease genes did not affect the viability of the host strains at the permissive temperature, but that expression of the genes at elevated temperatures killed the strains to varying extents. The location of the genetic circuit cassette in the chromosome was critical, and insertion at the *ycgH* site led to minimal cell death. Induction of the TS circuit in a growing culture led to a pre-mature leveling off of the optical density, and a shift in the number of cells that could exclude a dye that indicated cell viability. Incubation of cells initially grown at low temperature and then suspended in phosphate buffered saline at high temperature, led to about 100-fold loss of cell viability per day compared to minimal loss of viability for the parental strain. The Dual strain containing two different genetic circuits was found to have reduced escape frequency compared to single circuit

strains. However, strains carrying either one or two TS lethal circuits could generate mutants that survived high temperature. These mutants included start codon deletions as well as upstream deletions of the TetRD1 encoding gene as well as complete deletions of the lethal gene circuits.

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List of Abbreviations

ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
AU	Arbitrary unit
bp	Base pair
CAT	Chloramphenicol acetyl transferase
Cb	Carbenicillin
CFU	Colony forming units
CFU/ml	Colony forming units per millilitre
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EPOD	Extended protein occupancy domain
EthD-III	Ethidium homodimer III
Gm	Gentimicin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KBMA	Killed but metabolically active
Km	Kanamycin
LB	Lysogeny broth
nm	Nanometer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBS	Ribosome binding site
RE	Restriction endonuclease or enzyme
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
TA	Toxin-antitoxin
TetR	Tetracycline repressor
tetO	Tetracycline operator
TIR	Translation initiation rate
Tn10	Transposon 10
TS	Temperature-sensitive or sensitivity
*ts*EPOD	Transcriptionally silenced extended protein occupancy domain
YFP	Yellow fluorescent protein

Acknowledgments

Thank you to all members of the Nano lab past and present.
I couldn't have picked a better lab to work in.

Special thanks to Dr. Fran Nano for giving me the opportunity to work in his lab all these years.
Thank you for your modesty, optimism, guidance and insight.

CHAPTER 1: INTRODUCTION

1.1 Perspective on Synthetic Biology

Synthetic biology aims to solve real world problems with the engineering of biological parts, systems and organisms. Over the past two decades, the field has rapidly expanded to impact areas including molecular biology, biotechnology, therapeutics, energy and environment. Utilizing microorganisms for different purposes often presents unique biological problems – large or small – that require practical solutions such as the design of genetic circuits and switches that allow for the control over microorganisms. Controlling microbes has important commercial value in, for example, the production of cleaner and more renewable biofuels (d'Espaux *et al*., 2015), the bioremediation of polluted sites (Liu *et al*., 2015) and the design of new and improved drugs and vaccines (Ruder *et al*., 2011).

There are two main approaches for engineering microbes, rational design and directed evolution. Rational design posits that one can rationally design parts, systems and organisms using a wealth of knowledge from literature, bioinformatics and well-characterized genetic tools. In contrast, directed evolution employs multiple rounds of random mutagenesis followed by a screening and selection process. Directed evolution is almost always paired with high throughput capabilities to increase the likelihood of eventually encountering the desired property. The main advantage of directed evolution is that one does not need to fully understand the mechanism of the activity of interest (Giger *et al.,* 2013). Currently this makes it the more appealing approach for engineering microbes over rational design, as the latter has been troubled by the unpredictable behaviour of biological parts occurring together *in vivo* (Kwok, 2010; Way *et al*., 2014; Luo *et al*., 2013). This unpredictability can be linked to the complexity of biological

organisms and the incomplete understanding of gene regulation. Conversely, recent advances in the directed evolution field such as Multiplex Automated Genomic Engineering (MAGE) have increased its attractiveness. This technique allows for large scale programming and evolution of cells *in vivo* by being able to target multiple genomic sites simultaneously using specialized, automated machinery (Wang *et al*., 2009). However, few investigators have access to this type of machinery and thus its use is limited. Despite the difficulties of applying rationale design to genome engineering, it can be the preferred approach when a desired phenotype can be programmed with a simple genetic circuit, such as described in this work. Therefore the literature was drawn upon, employing many current synthetic biology techniques in order to rationally design genetic circuits in *E. coli* to render strains TS. These TS *E. coli* strains could have important applications in the design of TS attenuated vaccines and in the biocontainment of microorganisms.

1.2 Engineering temperature-sensitive microorganisms

Engineering a microbe's maximum and minimum growth temperature allows for control over spatial and temporal survival. All organisms have a natural upper and lower temperature limit for growth (Morita, 1975). However, in general these natural limits cannot be dramatically changed by using simple selection or mutagenesis procedures (Rudolph *et al.*, 2010) such as random chemical mutagenesis or passaging. To overcome this, one can use psychrophilic essential genes, directed evolution TS essential genes, or genetic elements to impose artificial temperature limits on the growth of microbes.

Historically the first TS microbes were isolated following chemical mutagenesis, a process which leads to random deoxyribonucleic acid (DNA) mutations that occur throughout

the genome. Similarly, TS viruses have also been isolated by repeated passaging at lower temperatures over the span of several months (Maassab and DeBorde, 1985). This results in an organism that is cold-adapted, and sometimes these are also unable to replicate at higher temperatures. Chemical mutagenesis and passaging can easily create TS organisms; however, the number, location and stability of mutations are undefined. This unpredictability is a major drawback for engineering reliable and stable TS microorganisms.

Another strategy for creating TS microbes is by using psychrophilic essential genes or essential genes from mesophiles that have been mutated to produce a TS product. An essential gene is one that is required for an organism's viability under all growth conditions. A psychrophilic essential gene is an allelic equivalent isolated from a cold-loving bacterium. These genes are valuable targets for attenuating microbes because they have been extensively researched and it is known that many essential genes are conserved across bacterial species (Duplantis *et al*., 2011). When some psychrophilic essential genes are swapped for their native homologues they can impart temperature-sensitivity on the host organism (Duplantis *et al*., 2010; Pinto and Nano, 2015; Pankowski *et al*., 2016).

There are many examples of creating TS proteins through mutagenesis of the encoding genes, and this applies to essential gene products. For example, R. McWhinnie in the Nano group used error-prone PCR mutagenesis to generate 39 temperature-sensitive mutants of the tetracycline repressor (TetR) protein. Additionally, the Arnold group used directed evolution to evolve an esterase to become thermostable (Giver *et al*., 1998). These studies demonstrated the applicability of this method to evolve proteins to impart a temperature phenotype.

TS essential proteins are valuable tools for engineering TS organisms, since without their activity the organism dies. The advantage of this approach is that temperature-sensitivity

originates from only one source and that source cannot be lost without resulting in the death of the organism. Conversely, a disadvantage of using TS essential genes includes the difficulty in replacing native essential genes because of their essentiality and homology to the TS counterpart. Furthermore, there is no guarantee that when a psychrophilic or TS essential gene is swapped for its native essential gene that it will confer temperature-sensitivity on the organism (Duplantis *et al*., 2010). Thus this technology is currently in a proof of concept stage and cannot be used to reliably produce TS microorganisms.

Lastly, one can use TS genetic elements such as repressor proteins to impart a TS phenotype on a microbe. These TS elements can be coupled to essential gene products, toxins or antitoxins within a genetic circuit and can result in microbial death. Genetic elements such as TS repressor proteins or TS riboswitches typically work by relieving transcriptional or translational repression upon increase in temperature. Riboswitches, which are regions of secondary mRNA structure that sequester the ribosome binding site (RBS), have been found to have TS activity (Narberhaus *et al*., 2006). Similarly, a number of natural repressor proteins have been mutated in the past to produce TS isolates, including the lambda repressor (cI857) (Hecht *et al*., 1984), lac repressor (Chao *et al*., 2002) and TetR repressor (Wissman *et al*., 1991; mentioned in this work). These repressor proteins can also be subjected to directed evolution if one desired to change their approximate induction temperature, making them versatile. Additionally, coupling TS repressors to the expression of toxic genes provides a universal method of killing microbes as many of these target ubiquitous biological components, including RNA, double stranded DNA (dsDNA), cell membranes and DNA gyrase. The use of TS genetic elements avoids many problems inherent in other approaches. For example, the genetic circuits are well-defined, they are modular and allowed a flexible design, and they can be inserted at many chromosomal loci without the

problem of disrupting an essential gene Here, it is demonstrated how temperature limits on a microbe can be set arbitrarily by genetic elements that can be transposed to a broad range of organisms.

1.3 Rationale for the design of temperature-sensitive lethal genetic circuits

In this work, genetic circuits were designed and integrated into the *E. coli* chromosome to render it temperature-sensitive lethal. These genetic circuits are made up of a variety of genetic elements such as promoters, ribosome binding sites and genes which when assembled together form a functional unit. In the circuits, TS repressor proteins block transcription of a lethal restriction enzyme (RE) gene at a lower temperature allowing the microbe to survive. When the temperature rises, the TS repressor protein relieves repression, leading to the expression of the lethal restriction enzyme gene which results in microbial death.

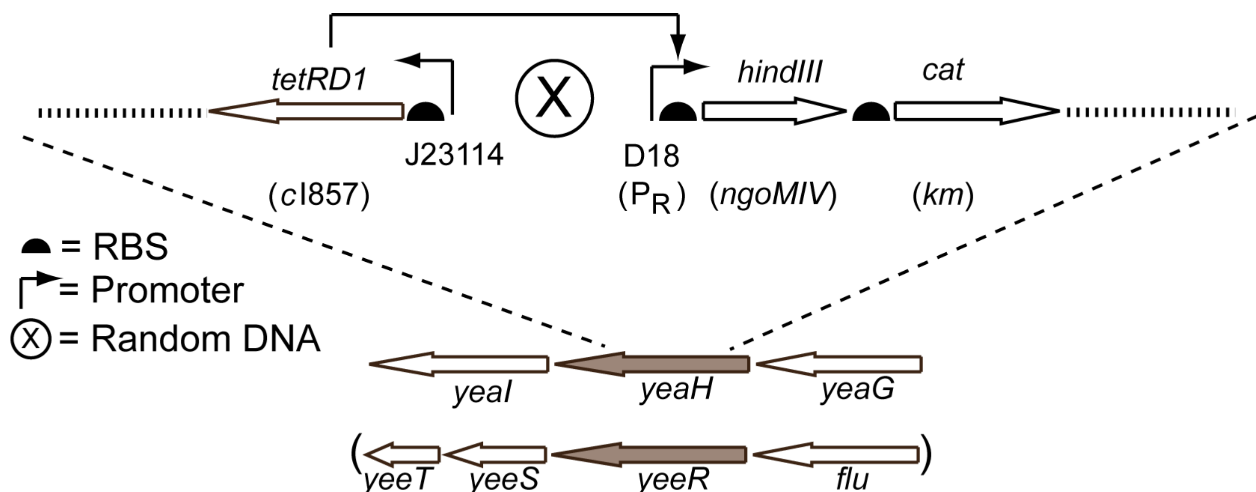

Figure 1.Synthetic temperature-inducible lethal genetic circuit design and chromosomal integration loci. Lower temperatures (30°C) result in blocked transcription of the restriction enzyme (RE) gene by a TS repressor protein. Increasing the temperature (≥37°C) causes the TS repressor to dissociate from its operator allowing for expression of the lethal RE gene. Circuits were integrated into *yeaH* or *yeeR* loci.

The TS lethal circuit is made up of many genetic elements which were selected to specifically minimize cell death at low temperatures and maximize cell death at higher temperatures while at the same time reducing reversion frequency.

1.3.1 Temperature-sensitive repressor proteins

Repressor proteins are those that either bind DNA to block RNA polymerase (RNAP) from attaching to a promoter, or bind mRNA and block attachment to the ribosome binding site, both of which obstruct the expression of genes. A number of repressor proteins exist that are commonly used in molecular biology to control gene expression, including the lac repressor, lambda repressor and tet repressor. Most of these require induction by addition of a small molecule (i.e. Isopropyl β-D-1-thiogalactopyranoside [IPTG] for lac operon). By using TS repressors one can eliminate induction costs associated with small molecules. Thus TS versions of TetR and lambda were chosen as the repressor proteins in the genetic circuits.

The bacteriophage lambda repressor was chosen in this work because it is historically one of the most well characterized systems for studying gene regulation. Its key component is the cI repressor which transcriptionally controls the expression of lytic genes, dictating whether or not phage should switch from lysogenic to lytic growth. Naturally, cI can bind to multiple operator sites (Stayrook et al., 2008) which are located on its left and right operon and thus is able to regulate expression from both the P_R promoter (lytic related promoter) and P_{RM} promoter (lysogenic related promoter).

A study in 1967 (Horuichi and Inokuchi, 1967) identified temperature-sensitive mutants of bacteriophage lambda. They found that these TS mutants caused clear plaques only when incubated at 42-43°C. Complementation experiments confirmed that the mutations responsible

for this change were indeed in the cI gene. The most well characterized TS cI mutant is cI857 which possesses two missence mutations A66T and K118E. cI857 tightly represses expression at 30°C, whereas at 42°C the protein becomes unstable and is no longer able to bind its operator sites, leading to gene expression. Additionally, the repressor has been used for decades and can function in variety of different bacteria including: *Escherichia, Salmonella, Erwinia, Serratia* and *Bacillus* species (Leemans *et al*., 1987; Jawale *et al*., 2012; Breitling *et al*., 1990) indicating functionality in a range of organisms. In the genetic circuit, cI857 tightly controls expression of a lethal RE gene, causing cell death at 42°C.

The Tet repressor was also chosen for this work as it has been widely utilized in the field of molecular biology. It is a homodimer consisting of two DNA binding domains and a regulatory core involved in ligand binding and dimerization (Ramos *et al.,* 2005). The DNA binding domain of TetR binds with high specificity to the tet operator (*tetO*) site, whereas the ligand binding domain binds to tetracycline complexed with a magnesium ion. Naturally this protein is involved with the regulation of tetracycline resistance by controlling expression of the TetA protein which encodes an antiporter efflux pump (Berens and Hillen, 2003). This repressor is often chosen in molecular biology as it demonstrates high binding affinity to *tetO* and high sensitivity to tetracycline (Epe and Woolley, 1984). Additionally, the TetR family of repressors has been detected in 144 microbial genomes associated with 80 genera of gram positive and gram negative bacteria, cyanobacteria and archaea (Ramos *et al.,* 2005). Furthermore, the repressor can be modified to function in eukaryotes (Yan *et al.,* 2000) and as a result, TetR is considered to have broad host range.

A library of TS TetR mutants was previously isolated in the Nano group by using random polymerase chain reaction (PCR) mutagenesis followed by a screening and selection process

using a chloramphenicol acetyl transferase (CAT) reporter cassette. Two mutants were chosen for this work: TetRG2 and TetRD1. TetRG2 has a single point mutation W75R, whereas TetRD1 has three point mutations E107G, C144R and F186S (Fig 2). These substitution mutations often result in entropically unfavourable interactions which in turn affect the ability of the protein to fold correctly at higher temperatures (Goldenberg, 1988). TetRD1 was chosen for the bulk of this work for two reasons: the lower chance of intragenic suppressor mutations and because its three mutations are stable and result in temperature-sensitivity at and above 37°C. Within the genetic circuit, the TS repressor protein TetRD1 acts to control the expression of a lethal RE gene.

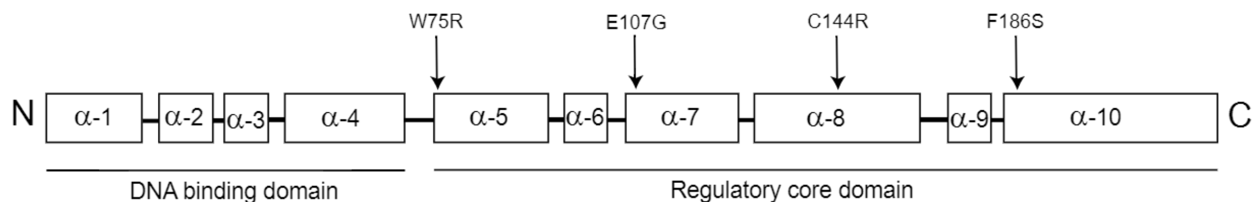

Figure 2. Location of mutations in wild type TetR protein that result in temperature-sensitive mutants TetRD1 or TetRG2. TetRD1 contains mutations E107G, C144R, F186S; TetRG2 contains the single amino acid substitution W75R. Boxes indicate alpha helices 1 through 10.

1.3.2 Promoters

In order to control the expression of a gene, one strategy is to change the upstream promoter. Many factors can affect a promoter's relative strength including transcription factor abundance and affinity as well as aspects related to its overall architecture including number and positioning of operators (Ezer *et al.*, 2014). In general, switching a promoter does not result in a dynamic change in gene expression (i.e. ~10-10,000 arbitrary units [AU]), but it can amend factors such as promoter "leakiness". In the genetic circuits different constitutive and controlled promoters were used, the latter having important implications with respect to the controlled expression of toxic or lethal genes.

When controlling toxic genes, it is important to ensure minimal expression (leakiness) in the uninduced state. Too much leaky expression of foreign or toxic genes can cause premature stress on the cell, leading to differences in growth, metabolism or cell death (Handa *et al*., 2000; Glick 1995). To prevent this, tightly controlled tet and lambda promoters were chosen to be used in the circuits. For TetR, the D18 promoter was selected based on a study by Cox III *et al.,* 2007, which was reported to have a very high regulatory range (ratio of the induced to uninduced activity). On the other hand, for cI857 repressor, the wild type P_R promoter was chosen consisting of operator sites O_{R1} and $O_{R2.}$ Previous research indicated that the lambda cI repressor is able to bind to promoters P_{RM}, P_R and P_L. It is well documented that cI controlled expression can be achieved using any of these three promoters (Lewis *et al*., 2011). It was rationalized that P_R would have the tightest repression since this is the promoter that drives lytic gene expression of which cI is said to tightly control. In the genetic circuits, TetRD1 binds to a single tet operator site within the D18 promoter and cI857 binds to two operator sites within the P_R promoter that both act to control lethal gene expression. Lastly a constitutive weak promoter J23114 (Anderson constitutive promoter collection) was used to drive expression of the repressor proteins TetRD1 and cI857.

1.3.3 Lethal restriction enzyme genes xbaI, ngoMIV, bglII *and* hindIII

Lethal genes are often used in molecular biology as counter selection agents causing the death of transformants that did not undergo the desired recombination. Some lethal genes such as *ccdB, hok* and *barnase* are isolated from naturally existing toxin-antitoxin (TA) systems (Afif *et al*., 2001; Faridani *et al*., 2006; Hartley, 1989). In prokaryotes plasmid encoded systems are generally part of postsegregational killing which ensures that the TA system is stabilized and

maintained within a population (Mruk and Kobayashi, 2013). Chromosomally encoded TA systems are less understood. Studies have linked them in the stabilization of superintegrons, resistance to phage and persister formation (Szekeres *et al*., 2007; Moyed and Bertrand, 1983; Pecota and Wood, 1996). Currently, there is no consensus on the biological significance of TA systems (Mruk and Kobayashi, 2013); however their toxin genes provide us with useful tools for controlling the death of microbes.

One TA system which has been studied for decades is the restriction modification (RM) system. This system is made up of a restriction enzyme (toxin) that targets and cleaves dsDNA, and a modification enzyme (antitoxin) which prevents cleavage by methylation. There are four types of restriction enzymes which differ in their subunit structure and recognition pattern which are called Type I, II, III and IV. The Type II restriction enzymes can be further classified into eleven subtypes (Roberts *et al*., 2003). The most widely used subtype is Type IIP in which common enzymes like EcoRI and HindIII are grouped. These systems are long thought to act as a cellular defence system for prokaryotes, where the RE toxin will only cleave dsDNA from incoming bacteriophages, transposons and plasmids, and not cleave its own DNA due to protection from the cognate methyltransferase (Vasu and Nagaraja, 2013).

There are over three thousand Type II REs (Roberts *et al*., 2015) and analyses indicate that they have low sequence identity and thus are a highly diverse group of enzymes with differences in their recognition sequences and mechanisms of cleavage (Vasu and Nagaraja 2013; Pingoud *et al*., 2005). Most Type II REs are homodimeric (ie HindIII, EcoRV) or tetrameric (ie NgoMIV) containing catalytic centers that recognize and cleave a specific 4-8 base pair (bp) palindromic DNA sequence. In 1996, Stahl *et al* conducted a study on EcoRV heterodimers where one dimer contained mutated residues involved with either recognition or catalysis. They

found that mutating residues in one subunit's catalytic center did not affect cleavage activity of the other, whereas mutating residues in one subunit involved with sequence recognition dramatically reduced overall cleavage activity. This further demonstrates a restriction enzyme's tremendous specificity to only dsDNA containing their individual recognition site, and this is an important factor when predicting the level of lethality within a given microbe.

The specificity of Type II restriction enzymes towards dsDNA can be exploited as a broad host range toxin since all microbes contain DNA. It was hypothesized that since different restriction enzymes recognize different sites and a given microbe's genome contain X number of those sites, it could be possible to "titrate" the amount of lethality imposed on the target microbe. A higher number of possible restriction sites within a microbe's genome would likely lead to a higher level of lethality since the genome would undergo increased fragmentation and disruption of essential genes, consequently overwhelming the host's DNA repair systems.

For these reasons Type II restriction enzymes were chosen as the lethal gene in the circuits that were constructed in this work. These enzymes are ideal since they are well characterized, are separate from their methylase protein and exhibit extreme specificity, cleaving only within a certain recognition site.

Table 1.Titration of *E. coli* MG1655 lethality by differences in RE recognition sites.

Restriction enzyme	**Originating organism**	**Recognition site**	**Number of restriction sites in *E. coli* MG1655**	**Level of predicted *E. coli* MG1655 lethality**
XbaI	*X. badrii*	TCTAGA	35	Low
NgoMIV	*N. gonorrhea*	GCCGGC	259	Medium
HindIII	*H. influenzae*	AAGCTT	508	Medium
BglII	*B. globigii*	AGATCT	660	Medium
SonI	*S. oneidensis*	ATCGAT	1311	Medium-high
RsaI	*R. sphaeroides*	GTAC	11500	High

Higher levels of toxin gene expression can often be detrimental when implementing defined genetic circuits in microbes. Most if not all repressor systems are never repressed 100% of the time leading to background expression in the uninduced state. Background expression of a toxin gene can have a negative effect on the cell's growth and metabolism depending on how toxic the gene is. For example background expression of RsaI (11500 recognition sites) could have more impact on a given cell compared to XbaI (35 recognition sites). Increased toxicity and leakiness can also apply evolutionary pressures (ie natural selection or genetic drift) on the cell resulting in inactivation of the toxin gene(s) which otherwise offer little fitness advantage (Mira *et al*., 2001). Thus it is important to minimize the amount of toxicity needed to effectively kill the microbe. This would decrease the chance of unfavourable effects such as reversion or changes in growth or metabolism. The use of different restriction enzymes offers a potential advantage in which the level of toxicity can be titrated for a given microbe. A variety of different enzymes were chosen in order to test this hypothesis, including XbaI, NgoMIV, BglII and HindIII which are controlled by the TS repressors TetRD1 and cI857.

1.3.4 Ribosome binding sites

A second strategy for fine tuning gene expression lies within the RBS sequence. This sequence lies upstream of a gene's start site and is responsible for the recruitment of the ribosome to the mRNA. In prokaryotes, altering this sequence allows for the dynamic (i.e. 1-1,000,000+ AU) control over the translation initiation rate (TIR) and therefore protein expression. There is mounting evidence that changing the RBS sequence can dramatically change the expression of a target gene. Studies indicate that site accessibility and formation of RNA secondary structures plays a large role in determining the protein expression level as these can occlude ribosome attachment (Salis *et al.*, 2009; Goodman *et al.*, 2013; Borujeni *et al.*, 2013). There are now several programs where one can either design a new synthetic RBS sequence for a given gene, or calculate the approximate strength of an existing RBS. Programs such as the RBS Calculator (Salis, 2011) combine a biophysical model of translation initiation and an optimization algorithm in order to generate the desired proportional TIR. Since the RBS translation initiation rate is dynamic, RBSs were designed for the restriction enzyme genes with TIRs resembling their natural counterparts. The natural RBS within the originating organism was first calculated and then redesigned with that approximate strength into the genetic circuits. It was postulated that the wild type systems would have evolved an appropriate expression level of the toxin gene and thus RBSs were designed to mimic this. In the lethal genetic circuits, the RBSs help fine tune the protein expression levels of the restriction enzymes XbaI, NgoMIV, BglII and HindIII.

1.3.5 Plasmids, chromosomal loci and E. coli *strains*

The copy number and genetic background of the TS circuits are important factors to consider when using toxic genes as these can impact the resulting lethality of the lethal gene circuit within the microbe. Plasmids are extrachromosomal circular DNA that are often multi-copy within a cell. The copy number of a plasmid is determined by its origin of replication. Some origins are considered "relaxed" yielding hundreds of copies per cell (i.e. ColE1) whereas others are "stringent" only allowing ~5 copies per cell (i.e. pSC101). The TS genetic circuits were first built on a stringent plasmid as a quick, efficient method of determining if the circuit was functional or not. Stringent plasmids are necessary to ensure fewer replicates of the lethal gene per cell. A relaxed plasmid would be unsuitable as there could be hundreds of plasmids per cell each carrying a copy of the lethal gene. In other words, the toxic effects observed would be biased to the actual effect of a single copy per cell. Additionally, plasmids are generally unstable and can be lost upon cell division (Sorensen *et al*., 2005), this is especially noted under longer culturing conditions without antibiotic selection.

Alternatively, integration of a genetic circuit within the chromosome results in a single copy that should be much more stable, and thus allow for integration of more than one circuit into a cell. Integrating two or more independently acting lethal circuits imposes multiple barriers on the cell, making it more difficult to overcome and therefore escape or revert (Gallagher *et al*., 2015; Cai *et al*., 2015.). For these reasons two circuits were chosen: one controlled by TetRD1 and the other controlled by cI857, in effect a "Dual" circuit with each containing a different restriction enzyme gene.

In prokaryotes, gene expression is known to vary with chromosome position (Schmid and Roth, 1987; Sousa *et al*., 1997), and therefore the two genetic circuits were first integrated at

multiple different loci (Table A1) to ensure adequate expression prior to construction of the dual strains. These loci were chosen on the basis that they were 1) non-essential, 2) not in the vicinity of essential genes, 3) previously shown that knock outs had little to no effect on growth or transformation efficiency (Posfai *et al*., 2006) and 4) loci used for dual circuits were approximately 50 kb apart. This spacing was to safeguard against a single horizontal gene transfer event in which a large DNA fragment could potentially recombine in the chromosome and simultaneously destroy both lethal genetic circuits. Keeping the two lethal circuits in regions lacking essential genes and far apart from each other on the chromosome would guard against unfavourable disruption of essential genes and potential horizontal gene transfer events.

Lastly, the genetic background of the *E. coli* strain could also impose differences in lethality effects. All initial construction and testing of the lethal genetic circuits were done in the cloning strain DH10B (Table A2). Notably, the strain is deficient in homologous recombination because of its *recA1* mutation. RecA is an important protein involved in repairing double stranded breaks as it binds to single stranded and double stranded DNA, facilitating strand invasion and homologous recombination. For quantitative experiments, the *E. coli* strain LE392 (Table A2) was mainly used. This strain is restriction negative (lacking its native restriction endonuclease) and also has a minimal number of other mutations, making it representative of wild type strains. Two other recombination deficient strains PMC103 and PMC107 (Table A2) were also used to determine if reduced DNA repair capacity would affect the strain's survival at high temperature.

1.4 Applications for the TS lethal genetic circuits

1.4.1 Engineering upper and lower temperature limits of microbes as a form of intrinsic biocontainment

Regulating the temperatures at which a microbe can survive in offers a novel approach for a biocontainment strategy. Biocontainment is typically defined as the physical containment of pathogenic microbes in secured facilities to prevent their accidental release either into the environment or into the people working with them. The same idea is applied here, except the microbes are intrinsically contained by carrying the TS lethal genetic circuits within their chromosomal DNA. Changes in a microbe's surrounding temperatures can trigger survival or death. An upper temperature limit is as described in the above sections – higher temperatures cause de-repression of TS repressors leading to expression of a restriction enzyme which kills the cell. Whereas the lower temperature limit could be implemented by TS repressor or TS riboswitch control of either the antitoxin or an essential gene. Lower temperatures would lead to the repression of the antitoxin or the essential gene also causing the death of the microbe.

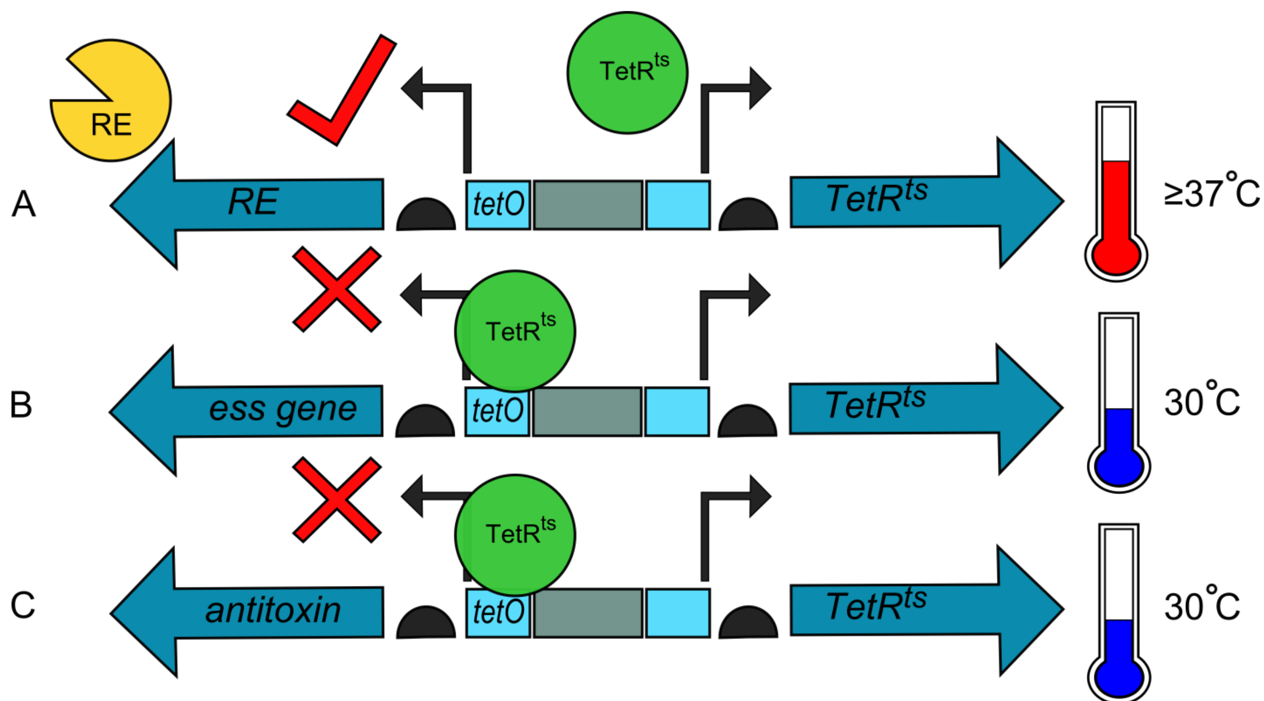

Figure 3. Intrinsic biocontainment by TS lethal genetic circuits at different temperatures. A. Upper temperature limit: increased temperatures cause the expression of a lethal RE gene, killing the organism. B. Lower temperature limit: decreased temperatures cause the repression of an essential gene, leading to the death of the organism. C. Lower temperature limit: decreased temperatures cause the repression of the antitoxin, leading to accumulation of the toxin, killing the organism.

The intrinsic biocontainment strategy can be expanded from pathogenic microbes to a broad range of industrial or recombinant microbes. These are ubiquitous in the biotechnology and pharmaceutical industries as they mass produce high value products such as chemicals and therapeutics. It is generally recognized to be undesirable for these strains, as well as pathogenic strains to be accidentally released in the natural environment, where they could be disruptive to native organisms or cause disease. To protect against this, industrial or recombinant strains can be implemented with an intrinsic biocontainment mechanism. Setting the maximum and minimum growth temperatures of an organism allows for spatial and temporal control over a microbe's survival.

These mechanisms have the potential to improve the overall safety profile of numerous pathogenic microbes. For example, biosafety containment levels (BSL) are assigned when working with pathogenic microbes, and these require facilities to be properly equipped to the standards of that containment level. The average cost of building a higher level (600ft2) BSL3 facility is upwards of 1.5M USD and around three times the amount for annual maintenance (Berger *et al*., 2009). This greatly limits the number of researchers that have access to working with these BSL-3 microbes as most institutions cannot afford such facilities. It is possible that by implementing containment systems into certain pathogens could result in improved safety profiles (i.e. BSL3 to BSL2 pathogen). This kind of reclassification could dramatically improve accessibility to certain pathogens thus increasing research productivity.

Similarly, biocontainment mechanisms could also help improve the safety profile of existing environmental vaccines and therapeutics (Rovner *et al*., 2015). These include veterinary vaccines that are administered into drinking water, or the release of beneficial recombinant microbes for agricultural or environmental purposes (Meeusen *et al*., 2007; Pieper and Reineke, 2000; Steidler, 2003). These recombinant microbes have no means of containment as they are readily released into the environment. Unchecked, these microbes could possibly disrupt the integrity of natural microbes by displacing them or exposing them to recombinant DNA (Schmidt and de Lorenzo, 2012). An intrinsic biocontainment mechanism would limit the ability of these microbes to disseminate into the environment.

Many ideas have been proposed to contain recombinant organisms, but the majority of them focus on two principles: auxotrophy and kill switches. Unfortunately, many of these earlier strategies lacked multiple barriers or overlooked key aspects which contributed to their escape (CDC, 2011; Torres *et al*., 2003; Ahrenholtz *et al*., 1994; Contreras *et al*., 1991). Recently there

has been renewed interest in biocontainment where several groups have designed innovative containment strategies with extremely low escape frequencies in the range of ~1x10^{-11} escapee per CFU (colony forming unit) (Mandell *et al*., 2015; Rovner *et al*., 2015). However impressive, this relied on drastically changing the genetic makeup of the organism by completely recoding its genome, forcing it to depend on incorporation of a synthetic amino acid at TAG stop codons in order for survival. Such drastic genomic changes require extensive work in order to apply to other organisms in addition to imparting metabolic differences compared to wild type. Lastly it can also be very costly to implement this on a large scale as the organism relies on supplementation with an uncommon synthetic amino acid. For example, 1 g of synthetic p-acetyl-L-phenylalanine can cost 500 times more than the non-synthetic form (Ark Pharm, Inc. 2012).

Future ventures of biocontainment strategies will therefore rely upon the safety, general acceptance, wild type behaviour, ease of transferability and ultimately cost of constructing and maintaining strains. Our technology should be applicable to a variety of organisms because 1) all synthetic genetic information is contained within convenient, small (<2 kb) circuits which ensures minimal disruption to wild type behaviour and relative ease of integration, 2) genetic elements have been shown to function in different organisms, meaning circuits should be transferable, and lastly 3) temperature induction drastically reduces the cost compared to using expensive inducer molecules.

1.4.2 *Temperature-sensitive attenuated vaccines*

Controlling the temperatures at which a microbe can survive at can also be beneficial when designing TS attenuated vaccines. Many different types of vaccines are available each possessing certain advantages and disadvantages regarding safety and efficacy; however only one type of vaccine appears to be effective against intracellular pathogens and these are classified as live attenuated. These vaccines result in not only an antibody response, but also a cell mediated response, the latter being a key component to clearance of intracellular pathogens (Coward *et al*., 2014; Kaufmann, 1993). In the past, live viral vaccines have been largely successful, although their bacterial counterparts are still not as common due to their complexity and safety concerns. Traditional approaches to generating live bacterial vaccines are no longer considered ideal because of the random nature of chemical mutagenesis and passaging. Thus an alternative approach to traditional methods is attenuation by temperature.

Temperature-sensitive live attenuated vaccines have been used for decades as veterinary vaccines, but have otherwise been slow to be accepted for human use. These veterinary vaccines are preferred because of their low cost and have been used successfully to vaccinate sheep and poultry (Morrow *et al*., 1998; Jackwood *et al*., 1985). Whereas for humans, the two TS live vaccines approved for use include the Sabin polio vaccine (Chapin and Dubes, 1956) (no longer recommended in USA) and the FluMist (influenza) vaccine (Jin *et al*., 2003) both of which target viral infections and were generated by chemical mutagenesis or passaging. Recent efforts to extend TS live vaccines to bacterial pathogens have been ongoing in the Nano lab. Substitution of certain psychrophilic essential genes for their native homologues in *F. novicida*, *M. smegmatis*, *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis resulted in TS organisms (Duplantis *et al*., 2010; Duplantis *et al*., 2015). A variety of strains were attenuated at the desired

restrictive temperature and some were found to provide moderate protective immunity in mice and chickens when subsequently challenged. However, not all substitutions resulted in a TS phenotype and few of these provided acceptable reversion frequencies.

As opposed to psychrophilic essential gene substitution, TS lethal genetic circuits offer an alternative technique to generating TS organisms that can be used as vaccines in three ways. First, a microbial pathogen could be attenuated such that it dies rapidly when exposed to a modest temperature increase, for example a shift of 30° to 35°C. This type of sensitivity to temperature could be used in bio-processing to kill a pathogen without denaturing its protective antigens, and might be suitable in creating cost effective veterinary vaccines. Secondly, the TS strains could be used to generate a new class of vaccine called killed but metabolically active (KBMA). These vaccines are either whole pathogenic or attenuated strains which are typically inactivated by UV radiation however they retain enough metabolic activity so as to stimulate a sufficient immune response (Dubensky *et al*., 2012). The TS attenuated strains could work as KBMA vaccines by treatment with a psoralen cross-linking agent which completely blocks DNA replication. Additionally the strains may act as KBMA vaccines without a cross-linking agent since the initial expression of RE in naïve *E. coli* cells has been shown to halt division and cell motility (Fig A3) yet still exhibit transcription and translation (Asakura and Kobayashi, 2009). This results in cells that are unable to productively grow and replicate, but still possess metabolic activity. This type of attenuation could yield a potential KBMA vaccine which would provide a compromising median between killed vaccines and live attenuated vaccines. Lastly, the TS strains could be used as a means of peripheral vaccination. A TS live attenuated microbe could be used to vaccinate mammals or humans by injection into a cool region of the body. The microbe would be able to replicate and stimulate protective immunity at the periphery of the

body because of exposure to low ambient temperatures, but is unable to survive and disseminate into the core because of its temperature restriction (Duplantis *et al*., 2011, White *et al*., 2011). Importantly, the core body temperature of humans is stably maintained at 37°C, where 1-2 degree deviations will result in serious illness or death (Mekjavic *et al*., 1991), making it a universal feature of body temperature.

For the scope of this work two different repressor proteins were needed in order to create Dual strains. The TS repressor proteins chosen were TetRD1 that has an ideal induction temperature of 37°C, and cI857 which is induced at 42°C. 42°C is not a biologically relevant temperature, but for proof of concept was considered adequate here. By using directed evolution, this repressor protein (or any other repressor protein) could be evolved to possess an induction temperature of 37°C.

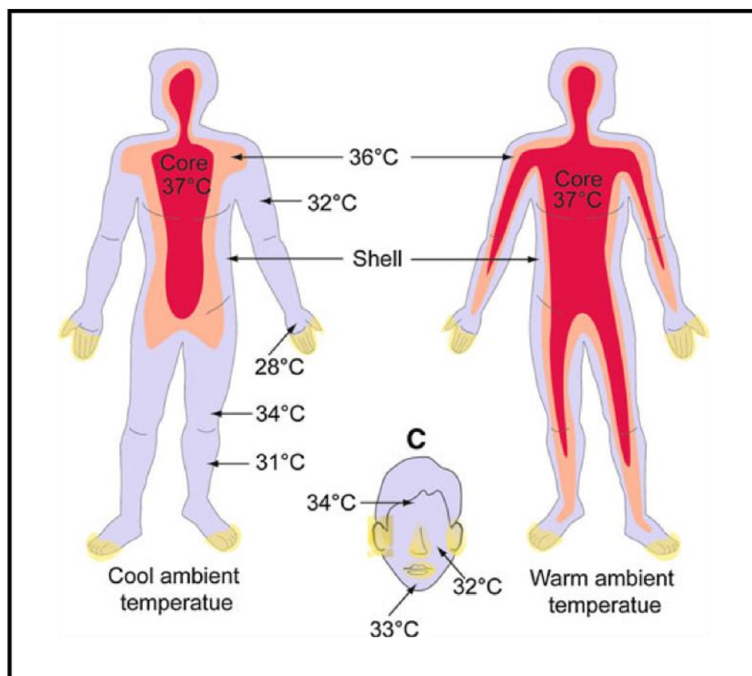

Figure 4. Core, cool and warm ambient temperatures of the human body. Adapted from White *et al*., 2011.

The peripheral vaccination approach using TS live attenuated bacterial strains offers the greatest advantage over conventional bacterial vaccines because of their effectiveness against intracellular pathogens. It is widely recognized that not all vaccines can provide protective immunity from an actual infection. Additionally it has been noted that immunity to certain diseases can be gained from a live vaccine but not an inactivated or subunit vaccine (Finco and Rappuoli, 2014). Live vaccines owe their efficacy to their resemblance to the wild type organism – intact, replicating, with a large complement of antigens. These vaccines have also been proven necessary for clearance and protective immunity to intracellular pathogens (Coward *et al*., 2014), whereas inactivated and subunit vaccines have poor effectiveness against these organisms. This is because these pathogens have the ability to hide within macrophages and epithelial cells, effectively escaping the phagosome and antigen detection. Thus the primary immune response is cellular, including the Type 1 Helper (Th1) T cells. Diseases caused by intracellular pathogens such as *S. enterica* and *M. tuberculosis* represent an enormous global health problem with non typhoidal *Salmonella* estimated to cause 93 million infections per year (Majowicz *et al*., 2010) and in 2014 alone 9.6 million people fell ill with tuberculosis (WHO, 2015). Furthermore, many other intracellular pathogens including eukaryote trypanosomes and plasmodium species still do not have optimal or effective vaccines. Thus TS live vaccines have the potential to address this outstanding issue.

Other advantages of TS lethal circuits for vaccines include faster construction and transferability to other organisms. Vaccine construction requires a single step of circuit integration into the target chromosome, drastically reducing the time needed for genome engineering as compared to traditional approaches. TS lethal circuits should also be transferable to a variety of organisms due to the use of a universal toxin and broad range repressor proteins.

Therefore TS lethal circuits could be used as a platform to assemble TS vaccines for many different pathogens, including intracellular organisms. Using this technology allows for a relatively simple, fast and transferable strategy of generating TS organisms as vaccines for animals or humans.

1.4.3 *Counter selection method*

Lastly the TS lethal circuits can also be utilized as an effective counter selection method. Counter selection refers to a powerful technique of selecting against bacteria that did not undergo the desired recombination. When coupled with positive selection (i.e. antibiotic resistance) allows for greater ability to find the desired clone. The lethal circuit would first be integrated in the locus of choice, and these clones would be selected for by the attached antibiotic cassette. Next, the gene product of interest would be introduced into this strain using a recombination method and then would be incubated at the higher temperature. Clones that underwent the correct recombination would survive the higher temperature since the lethal circuit would be removed. In contrast, clones that did not undergo correct recombination would die at the higher temperature as the lethal circuit would still be intact. This strategy has an advantage over others in that it does not require the addition of any small inducer molecules and relies solely on temperature induction, making it a cheaper alternative.

1.5 Potential caveats

The greatest caveat for this technology is reversion to either non-functional or non-temperature-sensitive phenotype. This can occur by several means including mutations, horizontal gene transfer events as well as genetic drift. Mutations can disrupt the circuit if they occur in the tet or P_R promoters, the TetRD1or cI857 repressors or in the restriction enzyme

genes. These can include missense, nonsense, insertions, and deletions which can lead to changes in transcription factor binding, protein stability or protein functionality. Horizontal gene transfer events can lead to incoming DNA recombining within the circuit locus thus disrupting or knocking it out, and additionally the random nature of genetic drift can lead to the eventual loss of the circuits. With these in mind, we have implemented many design characteristics into the TS lethal circuits including tight repressor control of RE genes, titration of killing with different RE genes, independent dual circuits containing different genetic elements and chromosomal spacing which should help reduce reversion frequency.

1.6 Summary

In this thesis, lethal genetic circuits were rationally designed for their integration into the *E.coli* chromosome to render the organism temperature-sensitive. The circuits were designed to be easily transferable to other organisms as well as minimize their frequency of reversion. These key aspects allow for a widely applicable method of generating TS organisms for use as an intrinsic biocontainment strategy, TS attenuated vaccine or counter selection method.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

E. coli DH10B (Invitrogen) was used as the host strain for cloning experiments whereas *E. coli* LE392 (Promega) was used as the parental strain for all characterization experiments. PMC103 and PMC107 (Doherty *et al*., 1993) were also used in select experiments because of their recombination deficient genotypes. These strains were grown in lysogeny broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or on LB agar unless otherwise stated. Antibiotic use included carbenicillin (Cb) at 100µg/ml, gentamicin (Gm) at 20µg/ml, kanamycin (Km) at 30µg/ml and chloramphenicol (Cm) at 10µg/ml unless mentioned otherwise.

2.2 DNA manipulations

High fidelity PCR was performed using PrimeSTAR GXL DNA polymerase (Clonetech-TaKaRa) and diagnostic PCR was performed with *Taq* DNA polymerase (NEB). All restriction digests and ligation reactions were done using NEB restriction enzymes and T4 DNA ligase (NEB). Multiple DNA fragments were assembled with a Gibson Assembly Master Mix solution which was made in the Nano lab using individual NEB reagents and was carried out according to the NEB Gibson Assembly® protocol E5510.

2.3 TS TetR mutant control of fluorescence expression

The repressor vectors were constructed as follows. TetR mutants *tetRD1* and *tetRG2* were inserted downstream of the *lac* promoter into vector pWSK29 (Wang and Krushner, 1991) using restriction sites ClaI and PstI; the *cI857* repressor gene was inserted into pWSK29 with PstI and

BamHI sites. These repressor genes were also amplified with and without the *E. coli* AAV degradation tag at the 3' end of the gene using primers that included the sequence as 5' tails on the complimentary region of 5' phosphorylated oligonucleotides. For the reporter vectors, mCherry (BioBrick part BBa_J06504) was PCR amplified with primers that added a new 18 bp RBS and XbaI and KpnI sites, and was then inserted into pSB3K3 (Registry of Standard Biological Parts). All promoters were amplified with BamHI restriction site ends and were ligated upstream of the mCherry gene. These were screened by PCR detection and sequenced to ensure correct orientation. Next, the reporter vectors were subjected to plasmid extraction and were then transformed into *E. coli* DH10B cells harbouring the pWSK29 repressor vectors.

E. coli strains were grown to mid-exponential phase in LB broth at 30°C and were diluted to an A_{595} of 0.05 in EZ Rich Defined Medium (Teknova) with 2% glucose supplemented with Cb and Km, in a clear bottom, black 96-well microtitre plate (Greiner Bio-One). Two identical microtitre plates were prepared, one incubated at 30°C and the other incubated at 42°C. Fluorescence at excitation wavelength 584 nanometers (nm) and emission wavelength 632 nm was measured for an integration time of 1 s using a SpectraMax M5 plate reader (Molecular Devices) every 2 h for 7 h as maximal fluorescence levels were observed at this point. The A_{595} was also measured at each time point and the fluorescence output was normalized against these readings.

2.4 Temperature-inducible lethal genetic circuit assembly

A 434 bp gene fragment was synthesized (IDT) and consisted of a 100 bp spacer sequence flanked by controlled promoter D18 (Cox III et *al*., 2007) and constitutive promoter J23114 (BioBrick part BBa_J23114) both directed outwards. Downstream of both promoters resides two

mini multiple cloning sites. The gene fragment was first inserted into pWSK29 using the SacI and KpnI sites. The *tetRD1* repressor gene was PCR amplified with primers containing a 12 bp RBS and restriction sites XbaI and KpnI and was subsequently ligated into pWSK29::*gene fragment*. Next, the lethal *hindIII* gene was PCR amplified with primers containing a 12 bp RBS and the sites EcoRI and PstI. This product was ligated into pWSK29::*gene fragment*::*tetRD1* and transformed in *E. coli* DH10B at 30°C, downstream of the D18 promoter to create the full length circuit.

A second gene fragment (246 bp) was synthesized (GenScript) to accommodate any controlled promoter which included the addition of several unique restriction sites. This gene fragment, similar to the first described, was digested with SacI and KpnI then ligated into pWSK29. The wild type lambda promoter P_R was synthesized from two overlapping single stranded DNA oligonucleotides by overlap extension which included restriction site ends. In brief, both oligonucleotides were added to a final concentration of 2 µM in 1X NEB buffer 2 and 25 µM each dNTP. These annealed together by boiling for 5 min and then allowing the mixture to cool slowly. Klenow fragment (3'→5'exo$^-$; NEB) was added when the mixture reached 37°C and this was incubated for 1 h at 37°C. Finally the mixture was inactivated and digested with XhoI. The P_R promoter was then ligated into pWSK29::*gene fragment*. Next the *cI857* repressor gene was PCR amplified with primers containing a 12 bp RBS and restriction sites XbaI and KpnI. This product was inserted into pWSK29::*gene fragment*::P_R. Lastly, the lethal *xbaI* gene was PCR amplified with primers containing a 12 bp RBS and the sites EcoRI and PstI. The product was subsequently ligated downstream of P_R in pWSK29::*gene fragment*::P_R::*cI857* and transformed into *E. coli* DH10B at 30°C to create the full length second circuit.

Additional lethal genetic circuits were constructed by using inverse PCR and Gibson Assembly by using the pWSK29::*gene fragment*::P_R::*cI857*::*xbaI* vector as template. The *xbaI* gene was removed by inverse PCR. Next, lethal restriction enzyme genes *bglII* and *ngoMIV* were PCR amplified using primers that included a 12 bp RBS and 20 bp flanking overlaps. The inverse PCR product and lethal genes were put together using Gibson Assembly and were then electroporated into *E. coli* DH10B and incubated at 30°C.

Flanking chromosomal overlapping regions and antibiotic selection cassettes were assembled onto the lethal genetic circuits in order for integration into the *E. coli* chromosome. 500 bp flanking regions were PCR amplified according to the target locus from MG1655 genomic DNA with primers that added 20 bp overlaps. The chloramphenicol cassette (source pBC SK+ [Stratagene]), kanamycin cassette (source pSEVA221 [Silva-Rocha *et al*., 2013]) and TS lethal circuits were all PCR amplified with 20 bp overlaps. Fragments were assembled together on a linear backbone pWSK29 by Gibson Assembly. This mixture was then electroporated into DH10B and selected on either Cm or Km at 30°C.

2.5 Chromosomal integration

TS lethal genetic circuits were integrated into the *E. coli* chromosome using a λ red recombination system. The *E. coli* strain LE392 was first prepared by transformation of the λ red plasmid pSC101ccdAgbaA (Wang *et al*., 2014). Next it was made electrocompetent as previously described (Wang *et al*., 2014). TS lethal circuits were PCR amplified out of plasmid pWSK29 and 1 µl was electroporated into induced electrocompetent *E. coli* LE392 carrying vector pSC101ccdAgbaA. Clones were selected on Cm or Km at 30°C, and resistant clones were

screened by PCR for correct integration. Positive clones were then sequenced for correct integration.

2.6 Growth assay

Performed at 30°C. E. coli strains were grown to mid-exponential phase in LB broth at 30°C and were diluted 1:50 in fresh LB broth with at least three replicates each in a clear 96-well microtitre plate. This was incubated at constant temperature at 30°C in an ELx808 absorbance plate reader (BioTek). The A_{595} was measured every 15 min after a 1 min shake for a total of 30h. *Performed at 42°C*. Absorbance readings at 42°C were found to be difficult to measure in the plate reader because of the use of small volumes and the eventual dense growth of the cultures. Instead, *E. coli* strains were grown to mid-exponential phase in LB broth at 30°C and were diluted to an A_{595} of 0.01 in 10 ml of fresh LB broth. Strains were grown at 42°C in triplicate with aeration for a total of 48 h. The A_{595} was measured approximately every 2 h by diluting a small volume of culture in LB broth and then measuring on a cell density meter model 40 (Fisher Scientific). The actual absorbance was calculated from multiplying by the dilution factor.

2.7 Survival assay

The most accurate method of determining cell death is to plate cells on agar and calculate the colony forming units per ml (CFU/ml). *E. coli* strains were first grown to mid-exponential phase in LB broth at 30°C and then were diluted to an A_{595} of 1.0 in phosphate buffered saline (PBS). Using saline reduces the likelihood that strains participate in continuous growth and death cycles which can occur when using a growth medium. Cultures were next diluted 1:10 in triplicate and suspended in 5 ml of PBS at 42°C for a total of 7 days with aeration. Every 24 h a small volume

of culture was removed, serially diluted, plated on LB agar and incubated at 30°C. Each day the CFU/ml was calculated.

2.8 Viability staining and flow cytometry

Using a combination of stains allows for the assessment of proportions of live and dead bacteria within a culture. *E. coli* strains were grown to mid-exponential phase at 30°C. Each strain was then diluted in duplicate to an A_{595} of 0.01 in LB broth, with one incubated at 30°C and the other incubated at 42°C with aeration for 24 h. Afterwards, each culture was diluted to an A_{595} of 1.0 and then serially diluted to approximately 1 x 10^6 cells/ml. 1 ml of each strain was then centrifuged, washed and resuspended in 1 ml of 0.85% NaCl solution. Cells were then stained with the fluorescent dyes DMAO and Ethidium homodimer III (EthD-III) which were either used alone or in combination according to the manufacturer's instructions of the Viability/Cytotoxicity Assay for Bacteria Live & Dead Cells kit (Biotium). Once stained, bacteria were analyzed with a FACSCalibur flow cytometry system (BD Biosciences) equipped with an argon laser (488nm) at 15mW. Green (live bacteria) and red (dead bacteria) fluorescence were collected in the FL1 (fluorescein isothiocyanate [FITC]) and FL2 (phycoerythrin [PE]) channels respectively. All parameters were collected as logarithmic signals. The population concentrations were estimated using the CellQuestPro and Flojo ver9.6.3 software.

2.9 Reversion frequency determination

Three independent cultures of the dual strain were grown from different individual colonies to mid-exponential phase at 30°C. Each culture was diluted to an A_{595} of 1.0 and a total of 1 ml was plated onto LB agar and incubated at 42°C for 48 h. The actual CFU/ml of the culture was

determined by plating serial dilutions on LB agar incubated at 30°C. The reversion rate was calculated by dividing the number of colonies arising on plates incubated at 42°C by the number of CFUs calculated from the plates incubated at 30°C. 20 CFUs from each independent culture were also streaked on LB agar at 30°C and 42°C in order to assess the proportion of real revertants.

CHAPTER 3: RESULTS

3.1 Characterization of TS responsive repressors and cognate promoters

With the intention on improving upon existing TS repressors, several variant TS repressors and cognate promoters were tested to see if one would yield a superior TS genetic circuit. Thus error-prone PCR was used to create TS variants of TetR (R. McWhinnie, Nano Lab). After generating 39 unique TS TetR proteins we identified two that appeared to provide good control over the reporter gene yellow fluorescent protein (YFP) (Fig A1).

In separate experiments using a distinct reporter protein, mCherry (a variant of red fluorescent protein [RFP]), TetRD1 and TetRG2 were tested for their ability to repress reporter gene expression at 30°C from three *tetO*-containing synthetic promoters (Cox III *et al*., 2007; McWhinnie and Nano, 2013); these were also tested with the AAV C-terminal tag (Andersen *et al*., 1998) that promotes rapid protein degradation (Table 2, Fig 5). For all three synthetic promoters we found more expression of RFP at 42°C than at 30°C, even when no repressor was present (Fig 5). However when the TetRD1 or TetRG2 variants were present there was greater repression at 30°C and induction at 42°C which ranged from about two to five fold. The addition of the degradation tag to the TetR variants did not improve the RFP induction at 42°C and variants with degradation tags were not used in subsequent experiments.

The ability of the TS λ bacteriophage repressor cI857 to regulate expression of RFP at 30° and 42°C was also tested (Fig 5). The cI857 control of gene expression was comparable to the best control by TetR variants. As with TetR, the addition of a degradation tag to cI857 did not improve the differential gene control at the two temperatures (Fig 5), and thus was not further used.

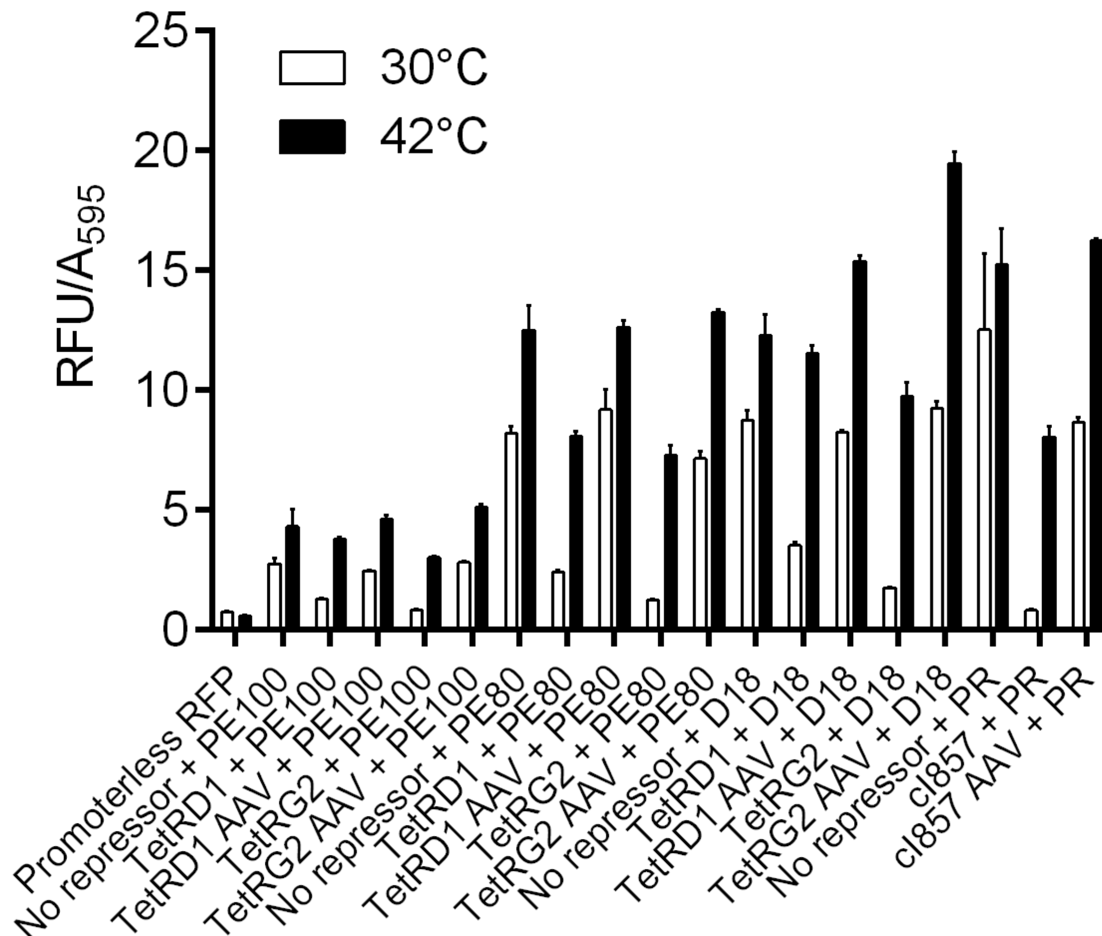

Figure 5. mCherry fluorescence expression controlled by TS repressors driven by various cognate promoters. Promoters PE100, PE80, D18 and P_R were controlled by either TetRD1, TetRG2 or cI857 repressors at 30° or 42°C. mCherry output was normalized by dividing the relative fluorescence units (RFU) by the A_{595}. Fluorescence and absorbencies were measured every two hours for a total of 7 h (last time point shown). Error bars indicate the standard error of mean from three or more replicate samples.

3.2 Testing restriction endonucleases as lethal gene products

We reasoned that restriction endonucleases could serve as a collection of lethal proteins, allowing one to customize the toxicity based on the number of recognition sites for particular endonucleases in a targeted genome (Table 1). The G+C content of the targeted genome serves

as a general guide for predicting the number of sites but is not always accurate. For example, two of the restriction enzymes used in this study, XbaI (TCTAGA) and BglII (AGATCT) have the same G+C content but XbaI has 35 and BglII has 649 recognition sites in the *E. coli* chromosome. Other factors could likely affect the lethality of a restriction enzyme encoding gene, including the amount of protein made and its stability.

We first tested potentially lethal gene circuits by assembling them in the pSC101-based low copy plasmid pWSK29. The circuits consisted of a synthetic constitutive promoter (Anderson promoter collection) driving expression of the TS repressor-encoding gene, and, in the opposite orientation, the gene encoding a restriction enzyme controlled by a promoter responsive to the repressor (Table 2). Expression of any of the genes encoding HindIII, XbaI, NgoMIV, or BglII in the plasmid constructs at the higher temperature reduced the viability of *E. coli* (Fig 6). On agar, the typical phenotype observed was reduced growth and single colony formation instead of lawn formation. Whereas in broth, reduced growth was observed from a more dilute A_{595} or the presence of visible cell debris (not shown). However, expression of cI-Xba led to unacceptably low levels of cell death (and this varied when the circuit was in plasmid or chromosome and when grown in broth or on agar) thus this circuit was not studied further.

Table 2. Components of four TS lethal genetic circuits.

	Restriction Enzyme	**TS Repressor**	**Controlled Promoter**	**Shorthand circuit name**
Circuit 1	HindIII	TetRD1	D18	TetD1-Hind
Circuit 2	NgoMIV	cI857	P_R	cI-Ngo
Circuit 3	XbaI	cI857	P_R	cI-Xba
Circuit 4	BglII	cI857	P_R	cI-Bgl

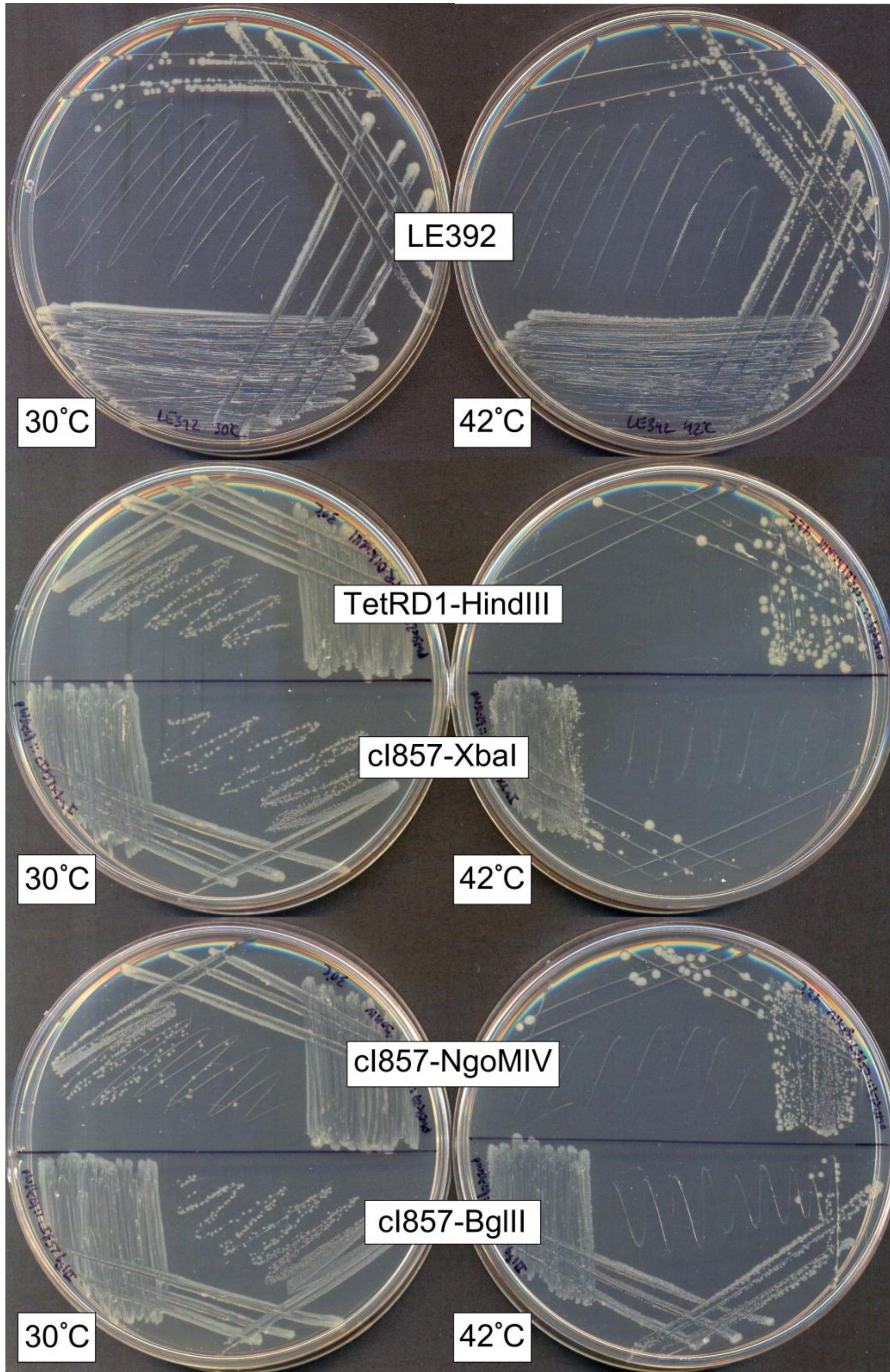

Figure 6. TS lethal gene circuits on low copy plasmid pWSK29 in *E. coli* DH10B streaked on agar at 30° and 42°C. Circuits consist of different restriction enzyme genes (*hindIII, xbaI, ngoMIV, bglII*) and TS repressors (cI857, TetRD1). Different combinations resulted in various levels of lethality, and this also varied when strains were either streaked on agar or grown in liquid broth.

For the majority of microbes, the most genetically stable approach for the introduction of foreign genes is by inserting them at a chromosomal location. Thus we inserted the TetD1-Hind, cI-Bgl and cI-Ngo circuits into one or more *E. coli* chromosomal sites previously determined to be non-essential (Table A1). We found that strains harbouring the circuits in every chromosomal locus grew similar to the parental strain LE392 at permissive temperature of 30°C (Fig 7).

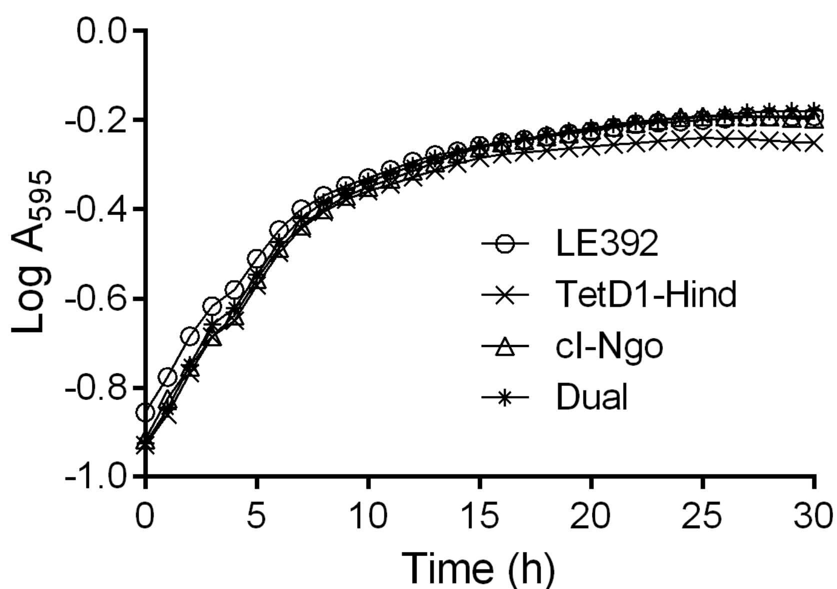

Figure 7. Broth growth of the TS *E. coli* strains at permissive temperature 30°C for a total of 30 h. Select three TS strains are shown: TetD1-Hind in the *yeaH* locus, cI-Ngo in the *yeeR* locus, Dual strain consisting of both TetD1-Hind in *yeaH* and cI-Ngo in *yeeR*.

The TetD1-Hind circuit was inserted at the *ycgH* and *yeaH* loci and upon heat induction the resulting strain showed a reduction in A_{595} and a steady loss of viability when inserted at the *yeaH* locus (Fig 8A and Fig 8B) but did not exhibit this phenotype when inserted at the *ycgH* locus (Fig 10).

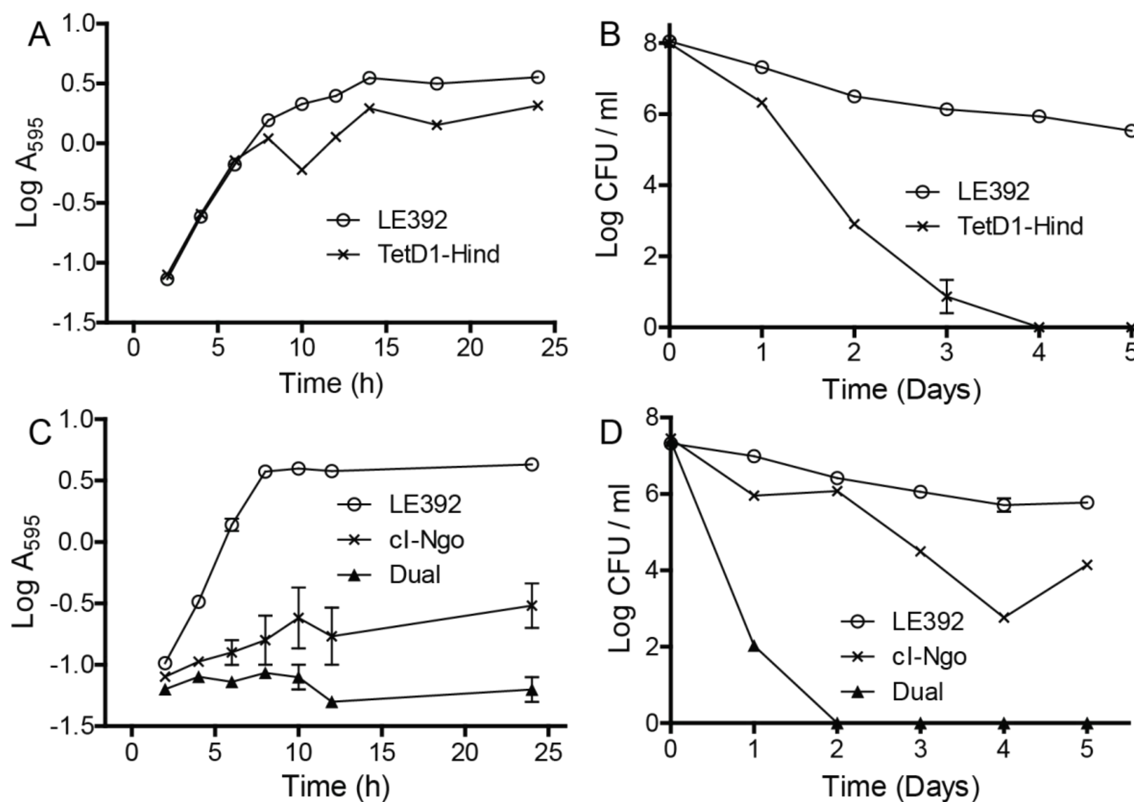

Figure 8. Growth and survival characteristics of the TetD1-Hind, cI-Ngo and Dual strains at 42°C. A and C. Broth growth of TetD1-Hind and cI-Ngo at 42°C for 24 h. B and D. Survival of TetD1-Hind and cI-Ngo at 42°C suspended in PBS as shown for 5 days. Error bars indicate the standard error of mean from three replicate samples.

This strain was also subjected to viability staining after 24 h incubation at 42°C, and exhibited a shift in the proportion of cells staining with green fluorescent dye only to green and red fluorescent dyes (Fig 9 Panel I and II) the latter scenario being indicative of dead or dying cells. This phenotype was not observed for the parental strain when subjected to identical conditions (Fig 9 Panel III and IV).

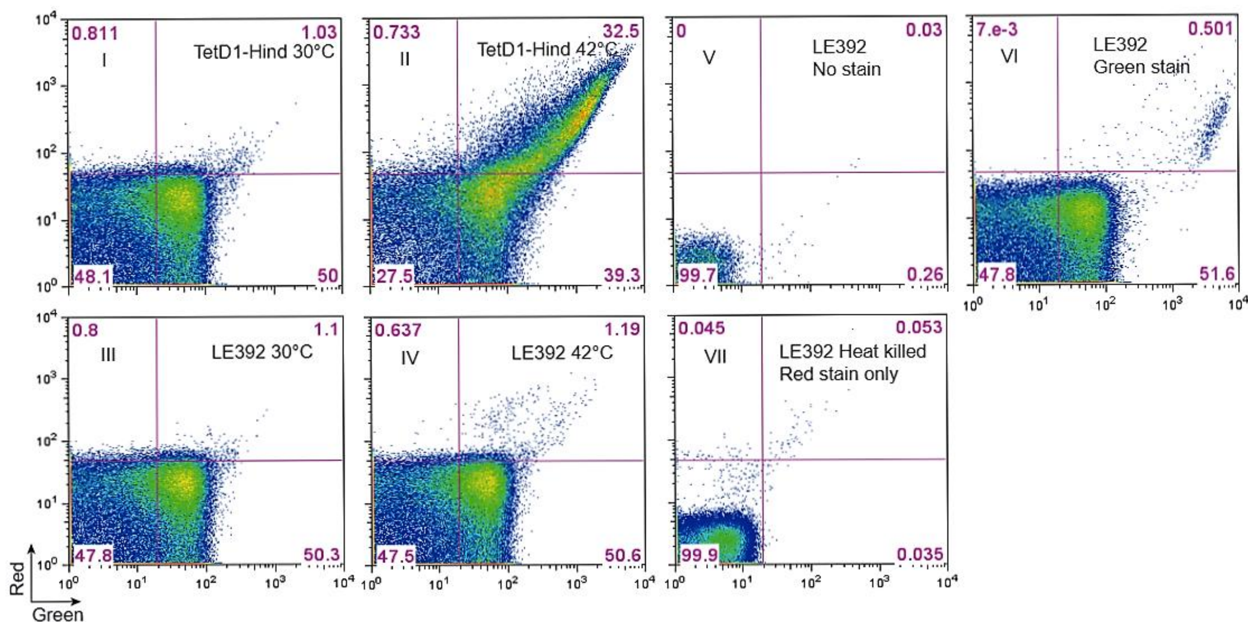

Figure 9. Viability staining and flow cytometry of the TetD1-Hind strain at 30 and 42°C.Distribution of presumed live (green stain) and dead (green and red stain) *E. coli* grown at permissive (I, III, V) and restrictive (II, IV) temperatures. (V) Parental strain LE392 with no staining. (VI) LE392 stained with green dye only. (VII) LE392 heat killed stained with red dye only. LE392 killed with heat or isopropyl alcohol (not shown) demonstrated weak uptake of the red dye.

A second lethal circuit was needed in order for the creation of a Dual strain. The first version of this circuit, cI-Bgl was inserted at the *ycgH*, *yeeR* and *insH1* loci. Strains created with this circuit at any of the three loci showed no TS phenotype (data not shown). This circuit was therefore not studied further.

To amend this, a second version of the circuit was created, cI-Ngo, and was inserted at the same loci: *ycgH*, *yeeR* and *insH1*. Strains with insertions at the *yeeR* and *insH1* loci were killed rapidly at 42°C (Fig 8C shown for *yeeR* locus) but when inserted at *ycgH* retained viability at high temperature (Fig 10).

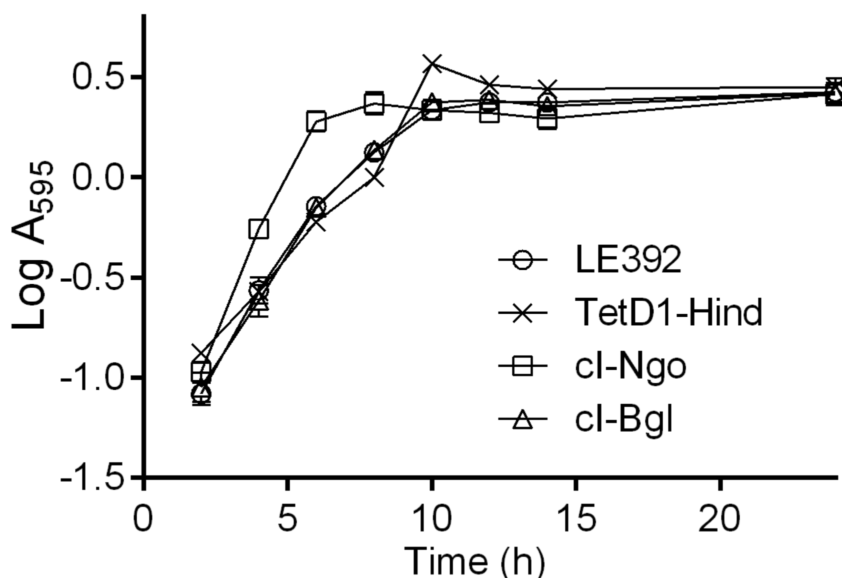

Figure 10. Broth growth of LE392-derived strains with lethal circuits TetD1-Hind, cI-Bgl and cI-Ngo integrated into the *ycgH* locus at 42°C for 24 h. Error bars indicate the standard error of mean from three replicate samples.

3.3 Stability of TS lethal genetic circuits

If a TS lethal circuit is to be practically useful it needs to be genetically stable with a low frequency of mutation to a temperature-resistant phenotype. It was found that the strains harbouring a single lethal circuit within the chromosome would occasionally revert during PBS survival experiments (i.e. one or more of three independent replicates). In an attempt to amend this, we integrated Circuit 1 (TetD1-Hind) into the *yeaH* locus of the existing strain harbouring Circuit 2 (cI-Ngo) at the *yeeR* locus, thus creating a "Dual" strain. The growth of this strain was further restricted at 42°C (Fig 8C) and the CFU/ml reduced quickly to 0 within 2 days and did not revert for the duration of 1 week (Fig 8D). Repetitions of this experiment revealed that this strain could revert, as only 1 independent replicate was found to revert in PBS. The actual reversion frequency was quantified using several independent replicates of the Dual strain. This

resulted in reversion frequencies ranging from 1.3×10^{-6} to 6.8×10^{-8}. Reversion frequencies were not calculated for strains with only Circuit 1 or only Circuit 2 because of the growth of a large number of CFUs on agar at the restrictive temperature. In order to determine if CFUs forming at the higher temperature were true revertants, about 100 CFUs collectively were streaked on agar at both 30° and 42°C. It was found that CFUs demonstrated a variety of phenotypes when streaked at 42°C; however it was clear that not all CFUs were true revertants since some did not grow at 42°C when re-streaked on agar. Lastly, Circuit 1 and Circuit 2 were sequenced from a variety of Dual strain mutants and established that they contained start codon deletions and upstream deletions of the TetRD1 gene or had complete deletions of both lethal genetic circuits.

In order to determine if a reduced DNA repair capacity would affect the survival of the TS strains at high temperature, the TetD1-Hind circuit was integrated into the recombination deficient strains PMC103 and PMC107 at the *yeaH* locus. Cleavage of dsDNA creates detrimental breaks which are repaired mainly by the RecBCD pathway or alternatively RecF. Mutations in these pathways could reveal a level of recombination used by cells during the expression of the restriction enzyme genes. TetD1-Hind in PMC103 resulted in a gradual decrease in CFU/ml similar to what was seen for the circuit in LE392 at 42°C, but was observed to not revert after 7 days (Fig 11). The TetD1-Hind circuit in PMC107 was found to rapidly reduce CFU/ml to 0 within 3 days at high temperature and also did not revert over the span of one week (Fig 11).

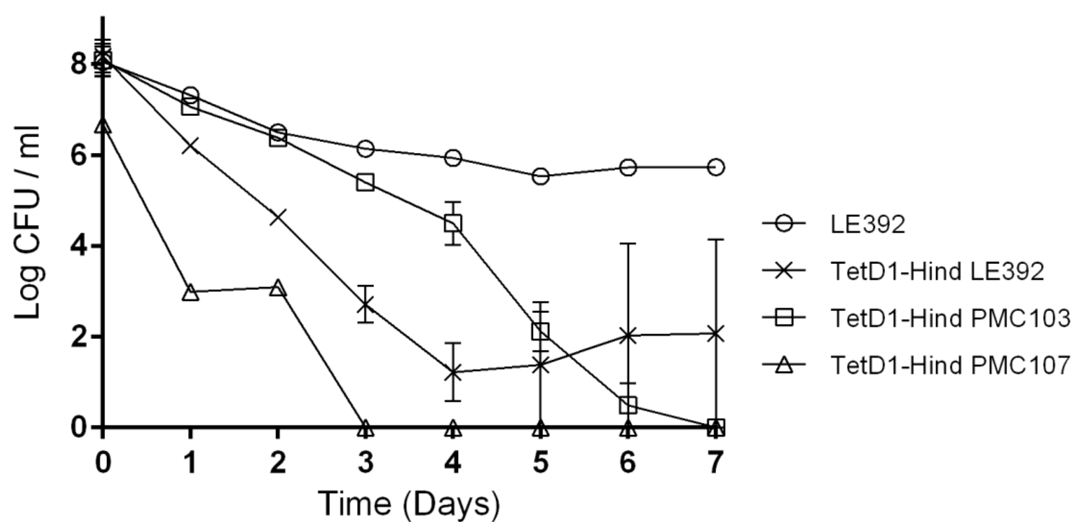

Figure 11. Survival of recombination deficient *E. coli* strains harbouring the TetD1-Hind circuit in the *yeaH* locus at 42°C. Strains were suspended in PBS at the high temperature for 7 days with aeration. Error bars indicate the standard error of mean from three replicate samples.

CHAPTER 4: DISCUSSION

In this work, a rational design approach was taken to create genetic circuits which impart temperature-sensitive lethality on the target organism. Broad host range elements such as a mutant TetR protein and restriction enzymes were incorporated into the circuits to allow for a widely applicable method for generating TS organisms. These TS organisms could have a variety of important applications including the creation of "safe" strains for laboratory or environment use (intrinsically contained) and also the creation of TS attenuated vaccines. Here, TS repressors and cognate promoters were first tested for functionality and were then assembled into the TS genetic circuits containing different lethal restriction enzyme genes. These were integrated into the chromosome and were characterized for acceptable TS lethality and escape frequency in recombination proficient and deficient *E. coli* strains.

4.1 TS TetR mutants function with various tetO promoters

To determine if TS TetR mutants functioned with other tetO promoters, we coupled their expression to mCherry reporter vectors containing various upstream tetO promoters. These included PE100, PE80 (McWhinnie and Nano, 2013) and D18 (Cox III *et al*., 2007) which have approximate strengths of weak, strong and strong respectively as confirmed in repressor-less controls (Fig 5). The slightly increased fluorescence of these controls at 42° vs 30°C can be attributed to differences in growth due to temperature. 42°C is closer to *E. coli's* optimal growth temperature of 37-39°C than 30°C leading to increased protein production which includes mCherry. TetR mutants D1 and G2 were able to effectively control mCherry fluorescence via all three promoters demonstrating increased repression when compared to repressor-less controls and increased expression at 42°C compared to 30°C. The cI857 repressor was also included in

the assay and was observed to exhibit tight control of fluorescence expression at both temperatures. The C-terminal addition of the AAV degradation tag (Andersen *et al*., 1998) to the TS repressor proteins was included to increase the induction ratio. The over expression of repressor proteins has been linked to decreased sensitivity of induction (Georgi *et al*., 2012), thus to prevent accumulation of TS repressors we included the AAV tag. However, it was found that repressors with the tag did not increase the induction ratio but rather increased fluorescence expression at both temperatures. One interpretation of these data is that the AAV tag is recognized more readily by *E. coli's* degradation machinery and this resulted in excessive degradation of the proteins leading to an insufficient number of repressors occupying the tetO sites. Because of this, the degradation tag version of the TS repressors was not included in further experiments.

4.2 Genetic circuits consisting of TS repressors TetRD1 and cI857 effectively control expression of lethal restriction enzyme genes in plasmid

When assembled on plasmid, these TS repressors and their promoters allowed for TS induction of restriction enzyme genes which in all cases led to reduced growth of *E. coli* DH10B. When different restriction enzyme genes were cloned into the low copy plasmid pWSK29, all lethal genes resulted in some TS killing of *E. coli* which was either observed from streaking on agar (Fig 6) or growing in broth at permissive and restrictive temperatures. This was expected since pWSK29 can have up to 5 copies per cell, which corresponds to more than one lethal gene per cell. Gene dosage effects paired with the fact that DH10B is homologous recombination deficient (*recA1*) likely contributed to a more noticeable TS phenotype. However, circuit cI-Xba demonstrated unacceptably low killing effects (in broth) and was thus not studied further. This could be due to low expression levels in combination with a low number of recognition sites (35)

in the *E. coli* chromosome. Circuit construction in plasmid is a rapid way to test functionality, however stability is not guaranteed and the killing effect is often not uniform because of the presence of multiple copies of the restriction enzyme gene. To improve this, two circuits that demonstrated good TS expression were chosen to be integrated into the chromosome of *E. coli* LE392. Unlike DH10B, this strain is recombination proficient making it more representative of a wild type-like strain.

4.3 TS genetic circuits demonstrate variable killing effects when integrated in the *E. coli* chromosome

4.3.1 TetD1-Hind circuit is functional in the chromosome at the yeaH *locus*

The TetD1-Hind circuit was integrated in the *yeaH* locus of LE392 and demonstrated a slower leveling off of A_{595}, a steady decline in CFU/ml and a shift in the proportion of cells staining green (live) to green and red (dead) when exposed to 42°C. An initial lag phase was observed in the growth curve for the first 8 h as seen in Fig 8A. There could be several factors contributing to this phenotype which include the structure and expression of the restriction enzyme, as well as the location and accessibility of the recognition sites. HindIII is a dimeric enzyme containing one catalytic site per subunit (Pingoud and Jeltsch, 2001). Each subunit of the dimer binds one half of its recognition sequence, cleaving one DNA strand each which translates to cleavage of one dsDNA recognition site. Therefore it can effectively cleave only one recognition site at a time, contrasting to tetrameric restriction enzymes which can cleave two sites at once. Additionally, a lower expression of HindIII could reduce its full potential to initially overwhelm the cell’s repair machinery. Furthermore, restriction enzymes may encounter barriers preventing access to their sites which include the folding and bending of DNA which

could make sites temporarily inaccessible. Lastly, the position of the recognition sites may play a role in the initial survival of the cell. If many recognition sites fall into less important or essential regions, this could allow enough time for adequate DNA repair to occur. Early experiments revealed *recA* expression could help to initially repair enough DNA allowing a proportion of cells to survive compared to a *recA*$^{-}$ strain (Fig A2). The observed constant decline in CFU/ml when the strain was suspended in PBS at 42°C (Fig 8B) could be attributed to an accumulation of HindIII enzymes. Restriction enzymes are more stable and have longer half lives compared to their cognate methyltransferases (Mruk and Kobayashi, 2013). This feature is a hallmark of post-segregational killing which ensures that Restriction-Modification (RM) modules are retained within a population. Together, these factors could all contribute to the initial lag phase and steady decline in CFU/ml of the TetD1-Hind strain.

The TetD1-Hind strain was also subjected to viability staining and flow cytometry which revealed a large proportion of cells stained green and red, indicative of dead or dying cells at the restrictive temperature (Fig 9 Panel II). This contrasted to the parental strain's proportion of cells which remained stained green at both permissive and restrictive temperatures which confirmed the TS phenotype of the TetD1-Hind strain. It was also noticed that the green stained fluorescence distribution of LE392 resembled that of TetD1-Hind at permissive temperature; however LE392 heat-killed cells stained poorly with the red dye only (Fig 9 Panel VII). The red dye stains only when it can bind to damaged cell membranes, which might suggest a difference in cell death mechanism which in turn affected the penetration of the red dye.

4.3.2 TS genetic circuits are non-functional in chromosomal locus ycgH

The chromosome position for integration of the circuits was an important factor as it was found that the *ycgH* locus was transcriptionally silenced. Three different circuits were integrated at *ycgH* (TetD1-Hind, cI-Ngo, cI-Bgl) and all were observed to have no killing effect in broth at 42°C (Fig 10), but both TetD1-Hind and cI-Ngo circuits inserted at other loci did have the effect which suggested silencing at this particular locus. Chromosome position has been known to affect gene expression especially in eukaryotes (Wilson, 1990), whereas this phenomenon is not well understood in bacteria. A 2009 study (Vora *et al*., 2009) mapped the protein occupancy landscape of the bacterial genome and termed certain regions as EPODs (extended protein occupancy domains) to which the majority were localized to transcriptionally silent loci (termed *ts*EPOD). The *ycgH* locus falls into a *ts*EPOD which explains the no killing effect for the circuits. These *ts*EPODs silence expression at the transcriptional level and are thought to serve as organization hubs involved in the structuring of chromosomal macrodomains and loops, however these mechanisms are currently unknown (Bryant *et al*., 2014).

4.3.3 cI-Bgl circuit is non-functional in chromosomal loci ycgH, yeeR *and* insH1

The first version of the second genetic circuit consisted of cI-Bgl but was revealed to be unsatisfactory when integrated into the chromosome. The cI-Bgl circuit was integrated into the *ycgH, yeeR* and *insH1* loci and was found to be non-functional in all positions. Several clones were sequenced which contained a recurring amino acid substitution A11D in the *bglII* gene. The mutation could have arisen during the Gibson assembly reaction and was subsequently carried forward in the chromosomal integration experiments. This could explain why the circuit was

functional in plasmid, but not in chromosome as different flanking regions were assembled after the fact. A single substitution within the *bglII* gene could abolish its activity if it occurred within a residue involved with catalytic function (Tang *et al*., 2000). However, this mutation resides near the beginning of the gene, indicating that the substitution might affect the ribosome binding site therein affecting the translation of BglII. Changing residues just upstream or downstream of the start codon can drastically affect the strength of the RBS (Salis *et al*., 2009) and this was confirmed by inputting the mutated sequence into the Salis RBS Calculator. The RBS of *bglII* was originally designed to have a TIR of 1500 AU; after the mutation the TIR was calculated to have 166 AU, a ten-fold reduction. It is possible that this change led to decreased expression of *bglII* enough that the TS phenotype was not noticed. Because of this, the cI-Bgl circuit was not studied further.

4.3.4. cI-Ngo circuit is functional at the chromosomal loci yeeR *and* insH1

The second version of the lethal circuit, cI-Ngo demonstrated a rapid killing effect of *E. coli* at the high temperature when integrated into the *yeeR* and *insH1* loci. In broth, the strain's growth was impeded immediately upon exposure to 42°C and remained at a low A_{595} for 24 h (Fig 8C). In PBS, the cI-Ngo strain steadily declined in CFU/ml until it reached 4-5 days of incubation (Fig 8D). This faster inactivating effect is likely due to the different cleavage nature of homotetrameric restriction enzymes. NgoMIV, like other tetrameric enzymes such as SfiI, consist of four catalytic sites meaning that these can simultaneously cleave two recognition sites (Deibert *et al*., 2000). This is achieved by DNA looping and can occur on separate DNA strands upwards of 1 kb away (Katiliene *et al*., 2003). There is also evidence that cleavage by these

enzymes occurs much more rapidly (i.e. up to 40 fold) when it can bind simultaneously to two sites (Embleton *et al*, 2001; Wentzell *et al*., 1995). Within the *E. coli* chromosome there exists 259 NgoMIV recognition sites, thus this allows NgoMIV abundant access to rapidly cleave two sites at once.

4.3.5 TetD1-Hind circuit integrated in the yeaH *locus in* E. coli *strains PMC103 and 107 results in differing phenotypes at 42°C*

The TetD1-Hind circuit was also integrated into two recombination deficient strains in order to assess survivability since restriction enzymes cause dsDNA breaks which in turn activates repair by recombination. PMC103::TetD1-Hind was observed to gradually decrease in CFU/ml approximately 10-fold each day and this strain did not revert after 1 week at the high temperature (Fig 11). Whereas the PMC107::TetD1-Hind strain was observed to rapidly decrease to 0 CFU/ml within 2-3 days incubation at 42°C and also did not revert over the span of 1 week (Fig 11). This contrasted to the TetD1-Hind circuit in LE392 which did on occasion revert after 4-5 days. The phenotype of PMC103 was less severe than that of the PMC107 and this can be attributed to the differences and number of mutations harboured by the two strains. PMC103 is not truly recombination deficient, as it contains intact RecBC and RecF pathways. This strain does contain a *recD* mutation as well as the *sbcC* mutation. *recD* mutations result in no exonuclease activity of the RecBCD complex, thus RecBC must rely upon other exonucleases (Finch *et al*., 1986). One of those nucleases is encoded by *sbcC*. It is possible that by delineating the natural pathway for recombination could render it less recombination proficient. Interestingly, mutations in *recD* alone result in viable cells which are resistant to DNA damaging agents (Biek

and Cohen, 1986) again relying heavily on the activities of other exonucleases (Dermic, 2006). This could explain why this strain initially survived better than the recombination proficient LE392 strain. The results suggest that the recombination ability of PMC103::TetD1-Hind must be slightly reduced, since this strain was not observed to revert after 7 days at the higher temperature. The second recombination deficient strain PMC107 contains mutations *recB*21, *recC*22, *recJ*154 and contains the suppressor mutations *sbcB*15 and *sbcC*201 which affect multiple recombination pathways. *recB* and *recC* encode DNA helicase and exonuclease activities of the RecBCD enzyme, and without these terminates activity of the RecBCD pathway. However, the strain also contains *sbcB*15 and *sbcC*201 which suppress the *recBC* phenotype, by suppressing Exonuclease I (Kushner *et al*., 1971). Both of these suppressors together activate the RecF pathway of recombination, although this pathway becomes impaired due to a further mutation in *recJ*, which encodes a 5′→3′ exonuclease involved in the RecF pathway. Mutations affecting this protein lead to decreased recombination from 1000 to 10,000 fold (Kowalczykowski, 1994). Furthermore, RecBCD can only process blunt end (or near blunt) dsDNA breaks. Cleavage by HindIII and NgoMIV create “sticky ends,” leading to an additional processing step by ExoI or RecJ before RecBCD can bind. On the other hand, the RecJ exonuclease imparts activity only on non-blunted dsDNA (Lovett, 2014). This could suggest that the RecF pathway might play a larger role in the repair of dsDNA breaks by restriction enzymes as cleavage sites do not require processing in order for RecJ activity. The mutations in PMC107 likely result in a reduction of recombination repair via the RecF pathway which leads to a significant decrease in the cell’s ability to repair dsDNA breaks. The results from Fig 11 may point to the importance of a functional alternative recombination pathway on the cell’s ability to repair DNA and survive expression of lethal restriction enzyme genes.

4.4 Two independent TS genetic circuits integrated into the same strain reduces reversion frequency

The idea of coupling two or more independent circuits within the same cell has been shown to significantly reduce escape frequency (Cai *et al*., 2015; Gallagher *et al*., 2015). The TetD1-Hind and cI-Ngo circuits were integrated into the *yeaH* and *yeeR* loci to create a Dual circuit strain which demonstrated reduced reversion frequency compared to each single circuit strain. Individual replicates of single circuit strains were observed to occasionally revert when suspended in PBS at 42°C over 1 week, whereas the Dual circuit strain showed reduced reversion frequency as its growth was rapidly restricted (Fig 8C) and CFU/ml reduced to 0 when incubated at 42°C for 1 week (Fig 8D). This was expected since two independent circuits effectively increase the number of barriers that a cell must overcome to escape. However, certain repeats of the experiment revealed that a single replicate of the Dual circuit strain could revert after 3-4 days of incubation at 42°C. We also calculated the reversion frequency of the Dual strain only and found the escape frequency varied from $1.3x10^{-6}$ to $6.8x10^{-8}$ depending on different replicates and experiments. These numbers were not quite within the NIH's standard for escape frequency of $1x10^{-8}$ and several replicates revealed a 10 and 100-fold difference in escape frequency which is undesirable for a marketable strain. We did not calculate the frequencies for single circuit strains because plating ~$1x10^{9}$ cells on agar at 42°C resulted in the growth of hundreds to thousands of different sized colonies. When CFUs from these experiments were streaked on agar at permissive and restrictive temperatures, they were found to have variable phenotypes ranging from absolutely no growth to wild type-like growth at the restrictive temperature. This made it clear that not all CFUs were indeed revertants and that the calculated reversion frequencies were not accurate or precise.

4.5 *E. coli* with integrated TS genetic circuits demonstrate differences in survival and death at the restrictive temperature

These inconsistencies in lethality and survival could be the result of a combination of unfavourable conditions evoked from the experiments at high temperatures. These experiments elicit a high stress response in the cell triggering SOS genes (DNA damage), stringent related genes (starvation), *rpoE* regulated genes (mild heat shock) and *rpoS* regulated genes (general stress response). Each of these modules is known to control many genes, for example the SOS response controls ~40 genes (Courcelle *et al*. 2001), whereas *rpoS* can modulate up to 500 genes (Weber *et al*., 2005). Together, this large amount of change can trigger heterogeneous cells and subpopulations (Makinoshima *et al*., 2003) leading to differences in lethality and survival. Because the lethal genetic circuits result in cleaved dsDNA, the discussion will focus mainly on the SOS response.

The SOS response is a regulatory network that is activated in response to damaged DNA; however its induction has also been implicated with increased mutation rates and bacterial adaptation and survival (Baharoglu and Mazel, 2014). The heterogeneous phenotypes in lethality and survival could be explained by the non-uniform expression of SOS genes. For example, SOS gene expression has been shown to be non-uniform among genetically identical cells growing in the same culture (McCool *et al*., 2004). Additionally, results from Friedman *et al*., 2005 suggested that SOS genes are expressed in multiple rounds in response to different doses of DNA damage, indicating that certain genes are periodically switched off and then on. This could explain the variable phenotypes seen at 42°C as not all cells are undergoing the same progression of SOS gene induction. The textbook progression of the SOS response outlines the ideal order of repair mechanisms: 1) Nucleotide excision repair, 2) RecA mediated homologous recombination,

3) Error prone DNA polymerase expression. In the case of restriction enzymes, the entire chromosome is under attack at hundreds of different positions, and it is likely that mechanism 2 and 3 are mainly carried out at this point. In the recombination proficient strain LE392, the presence of RecA allows for homologous recombination. This process involves the joining of two DNA fragments containing similar but not identical sequences. Under such circumstances, the presence of many cleaved DNA fragments could effectively act as substrates for recombination, which would further result in more heterogeneity. Lastly, induction of mechanism 3 can lead to increased mutation rates because of the expression of error prone DNA polymerases IV and V. These enzymes are able to permit DNA replication across lesions which would otherwise stall DNA polymerase III. The expression of these error prone polymerases can introduce mutations at the lesion site or elsewhere in the genome (Michel, 2005) which could contribute to a cell's escape by either disrupting or altering important pathways. Furthermore the SOS response has been associated with rapid bacterial adaptation giving rise to antibiotic resistance and virulence (Beaber *et al*., 2004; Levin, 2004; Zgur-Bertok 2013; Maiques *et al*., 2006). For example, the resistance to fluoroquinolones has been linked to the expression of the SOS response (Qin *et al*., 2015). These antibiotics act by inhibiting DNA synthesis by stabilizing dsDNA breaks created by DNA gyrase and topoisomerase IV (Hooper, 2001). Resistance to these antibiotics has been linked to mutations occurring in DNA gyrase, topoisomerase IV, and also in *recA* (Urios *et al*., 1991). From these studies, it is possible that triggering the SOS response could result in bacterial resistance to restriction enzymes by a number of different mechanisms.

Alternatively, the observed revertants could not all be mutants but instead be persister cells which are phenotypically distinct from wild type cells. This type of cell is synonymous with

"antibiotic resistance" and has been widely studied in this area. However, the use of antibiotics (a toxin) on bacteria is not unlike the scenario of expressing restriction enzyme toxins in a cell. Persister cells have been proposed to either be preexisting (Pedersen *et al*., 2002) or can be induced by the over expression of toxins which trigger the SOS response (Dorr *et al*., 2009; Wu *et al*., 2012; Correia *et al*., 2006). During certain survival experiments the CFU/ml was observed to decrease to 0, but could eventually recover (data not shown) following several days. An explanation for this could be that the majority of cells died leaving behind a persisting population which could then switch back to a normal cell state (Hofsteenge *et al*., 2013) when conditions became favourable. These cells could initially remain undetectable since the SOS response has been linked to promoting a senescence-like state in which cells are still viable but are unable to form colonies (Pennington and Rosenberg, 2007). This evidence might indicate that some cells escaping death at 42°C are persisters instead of revertants.

From sequencing a variety of escape mutants, we found that they contained mutations in or upstream of the TetRD1 gene or completely lacked both TS genetic circuits. TetRD1 revertants contained deletions of the first 3-4 codons and also 12-15 bp upstream of the gene. These mutations effectively abolish repressor expression as the RBS and start codon is absent, thus rendering the strain non TS. In bacteria, new mutations are biased towards deletions, thus unless a gene is maintained by selection, it will become lost (Mira *et al*., 2001). The lethal circuit expressing restriction enzymes offers limited benefit to the cell and will therefore not likely be maintained by selection. The effect could further be explained by genetic drift. Increased levels of drift can arise from a reduction in effective population size (Kuo *et al*., 2009) which actively continues the longer cells are exposed to the higher temperature. Genetic drift promotes genome reduction which could explain the complete loss of both circuits over time. Interestingly, certain

Dual strain revertants contained the TetRD1 mutations, but no genetic changes were detected in the cI-Ngo circuit. This could suggest an extragenic suppressor mutation acting on elements of the cI-Ngo circuit. Extragenic suppression suppresses the effect of one gene by a mutation occurring in another gene elsewhere in the genome. Such effects might include over-expression or mutations in chaperone proteins or in DNA repair elements such as the RecA protein (Urios *et al*., 1991). Sequenced revertants were found to contain mutations in the circuits and also likely possessed mutations outside of the circuits. Additionally it cannot be discounted that persister cells could be present, but otherwise cannot be identified since they sometimes cannot form colonies. These results further highlight the heterogeneous nature of bacterial stress responses and their ability to overcome unfavourable circumstances.

4.6 Future directions

Reversion frequencies of the Dual strain were determined to be inadequate and thus the technology would benefit from incorporating other elements to further restrict a bacterium's ability to revert. One additional element includes utilizing an uncleavable version of the LexA protein. LexA is the controller protein for the SOS response and its initiation is signaled by the autocleavage of the protein. If the protein cannot autocleave, this effectively abolishes the SOS response which can heavily influence a bacterium's ability to adapt and survive when exposed to unfavourable elements. Several studies have shown that using an uncleavable version of LexA could prevent the evolution of bacteria to resistance (Zgur-Bertok, 2013; Levin, 2004; Miller *et al*., 2004). Alternatively, a cell with no means to repair DNA damage could be detrimental to its growth and wild type like behavior, since at the permissive temperature there is almost certainly some background expression of the restriction enzymes genes. Another element that could be added to the TS genetic circuits could be the addition of different lethal genes which target

different biological components. In this work, both circuits encode restriction enzyme genes which target dsDNA. It is possible that two circuits both targeting DNA could cause the cell to direct the majority of its resources towards repairing DNA. Whereas if one circuit targeted DNA and the second circuit targeted RNA or an essential protein, this could divide up the cell's resources, making it increasingly difficult to overcome not only damaged DNA, but damaged RNA and or a reduction in an essential protein. A recent study conducted by the Isaac's group confirmed that incorporating multiple different safeguards into the same strain could drastically reduce the escape frequency to as low as 1×10^{-12} (Gallagher *et al.*, 2015). Furthermore, the TS genetic circuits could act as a backup module for other forms of intrinsic biocontainment in order to decrease the likelihood of reversion. The Nano lab is working on creating TS forms of bacteria by swapping native essential genes for TS homologues. To complement these efforts, a TS lethal genetic circuit could also be integrated in a non-essential region of the organism's chromosome effectively acting as a substitute if the TS essential gene fails to demonstrate its TS phenotype.

4.7 Conclusions

In this work a variety of TS lethal genetic circuits were designed, constructed and integrated into the chromosome of *E. coli* strains which rendered them TS. The circuits were designed with broad host range components (TetR and the restriction enzymes) to allow for the technology to be used as widely applicable approach to generating TS organisms. TS organisms can be utilized as “safe” strains or intrinsically contained strains for use in industrial or environmental settings. Alternatively TS organisms can be used as TS attenuated or TS live attenuated vaccines in order to protect against, for example, intracellular pathogens, many of which currently do not have effective vaccines. The TS lethal genetic circuit strains demonstrated variable killing effects at the higher temperature but retained wild type like growth at the permissive temperature. It was found that integrating two independent circuits into the same strain reduced the reversion frequency; however refinements are needed to reduce the reversion rates even further. Therefore, acting alone, these genetic circuits would benefit from additional elements to reduce escape frequency; conversely, TS genetic circuits might not need to be altered if they acted in concert with other forms of intrinsic biocontainment such as TS essential genes.

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Appendix

Table A1. *E. coli* chromosomal loci, position and predicted encoded products where lethal genetic circuits were integrated.

Chromosomal loci	Position (kb)	Encoded product
yeaH	1866979 - 1868262	Hypothetical UPF0229 protein
ycgH	1218824 - 1221471	Pseudogene; putative ATP-binding component of a transport system
yeeR	2072803 - 2074335	Prophage; predicted membrane protein
insH1	2064329 - 2065345	Insertion sequence; transposon related

Table A2. *E. coli* strains and their genotypes.

E. coli **strain**	**Genotype**
DH10B	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–
LE392	*hsd*R514(rk-, mk+), *gln*V(supE44), *try*T(supF58), *lac*Y1 orΔ(lacIZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55
PMC103	*mcr*A A(*mcrBC-hsdRMS-mrr*)102 *recD sbcC*
PMC107	*mcr*A A(*mcrBC-hsdRMS-mrr*)102 *recB21 recC*22*recJl*54 *sbcB*l5 *sbcC*20 I

Table A3. Summary of all functional and non-functional TS lethal genetic circuits constructed and integrated into the *E. coli* chromosome.

	Restriction enzyme	Repressor protein	Promoter	Locus	Functional?
1	HindIII	TetRD1	D18	*yeaH*	Yes
2	XbaI	cI857	P_R	*ycgH*	No
3	XbaI	cI857	P_R	*isrC*	No
4	BglII	cI857	P_R	*ycgH*	No
5	BglII	cI857	P_R	*yeeR*	No
6	BglII	cI857	P_R	*insH1*	No
7	NgoMIV	cI857	P_R	*ycgH*	No
8	NgoMIV	cI857	P_R	*yeeR*	Yes
9	NgoMIV	cI857	P_R	*insH1*	Yes

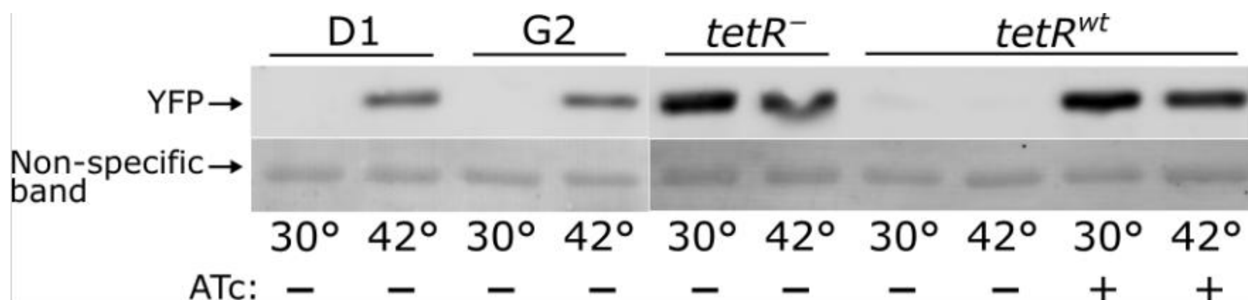

Figure A1. Western immunoblot of YFP controlled by TS mutants TetRD1 and TetRG2 via the upstream P_L-tetO promoter at 30° and 42°C. The wild type TetR inducer anhydrotetracycline (ATc) was also used as a control at both temperatures (R. McWhinnie, Nano Lab).

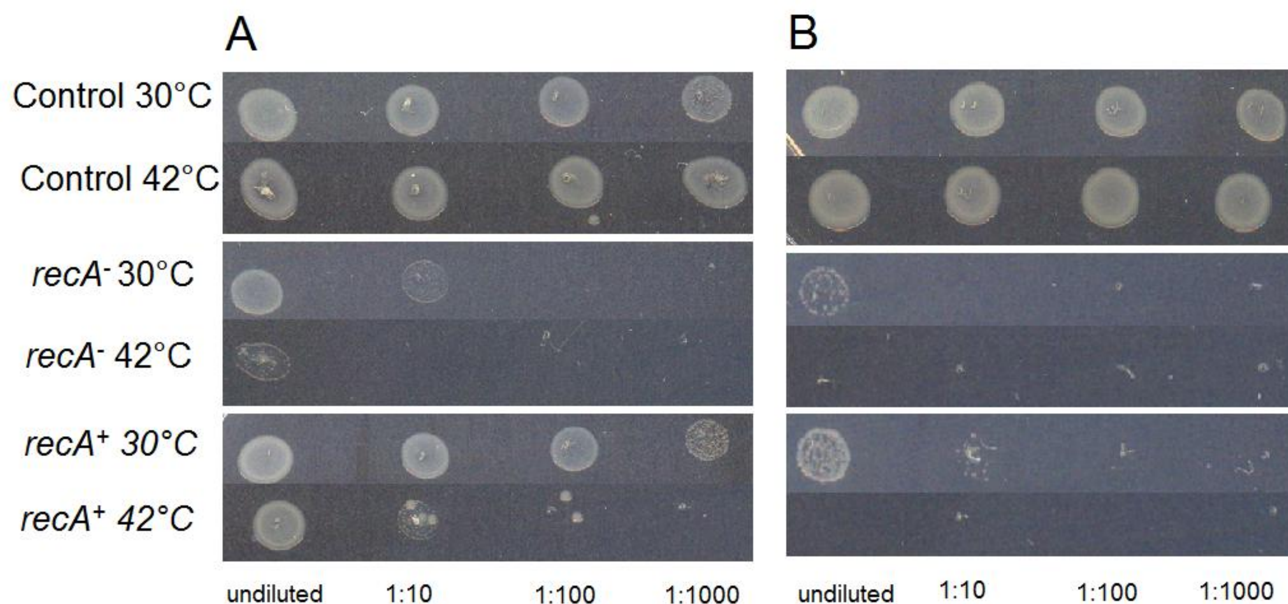

Figure A2. Survival of *E. coli* harbouring TetD1-Hind circuit in *recA*$^{+/-}$ strains. Control strain is DH10B; *recA*$^{-}$strain is TetD1-Hind in DH10B; *recA*$^{+}$ strain is TetD1-Hind in DH10B harbouring low copy pWSK29::*recA*, with *recA* inserted downstream of the *lac* promoter via SacI and KpnI sites. The *lac* promoter was not induced in order to avoid over expression. Strains were grown in LB broth at 30° or 42°C, diluted and then 2µl were spotted onto LB agar which was subsequently incubated at 30° or 42°C for A. 24 h and B. 48 h.

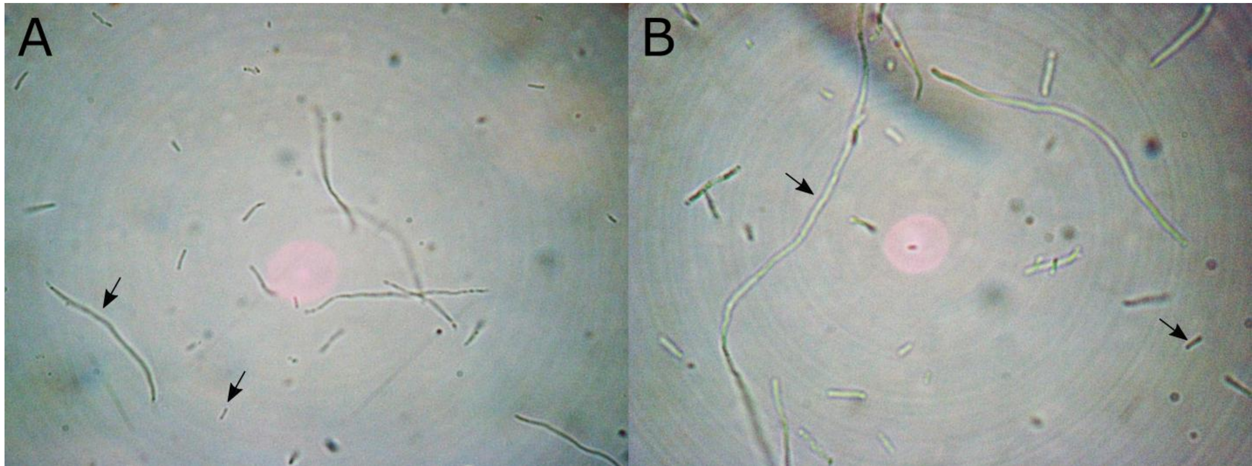

Figure A3. Light microscopy images of *E. coli* LE392 containing the TetD1-Hind circuit at 42°C. Panel A and B depict two different viewpoints. Arrows indicate either long filamentous or smaller normal cell morphologies. Non motile filamentous cells are indicative of DNA damage caused by RE resulting in disruption of cell division. Cells were viewed on a Nikon ALPHAPHOT-2 YS2-H light microscope at 1000X magnification and images were captured using a Sony Cyber-shot DSC-W120 digital camera.