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Development of a Thermosensitive Endonuclease to Act as a Plasmid Kill-Switch

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Abstract

Biocontainment is an integral part of biomedical research that aims to protect the environment and human health by containing hazardous or invasive organisms in the laboratory. Containment systems often rely on elaborate genetic circuits; however, cells may escape containment by developing mutations that render the genetic circuits inviable or resistant to killing mechanisms. The aim of this thesis was to create a site-specific nuclease for biocontainment of plasmids in the mammalian gastrointestinal tract. LAGLIDADG homing endonucleases would be good candidate nucleases for a biocontainment system as they are resistant to mutations in their coding sequence and their target sequence in comparison to other nucleases, such as Cas9. Screening mutagenic libraries of the I-OnuI nuclease yielded a single variant that displays thermosensitive properties *in vivo* that may prove useful for biocontainment purposes. Using thermosensitive LAGLIDADG homing endonucleases for this purpose may prove to be a new, more robust approach to biocontainment.

Keywords

- Biocontainment
- LAGLIDAG Homing Endonucleases
- Thermosensitivity
- Two-plasmid screen
- *In vitro* cleavage assay

Summary for Lay Audience

Biocontainment is an essential part of biomedical research that aims to protect the environment and human health by containing potentially hazardous organisms in the laboratory. Efforts to develop containment systems often rely on elaborate and expansive DNA circuits. The methods used previously also raise the concern that the cells may be able to escape containment, either through mutations that break the DNA circuits or by the evolution of resistance to killing mechanisms. Nucleases, which are enzymes that cut DNA, are attractive candidates for use as a containment mechanism. DNA cutting proteins that can target internal DNA sequences are called endonucleases (in comparison to exonucleases which target the ends). LAGLIDADG homing endonucleases are small, yet highly specific DNA cutting proteins that are resistant to mutations in their DNA sequence and the particular sequence they cut (in comparison to other DNA cutting proteins). As a result of their resilience and specificity, homing endonucleases from the LAGLIDADG family were chosen as the candidate enzymes for developing a biocontainment mechanism. Screening libraries of LAGLIDADG DNA cutting enzymes yielded a single type that displays temperature-sensitive properties when used in a bacterial cell. Temperature sensitivity is useful as a biocontainment mechanism as it means the bacterial cell can only survive in a specific temperature range. These properties may prove helpful for biocontainment purposes in that it could be used as a new, more straightforward, more robust approach to biocontainment.

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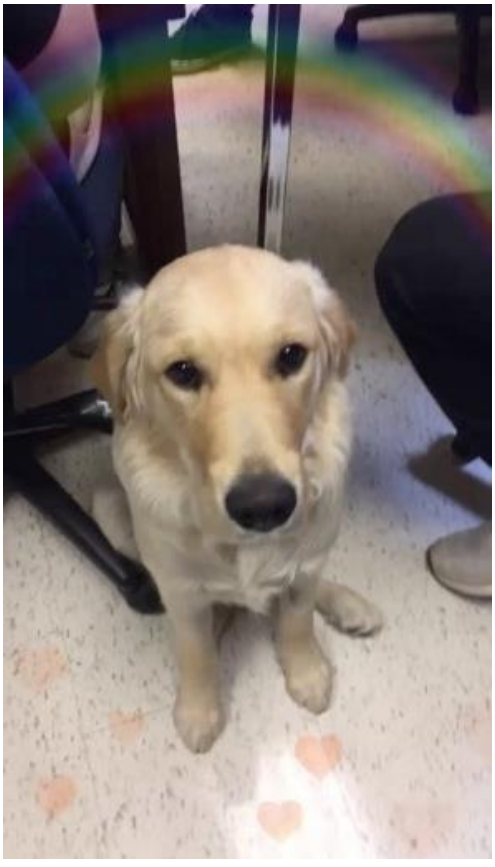
Dedication

I want to dedicate this work to my favourite member of the Edgell Lab, Oliver Edgell. I am confident that someday you will get as much joy out of shredding this thesis as I got out of completing it.



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Chapter 1

1 Introduction

1.1 Objective Statement

The goal of this thesis is to develop and test thermosensitive site-specific LAGLIDADG homing endonucleases that target specific sequences in synthetic plasmids and chromosomes for use as genetic kill switches. The existing systems are of limited utility across all potential applications that require genetically programmed means of biocontainment, and this will be expanded upon below. Employing LAGLIDADG homing endonucleases as the containment method will allow for a more robust and modular application of this biocontainment strategy as they are relatively small (approximately 1 kb in size or less), yet reliable in comparison to other nucleases. The use of temperature as a mediating factor controlling activity will circumvent the pitfalls associated with molecule-based signals and allow the application of this method to be expanded to elements in which their very application requires mobility, such as conjugative plasmids.

1.2 Biocontainment

1.2.1 Historical Perspective

Before 1951, there were no formal inquiries into the threat that biological laboratories may pose to their workers' health, let alone the surrounding communities or environment. The first survey of biological laboratories was done in the USA as a result of multiple case studies and reports around the world of lab workers acquiring infections from the materials to which they were exposed[1]. Thus, an attempt was made to gauge the risk involved with such work[1]. The first effort to formulate a protocol for dealing with biological agents was at a conference held in 1955 at Camp Detrick in Fredrick, Maryland[2]. At this conference, multiple health and safety directors, as well as scientists from military laboratories, met to share their knowledge of working with biological warfare agents to handle common safety problems collaboratively[2].

Although not formally founded until 1984, this would later become recognized as the first meeting of the American Biological Safety Association[2]. The American Biological Safety

Association promotes biosafety as an essential principle to reduce the likelihood of occupational illness using safety principles to minimize human exposure and the harmful impacts of research on the environment by the containment of biological agents to laboratories[2]. Employing the insight gained from the early American Biological Safety Association conferences, the director of health and safety at Camp Detrick, Arnold Wedum, published the first comprehensive biosafety guidelines in 1964[3].

The beginning of the discussion concerning the biocontainment of modified organisms is marked by the 1975 Asilomar Conference, which was held due to concerns about how the recent advent of genetic engineering may affect the biosphere and human health if host species with non-native DNA were to escape into the environment[4]. This conference outlined four levels of risk, which track largely with the biosafety or containment levels used in laboratories today[4]. However, these four levels of containment would not become set in place for another 9 years by the American Biological Safety Association with the publication of the first edition of the "Biological Safety in Microbiology and Biomedical Laboratories" text[4,5]. At the Asilomar Conference, changes to laboratory procedures, such as adopting mechanical pipettes over mouth pipettes, were recommended[4]. Scientists attending the conference also theorized about adopting genetic means of containing engineered organisms such as host strains or vectors incapable of surviving outside the laboratory[4].

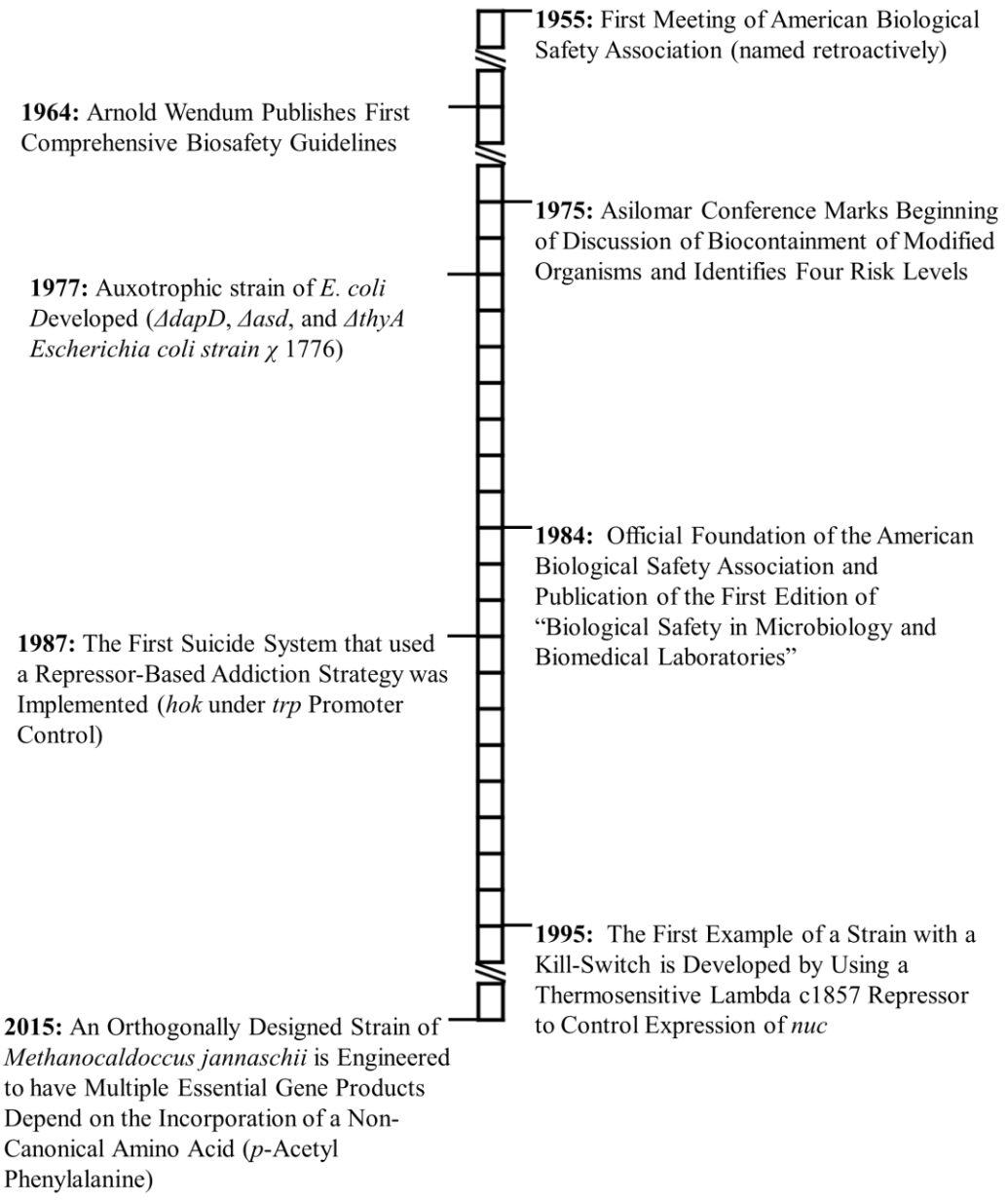

Figure 1. A timeline illustrating the key historical events pertaining to biocontainment. Each box illustrated on the timeline is equal to a year, and double slashes indicate scale breaks.

1.2.2 An Overview of the General Methods of Genetically Programmed Biocontainment

Modern systems for genetic containment are often elaborate and involve integrations or alterations to the host organism's genome[6–8]. Different strategies are combined to reduce the probability of host organism or vector escape[6–8]. An ideal design depends on the ability to control the system within the given context of use, prevent growth if the organism or vector were to escape that context, and allow for uninhibited growth within the intended context of application. In general, individual biocontainment strategies cluster into three distinct categories, as follows: addiction (which can be further broken down into auxotrophs and orthogonally designed systems), suicide systems, and kill switches. The efficacy of biocontainment systems is measured as escape frequency or colony-forming units that survived in non-permissive conditions per 10^x (i.e., if 1 in 1000 escaped, this would be given as 1 in 10^3 or $1x10^{-3}$).

1.2.2.1 Addiction

Addiction based containment strategies attempt to contain the organism by generating strains that are unable to grow in the absence of a nutrient necessary for the replication of DNA or the production of proteins.

1.2.2.1.1 Auxotrophy

Auxotrophy, or the inability for the cell to manufacture critical nutrients, is the oldest method of biocontainment and is still employed today. In 1977, *ΔdapD*, *Δasd*, and *ΔthyA Escherichia coli* strain χ 1776 was developed[9]. This strain is incapable of growing in media deficient in diaminopimelic acid, lysine, threonine, methionine, thymine, or thymidine[9].

Some studies have presented their *Lactococcus lactis ΔthyA* auxotroph's escape rate as below a detectable limit[10,11]. However, one major problem with using auxotrophic strains for biocontainment is that the nutrients exist everywhere in the biosphere outside of the nutrient-deficient media, and the strain is therefore readily able to escape containment when presented with a medium as simple as sterile soil[6]. The problem of nutrient availability is especially true for thymine and thymidine auxotrophs[6]. Additionally, auxotrophic strains display growth deficiencies compared to their prototrophic

counterparts (strains capable of manufacturing all critical nutrients from other organic molecules), even in supplemented media[9,12]. On a positive note, this does make auxotrophs unlikely to take over the ecological niche of their wild-type counterparts since even prototrophic lab strains of *E. coli* do not seem to possess the capability to outcompete wild-type strains. Overall, auxotrophic strains have limited value outside of avoiding the need for antibiotic-based selection in the lab[13].

1.2.2.1.2 Orthogonal Design

Some auxotrophic strains and vectors have been created that require the supplementation of factors that are orthogonal to conventional biology to avoid the ecological prevalence of nutrients that can supplement traditional auxotrophs. The first example of an orthogonally designed addiction system comes from Rovner *et al.* (2013) where they reassigned all the TAG stop codons to TAA stop codons, introduced a new tRNA:aminoacyl-tRNA synthetase pair for the introduction *p*-acetyl phenylalanine at TAG (UAG) sites, and then added TAG codons to multiple essential genes in *Methanocaldoccus jannaschii* to create a strain that is dependent on a non-canonical amino acid[14]. Other examples of orthogonally designed strains include ones that make use of unnatural bases, expanded genetic codes, and ligand-dependent essential gene modifications[15–18]. These approaches often exhibit exceptionally low escape frequencies (generally around 10^{-11} to 10^{-12}) and lack the growth deficiencies inherent to ordinary auxotrophs[14,16].

The first weakness of this approach is the possibly prohibitive cost of reagents used, although it is worth noting that this is not the case with the ligand-dependent essential gene modification approach[18]. Second, the reagents used in the orthogonal design process (and their resultant products) are potentially toxic to the environment, animals, or humans, thereby constricting the use of this approach to laboratories[19]. Third, this approach still involves the cumbersome creation of elaborate strains capable of using factors orthogonal to natural biology[14,18]. Finally, this approach does not allow for horizontal gene transfer, so it would not be a usable containment mechanism in any application dependent on horizontal gene transfer, such as the use of conjugative plasmids in a medical context for microbiome modulation[20]. Admittedly, the inability of orthogonal systems to undergo

horizontal gene transfers could potentially be an advantage of the system in some contexts[20].

1.2.2.2 Suicide Systems

Another method of genetically encoded biocontainment involves the use of single genes that can result in cell death[12,21,22]. These may be used in a toxin-antitoxin context in an attempt to prevent horizontal gene transfer, or in combination with a repressor-based addiction strategy. This strategy was first proposed in 1987, involving *hok* under the control of the *trp* promoter[12,21]. In the absence of tryptophan, Hok is expressed, causing membrane depolarization and cell death[12,21].

The negative aspect of this style of biocontainment strategy, notwithstanding any drawbacks to the addiction-like strategies employed to control gene expression (e.g. p_{Lac}), is that suicide genes are prone to deactivating mutations and therefore are generally ineffective[12,21]. As such, the suicide system strategy's rate of escape is generally in the 10^{-3} to 10^{-5} range[7,12,23]. Moreover, this strategy does not prevent the release of DNA to the environment when used in combination with an addiction strategy. The viability of the cell itself is targeted, not the DNA. This is problematic as DNA can persist in media such as soil, and the potential danger of release generally does not come from laboratory strains themselves, but the non-native or extrachromosomal DNA they may be carrying[13,24]. Finally, when used in an antitoxin-toxin sense to prevent horizontal gene transfer, even if an effective mechanism is devised, this containment method would not be viable for the containment of conjugative plasmid applications[20,25].

1.2.2.3 Kill-Switches

Kill-switch biocontainment mechanisms target the integrity of the genetic information carried, as opposed to the cell's integrity through other means[I]. Kill-switches can be broken

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[I] In the literature the terms suicide systems and kill-switches are used interchangeably. This may be due to the use of the term as it pertains to physical kill-switches (or emergency stops) in industry. However, given the parallels between synthetic biology and computer science, moving forward it should be used in a sense that aligns with the use of the term in software development, a program which prevents unwanted use and dissemination by rendering pirated software unusable. In this sense, the suicide system would be an emergency stop as it pertains to hardware (the cell), and the kill-switch would refer to a mechanism of preventing the unwanted spread of software (DNA).

down into three categories: non-specific nucleases, restriction enzymes, and highly targeted nucleases with long recognition sequences[7,26,27]. Like suicide systems, this method can be used to contain an organism or to prevent the escape of extrachromosomal elements, albeit, using this method has a significant advantage in the later context if combined with more specific nucleases to create a self-targeting system. The first published use of this containment strategy consisted of the non-specific nuclease *nuc* under the control of lambda p_L promoter in *E. coli* TGE900, which carried a thermosensitive lambda cI857 repressor[26]. At 42°C, the lambda repressor becomes unstable, and Nuc is expressed, which results in the destruction of any genetic information, including chromosomal DNA, causing cell death[26]. This strategy has since been improved upon by using genetic circuits to control expression that do not rely on high temperatures and use more specific nucleases, such as EcoRI or CRISPR[7,27]. Depending on the method chosen, the reported escape frequencies range from 10^{-5} (Nuc and EcoRI) down to below a detectable limit ("Deadman" and "Passcode")[7,26,27].

Again, such a system's downsides will mirror those of the addiction-like molecule dependent systems that are relied upon to control gene expression. As such, if a naturally occurring molecule is used to control expression, this will result in aberrant activity in non-permissive conditions selecting for mutations over time, which will inevitably lead to increased escape frequencies[7]. This approach's attractiveness is that if highly targeted nucleases with long target sites are used, the genomic DNA could be left intact while allowing the biocontainment system to target extrachromosomal elements exclusively.

1.2.2.3.1 Thermosensitivity: Leaving Addiction Behind

To overcome the drawbacks of activity controlled by an addiction strategy and retain the desirable feature of highly-targeted cleavage, one could potentially use a thermosensitive version of Cas9 to create a self-targeting kill-switch[28]. Thermosensitive Cas9 could prove an ideal strategy to prevent the escape of non-chromosomal genetic material (even conjugative plasmids) in the context of industrial, laboratory and medical applications of microbes.

Unfortunately, guide RNA expression levels have been shown to drop drastically when the temperature decreases to 30°C[29]. This raises the concern that a further drop to room

temperature may allow for a construct to escape containment. Additionally, the *cas9* sequence is relatively large (approximately 4 kb in length), reducing its attractiveness as a candidate nuclease to use as a plasmid-based kill-switch. A type II restriction enzyme would be smaller, and one example, AbaSI, exists with no activity at 37°C and performs optimally at 25°C[30]. Unfortunately, its target sequence is relatively frequent (CNNNNNNNNNNNNNNNNNNNG), and its activity is methylation-dependent[30]. Likewise, a temperature-sensitive variant of BamHI exists, but it is only active in the 30-60°C range (which is inside of the biologically relevant range) and also suffers from a short target sequence (GGATCC)[31]. Other thermosensitive type II restriction enzymes could be developed for such a purpose. However, this strategy would be quite prone to escape by single base-pair mutations in the target, as type II restriction enzymes are not tolerant to deviations in their target sequence[32]. The development of an ideal thermosensitive nuclease kill-switch system necessitates looking beyond nucleases currently employed as kill-switches.

1.3 LAGLIDADG Homing Endonucleases

LAGLIDADG homing endonucleases (LHEs) are a family of selfish genetic elements found as free-standing genes or encoded in introns as group I introns and inteins[33,34]. LHEs are primarily found in Archaea or the organellular genomes of fungal and algal species[35]. They function as either homodimers or two-domain monomers, and their name is derived from the conserved residues found between the core alpha helices[33,34,36,37]. The side chains of these residues interact with the opposing alpha-helix to form the domain interface[33,34,36].

They make an attractive candidate for the development of thermosensitivity-based kill-switches due to their inherent properties. Their long 22 bp recognition sequences make them precise despite retaining activity in the face of some variation in the target sequence[33,35,38–41]. This family of homing endonucleases is also resistant to point mutations within their coding sequence[42]. Additionally, these proteins display strong binding affinities[33]. LAGLIDADG homing endonuclease gene variants exist that are approximately 1 kb in length or less, such as I-OnuI, I-LtrI, and I-HjeMI[43,44]. These variants are derived from the ascomycetes *Ophiostoma novo-ulmi*, *Leptographium truncatum*, and *Hypocrea*

jecorina[II], respectively[43,44]. A selfish genetic element of fungal origin would be under a stabilizing selection to ensure activity around the organism's growth temperature. Given that all three of these LAGLIDADG homing endonucleases originate from Ascomyota involved in plant rot-diseases, they are likely active at temperatures of 30°C or lower[43,45].

While these variants are ideal candidates for use as kill-switches, they are active at 37°C (the preferred temperature of *E. coli* and other species in human gastrointestinal microbiomes), as well[38,44,46]. Therefore, some development is required before they can be feasibly used as thermosensitive kill-switches in a laboratory or medical setting.

1.3.1 Example Application

An instance where a thermosensitive kill-switch would be an essential component for containment is the conjugative *cas9* plasmid developed by Hamilton *et al.* (2019)[25]. The system Hamilton *et al.* (2019) are developing is a promising prospective approach to dealing with bacterial gastrointestinal infections because it spreads exponentially between bacteria in liquid culture and can efficiently target the chromosomes of *Salmonella enterica* via Cas9 nuclease variants leading to cell death[25]. Due to the programmability of the guide RNAs directing the Cas9 protein's sequence-specific targeting, a conjugative *cas9* system will also work well for targeting other species[20,47].

However, the same aspects that make this system an attractive prospect for use as an antibiotic (exponential transfer and efficient bactericidal activity) cause concern from a biocontainment perspective. Escape of a conjugative plasmid carrying a *cas9* construct from a patient's body could have wide-ranging effects from the target bacterial species being removed from other people's microbiome, at least, to a population bottleneck in the target bacterial species, at worst. Whether the large scale spread of a conjugative plasmid with *cas9* targeting an infectious bacterial species is desirable or not is best debated

[II] It would be more correct to refer to the source organism as *Trichoderma reesei*, as *H. jecorina* is an anamorph of *T. reesei*[68]. Note, this means I-HjeMI should be renamed to I-TreI to follow homing endonuclease naming conventions[69]. However, for the purpose of this thesis, I-HjeMI will be referred to as such, to avoid confusion when referring to the pertaining literature.

elsewhere[III]. However, it does showcase a great example of where a thermosensitive nuclease-based kill-switch would be necessary to prevent escape from a narrow application-specific perspective. Containing Hamilton *et al.*'s (2019) system to the human gut (37°C) would alleviate the ecological and ethical concerns that an application of such a system on an individual would raise, thus bringing their system closer to an application in the medical market where novel approaches to treating bacterial infections are in strong demand[25,48–50].

[III] Such a debate will likely be akin to the on-going discussions concerning the potential applications of gene drives to eradicate or control infectious disease spreading insect and rodent species[70].

Chapter 2

2 Materials and Methods

2.1 Media

2.1.1 2xYT Media

To make 2xYT media, 16 g of carbohydrate-free tryptone, 10 g of yeast extract, and 5 g of NaCl were added to 400 mL of ddH_2O in a 1 L graduated cylinder and stirred until dissolved. The volume was adjusted to 1 L with dd H_2O, and 15 g of agar A were added for solid media. The media was autoclaved at 120°C for 20 minutes.

2.1.2 Modified M9 Media

2.1.2.1 10x M9 Salt Solution

To make 10x M9 salt solution media, 70 g of $Na_2HPO_4 \cdot 7H_2O$, 30 g of KH_2PO_4, 10 g of NH_4Cl, and 5 g of NaCl were added to 400 mL of ddH_2O in a 1 L graduated cylinder and stirred until dissolved. The volume was adjusted to 1 L with dd H_2O. The media was autoclaved at 120°C for 20 minutes.

2.1.2.2 Selective M9 Solid Media

To make selective M9 solid media, 8 g of carbohydrate-free tryptone was added to 280 mL of ddH_2O in a 1 L graduate cylinder and stirred until dissolved. Then, 100 mL of 10x M9 salt solution and 20 mL of 50% glycerol were added to the graduated cylinder. The volume was adjusted to 0.996 L with ddH_2O, and 15 g of agar A were added. A magnetic stir bar was added to the media before autoclaving. The media was then autoclaved at 120°C for 20 minutes.

After autoclaving, 1.0 mL of sterile 1.0M $CaCl_2$, 1.0 mL of sterile 1.0M $MgSO_4$, 1.0 mL of 100 mg/mL carbenicillin suspended in 50% EtOH, 1.0 mL of sterile 20% arabinose, 400 µL of sterile 1.0% Thiamine, and 400 µL of sterile 1.0M IPTG were slowly dripped in media cooled to 65°C and immediately poured into plates.

2.1.2.3 Non-selective M9 Solid Media

To make non-selective M9 solid media, 8 g of carbohydrate-free tryptone was added to 280 mL of ddH_2O in a 1 L graduate cylinder and stirred until dissolved. Then, 100 mL of 10x M9 salt solution and 20 mL of 50% glycerol were added to the graduated cylinder. The volume was adjusted to 0.996 L with ddH_2O, and 15 g of agar A were added. A magnetic stir bar was added to the media and was autoclaved at 120°C for 20 minutes.

After autoclaving, 1.0 mL of sterile 1.0M $CaCl_2$, 1.0 mL of sterile 1.0M $MgSO_4$, 1.0 mL of 100 mg/mL carbenicillin suspended in 50% EtOH, 1.0 mL of sterile 20% glucose, and 400 µL of sterile 1.0% thiamine were slowly dripped in media cooled to 65°C and immediately poured into plates.

2.2 Gibson Assembly

2.2.1 5x Reaction Buffer

To make 5x reaction buffer, 0.2 g of PEG 8000, 0.5 mL of 1M Tris (pH 7.5), and 25 µL of 2 M $MgCl_2$ were rotated in a 1.5 mL Eppendorf tube for 2 hours to dissolve. Then 50 µL of 1 M DTT, 100 µL of dNTP mix (100 mM dGTP, 100 mM dATP, 100 mM dTTP, 100 mM dCTP), and 100 µL of 50 mM NAD were added. The volume was adjusted to 1 mL with ddH_2O. The 5x reaction buffer was stored at -20°C.

2.2.2 1.33x Reaction Mixture

To make 1.33x reaction mixture, 26.7 µL of 5x reaction buffer, 0.4 µL of T5 exonuclease (1.48 mg/mL), 4.8 µL Taq ligase (0.28 mg/mL), 0.7 µL of Pfu polymerase (2.5 U/µL), and 67.3 µL of ddH_2O were added to a 0.5 mL Eppendorf tube and mixed by pipetting The 1.33x reaction mixture was stored at -20°C.

2.2.3 Gibson Assembly Protocol

To begin the Gibson assembly, 2 µL of insert and 3 µL of vector were placed into a 0.2 mL PCR tube. The insert and vector must have approximately 40 base pairs of homology at their ends. Then, 15 µL of chilled 1.33x reaction mixture was added to the 0.2 mL PCR tube and mixed via pipetting. The 0.2 mL PCR tube was immediately transferred to a

thermocycler set to 50°C and incubated for 1 hour. Finally, 10 µL of the Gibson assembly reaction was transformed into 200 µL of $CaCl_2$ competent *E. coli* cells.

2.3 Libraries

2.3.1 LAGLIDADG Point Mutation Libraries

Mutagenized libraries of the LAGLIDAG homing endonucleases I-OnuI, I-LtrI and I-HjeMI, were created using the GeneMorph II EZClone Domain Mutagenesis Kitgene[51]. Directions were followed to obtain a high mutation frequency (9-16 mutations/kb). Mutagenized products were digested using NcoI and NotI, and ligated into NcoI/NotI digested pEndo using 3:1, 5:1, and 7:1 vector to insert ratios following the NEB: Protocol with T4 DNA Ligase (NEB catalogue #M0202)[52]. These were transformed into NEB5α cells, and 100 µL of the recovery mixtures were plated. Theoretical complexity was calculated by subtracting the number of colonies on no insert control plate from that on the ratio plates, and then multiplying that number by the amount of 100 µL aliquots remaining in the recovery mixture, and added to the sum of each library generated for that variant.

2.3.2 I-LtrI FLL_E Library

This I-LtrI library is randomized at positions 27, 58, and 66 and consists of 8000 possible combinations, and was identified as a coding network[46]. The library was ordered as a gene block from IDT and cloned into pEndo by Dr. McMurrough (unpublished data). F27 is in the N-terminal helix, I58 is in the second helix, and W66 is located at the edge of the N-terminal beta-sheet. All three positions are facing a hydrophobic pocket of the domain's core and are predicted to be involved in intra-domain stability, making this library a potential source of thermosensitive mutants. The FLL_E library has a complexity of 8,000 variants

2.3.3 I-LtrI H1-IR Library

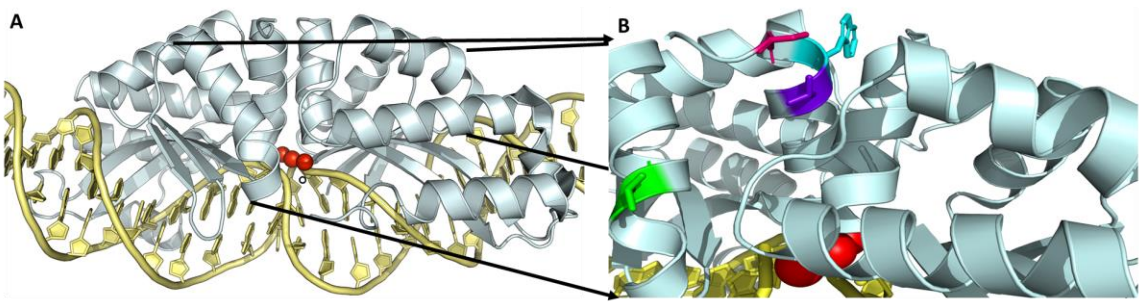


Figure 2. Structure of I-LtrI (silver, PDB 3R7P) to DNA (yellow) in the presence of divalent cation cofactors (red spheres). A) Plain view of I-LtrI's structure. B) I-LtrI's structure rotated 90° clockwise and zoomed in for emphasis to show the residues that were randomized in the H1-IR library. The following residues have their side chains displayed and are highlighted for contrast: S20 is coloured pink, W22 is coloured light blue, T23 is coloured purple, and T64 is coloured lime green.

This I-LtrI library is randomized to all 20 amino acids at positions 20, 22, 23 and 64, and was likewise ordered as a gene block from IDT and cloned into pEndo by Dr. McMurrough (unpublished data). Positions 20, 22, and 23 are located within the N-terminal alpha-helix of the protein and are near the interface between the two domains of I-LtrI. Position 64 is located in the second alpha-helix and is a conserved residue according to an alignment done by Baxter *et al.* (2012)[39]. These positions should play a role in inter-domain stability and intra-domain stability, similarly making this library a potential source of thermosensitive mutants. This library contains 160,000 variants.

2.3.4 H1-IR-RND and FLL_E-RND Combination Library

The point mutation library of I-LtrI library was combined by co-culturing with the I-LtrI H1-IR and FLL_E libraries to create a combined library that was passaged through the dual plasmid-selection screens.

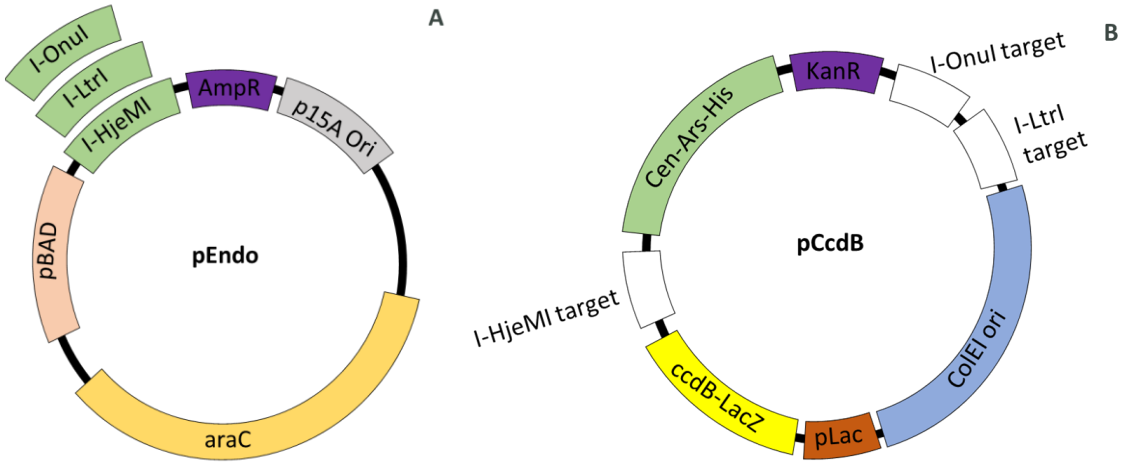


Figure 3. Maps of the plasmids used in the two-plasmid screen. A) pEndo (5.0 kb) construct containing required components for arabinose inducible LAGLIDADG homing endonuclease expression. This plasmid also contains the AmpR selectable marker for ampicillin resistance. B) pCcdB (4.5 kb) construct with the necessary components to act as an IPTG inducible CcdB based negative selection for the two-plasmid screen. This plasmid contains the KanR selectable marker for kanamycin resistance, as well as the LAGLIDADG homing endonuclease cut sites. Finally this plasmid also contains a CEN-ARS-His element from propagation in yeast. Diagrams are not to scale

2.4 Two-plasmid Screen

Thermosensitive variants are identified using an *E.coli* two-plasmid selection system where one plasmid (pEndo) expresses the LHE libraries (Fig1A). The pCcdB plasmid contains *ccdB* that binds to DNA gyrase to inhibit DNA replication[53]. This selection is, therefore, bacteriostatic. pCcdB also includes the sequence of the recognition sites for the endonuclease expressed from pEndo. Cells survive the selection if the endonuclease cleaves its target on pCcdB, linearizing the plasmid and promoting its degradation. This two-plasmid screen is an adaption of the version previously used by McMurrough *et al.* (2014); for the original version of this screen, see Chen & Zhao (2005)[46,54].

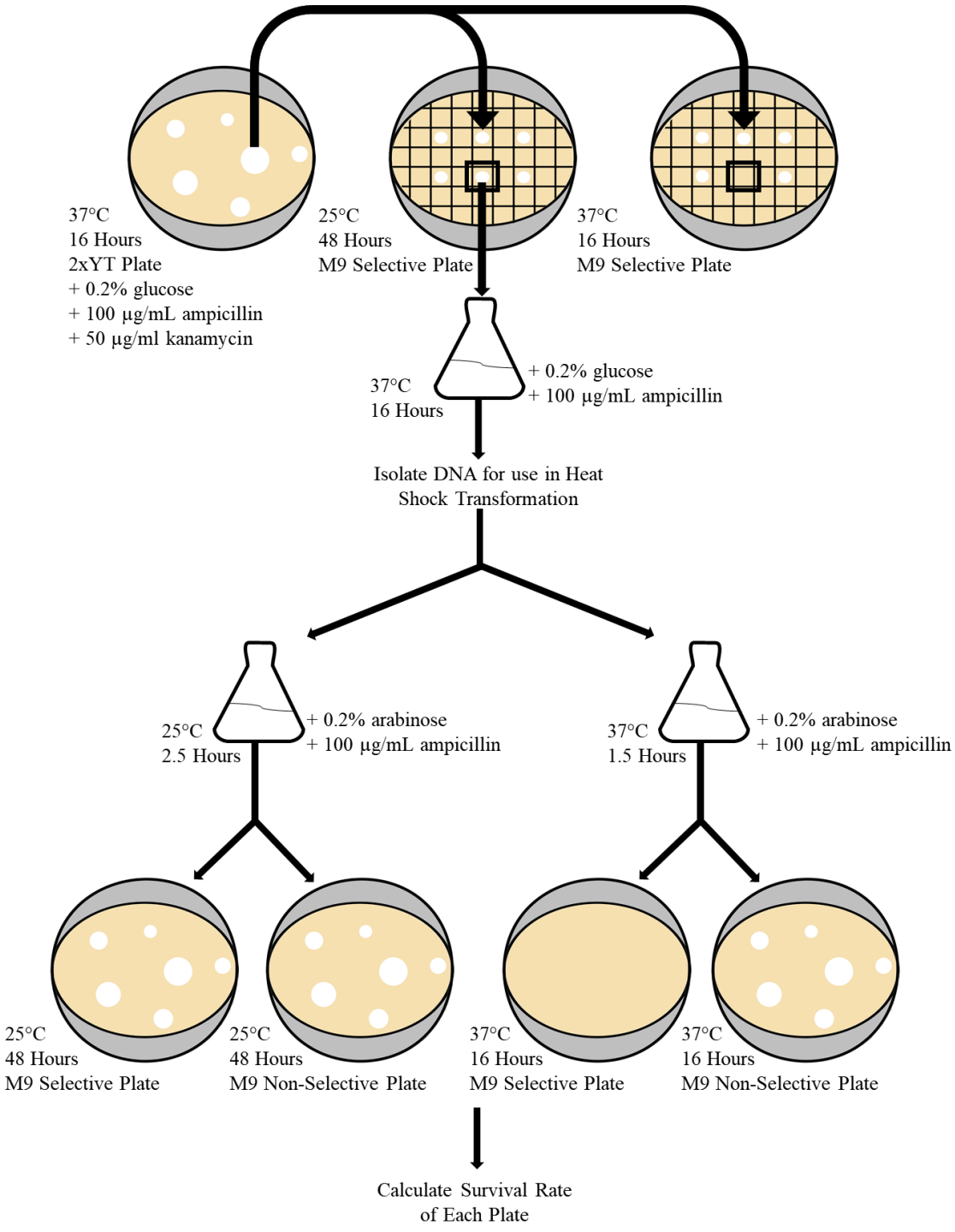


Figure 4. Workflow of the two-plasmid screen used to identify thermosensitive LHE variants. M9 selective plates contain 100 µg/ml carbenicillin, 0.2% arabinose, and 0.4 mM IPTG. M9 non-selective plates contain 100 µg/ml carbenicillin, and 0.2% glucose.

2.4.1 Initial Dual Plasmid Screen

50 µL of calcium chloride competent Nova XGF' *E. coli* cells harbouring pCcdB with appropriate target sites were transformed with 50 ng of the library by a standard 45 second 42°C heat shock protocol. The mixture was recovered for 1 hour at 37°C in 1 mL of 2xYT, and 100 µL of the mixture was plated on 2xYT plates containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and incubated overnight at 37°C. The wild-type positive control was plated on a 2xYT plate containing only 100 µg/ml ampicillin to obtain colonies (the positive control won't grow on double selection). *E. coli* transformants were screened by picking colonies from the 2xYT plate onto two selective M9 minimal media plates followed by incubation of one plate at 37°C and the second plate at 25°C. A fresh pipette tip was used to pick each colony. Putative thermosensitive endonuclease variants will promote *E. coli* growth at 25°C, but not at 37°C.

2.4.2 Confirmatory Dual Plasmid Screen

Putative clones were picked, grown overnight at 37°C in 2xYT media with ampicillin (100 µg/ml), as well as 0.2% glucose, and were re-screened to confirm temperature dependence. Plasmid DNA was isolated, and 50 ng was transformed into 50 µL of calcium chloride competent Nova XGF' pCcdB *E. coli* cells. Following a 1-hour recovery at 37°C, the culture volume was split in half. In one culture the endonuclease on pEndo was induced using 0.2% arabinose while under ampicillin selection (100 µg/ml) at 25°C for 2.5 hours, and 37°C for 1.5 hours, respectively. The other culture contained 0.2% glucose to repress expression. After induction, 100 µL of each mixture was plated on selective M9 plates, and a non-selective plate. The two plates corresponding to the 37°C culture incubated at 37°C for 16 hours, and the two plates corresponding to the 25°C culture incubated at 25°C for 48 hours.

2.4.3 Survival Rate Calculation

The survival rate was calculated for each temperature taking the ratio of the number of colony-forming units on the selective plate by the number of colony-forming units on the non-selective plate, multiplied by a hundred to obtain a percentage of survival for that temperature.

2.5 Repair of the pCcdB Origin

The pUC origin of replication from pMSP3535 (Addgene catalogue #46886) was amplified using primers that gave the insert 40 bp of overlap to that of pCcdB to create an origin that was reported to be capable of maintaining the same copy number at 25°C and 37°C[55]. The ColE1 origin of pCcdB was removed using an AattII (NEB catalogue #R0117S) and SacII (NEB catalogue #R0157S) double digest in NEB Cutsmart buffer for 1 hour at 37°C. These two fragments were then used for Gibson Assembly, as demonstrated in section 2.2.

2.6 Protein Purification

I-OnuI wild-type and I-OnuI G177A were previously cloned into pPROEX HTa (Invitrogen and Life Technologies) via its unique NcoI and NotI sites by Dr. McMurrough[38]. I-OnuI P14Q was cloned into pPROEX HTa using the same method. The three 6x-histidine-tagged I-OnuI variants were transformed individually into ER2566 cells (New England Biolabs). A 25 mL starter culture of each were grown overnight at 37°C to saturation in LB containing ampicillin (100 µg/ml) and 0.2% glucose. These starter cultures were then used to each inoculate 975 mLs of LB containing ampicillin (100 µg/ml) and grown to an OD_{600} of 0.6 at 37°C. Once an OD_{600} of 0.6 was reached, 1 mL of 1M IPTG (final concentration 1mM IPTG) was added to each of the cultures, followed by 16 hours of incubation at 16°C. The cells were then harvested via centrifugation at 6,000xg, 4°C for 15 minutes. The resultant pellets were resuspended (40 mL/g) in binding buffer (50 mM Tris·HCl, pH 8.0, 500 mM NaCl, 1 mM imidazole, and 10% glycerol), as well as supplemented with EDTA-free Protease Inhibitor Cocktail (Roche, 1 tablet per 50mL) and PMSF Protease Inhibitor (Thermo Scientific, final concentration: 0.4 mM.) The resuspended cell pellets were then lysed using an EmulsiFlex-C3 high-pressure homogenizer. The lysed cell solutions were then ultra-centrifuged at 29,000xg, 4°C for 30

minutes. The supernatants were loaded onto Ni^{2+}-NTA resin. The resin was washed with wash buffer (50 mM Tris·HCl, pH 8.0, 500 mM NaCl, 35 mM imidazole, and 10% glycerol), and the bound protein was eluted in 6x1 mL fractions using elution buffer (50 mM Tris·HCl, pH 8.0, 500 mM NaCl, 500 mM imidazole, and 10% glycerol.) The 6 fractions for each I-OnuI variant were pooled, dialyzed in dialysis buffer (50 mM Tris·HCl, pH 8.0, 250 mM NaCl, 30 mM imidazole, and 10% glycerol), and the N-terminal 6xhistidine tags were concomitantly cleaved using TEV protease (1:25 TEV to I-OnuI molar ratio.) This was done first for 1 hour at 25°C, followed by 16 hours at 4°C. The cleaved I-OnuI products in dialysis solution were then dialyzed into binding buffer (50 mM Tris·HCl, pH 8.0, 500 mM NaCl, 1 mM imidazole, and 10% glycerol) for 4 hours at 4°C. The resultant cleaved I-OnuI products with TEV were then loaded onto Ni^{2+}-NTA resin, and their flow-through's were collected and dialyzed into storage buffer (50 mM Tris·HCl, pH 8.0, 25 mM NaCl, 1 mM DTT, and 10% glycerol) for 16 hours at 4°C. Following dialysis into storage buffer, size-exclusion chromatography using an AKTA Superdex 200 Increase 10/300 GL column (GE product ID 28-9909-44) was performed at a flow rate of 0.2 mL/minute, and 1 mL fractions were collected. These final fractions were then stored at -80°C.

2.6.1 Second Purification of I-OnuI P14Q

I-OnuI P14Q was purified a second time as above with some minor changes. The wash solution used contained 50 mM Tris·HCl, pH 8.0, 2 M NaCl, 35 mM imidazole, and 10% glycerol. Also, 6 x 2 mL fractions were collected. Finally, size exclusion was not performed following Ni^{2+}-NTA immobilized metal affinity chromatography (IMAC).

2.7 *In Vitro* Cleavage Assay

I-OnuI WT, G177A, and P14Q variants were diluted to 4 µM (10x) working concentrations using storage buffer. A 4x reaction mixture (200 mM Tris·HCL (pH 8.0), 400 mM NaCl, 40 mM $MgCl_2$, 4 mM DTT) was mixed with the appropriate volume of pCcdB and sterile double deionized H_2O to bring the reaction mixture to 1.11x and the concentration of pCcdB to 11.1 nM. Aliquots of the reaction mixture with pCcdB and the 10x protein samples were then incubated separately at 25°C and 37°C for 5 minutes. This assay was performed in parallel at 25°C and 37°C at final concentrations of 10 nM of pCcdB, and

400 nM of either I-OnuI WT (fraction 20), I-OnuI G177A (fraction 48), and I-OnuI P14Q (fraction 72) for the first 3 replicates. The 4th replicate was performed the same way except that the final fraction from the second purification of I-OnuI P14Q was used. The final composition of the reaction mixture was 50 mM Tris·HCL (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. The assay was stopped at 5 time points using a 2x stop solution (200 mM EDTA, 30% glycerol, 0.2% SDS, and bromophenol blue) and incubated at 50°C for 5 minutes.

Reactions performed at 25°C were stopped at 0 minutes, 15 minutes, 30 minutes, 60 minutes, and 120 minutes. Reactions performed at 37°C were stopped at 0 minutes, 5 minutes, 15 minutes, 30 minutes, and 60 minutes. The percent coiled, open circle, and linear products were measured based on the corresponding band intensity of a 1% agarose as determined using the BioRad Image Lab Software v6.0.1.

2.8 Golden Mutagenesis

The three I-OnuI mutants (GGA2, CCG14CAG), (GGA2GGC, CAG14), and (GGC2, CAG14CCG) were created sequentially from WT I-OnuI (GGA2, CCG14) via golden mutagenesis. This was done using PCR to insert the mutation and a type IIS restriction enzyme site into the region where the desired mutation is to be created in a circular vector. (The primers used can be found in Appendix A.) Following PCR, 300 ng of the resultant linear DNA piece is added to a solution containing 2 µL of T4 DNA ligase buffer (NEB catalogue #M0202), 1 µL of T4 DNA ligase (NEB catalogue #M0202), 2 µL of 1 µg/µL BSA, 1 µL of BsmBI (NEB catalogue #R0580) in a fresh PCR tube on ice. This mixture is then filled to a total volume of 15 µL using sddH_2O and mixed via gentle pipetting. The PCR tube containing all the above components was transferred to a thermocycler. The thermocycler protocol used was 37°C for 2 minutes, followed by 20°C for 5 minutes. These two steps were repeated 40 times and finished with a heat deactivation at 80°C for 20 minutes. Finally, 2.2 µL of the golden mutagenesis reaction was transformed into 50 µL of $CaCl_2$ competent cells via a standard heat shock transformation protocol. This is a modified protocol of the one described by Püllmann *et al*[56].

2.9 Data Analysis

All graphs and charts presented were generated in R studio. Means and sample standard deviations (represented by the error bars) shown on the *in vitro* cleavage charts were calculated in Excel. R studio's base functions were used for performing one-way ANOVA and Tukey post hoc tests, and the data used for these tests was the raw data (not the mean or standard deviations calculated in Excel). In all cases, the α value chosen was 0.05.

2.10 Protein Structure Models

The protein structure models presented in this study were generated using PyMol to render the individual Protein Data Bank (PDB) files specified in each figure legend.

Chapter 3

3 Results

3.1 Development of a Two-Plasmid Screen that Works at 25°C and 37°C

3.1.1 Identification of a Problem with pCcdB

To identify thermosensitive LAGLIDADG homing endonucleases, a two-plasmid based survival assay based on the bacteriostatic properties of CcdB was selected based on previous applications to LAGLIDADG homing endonucleases[38,46]. To test the functionality of this assay at 25°C and 37°C pEndo I-Ltr and pCcdB in control experiments anticipating that I-LtrI WT should function at both 25°C and 37°C. As shown in Figure 5, I-Ltr WT survived at both temperatures. However, the negative controls (I-LtrI G185Aand the empty vector) could survive on selective media at 25°C but not at 37°C (Fig. 5). This meant that thermosensitive variants could not be distinguished from background growth. Sequencing of the CcdB regions of pCcdB revealed a G→A transition at position 229, resulting in a glycine to glutamate substitution mutation. Isolation of a clone of pCcdB without the G229A mutation did not resolve the issue of I-LtrI G185A and the empty vector surviving at 25°C (Fig. 6).

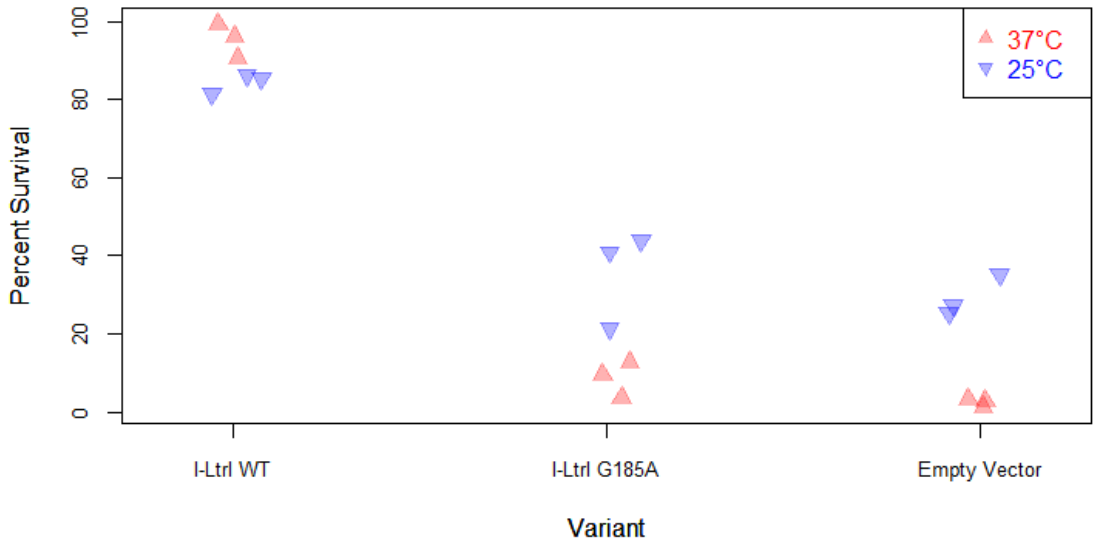


Figure 5. Two-plasmid selection using pCcdB (G299A) illustrates a problem with the screen at 25°C. Two-plasmid selection was performed using Nova XGF' *E. coli* cells carrying pEndo I-LtrI WT, G185A, or an empty pEndo backbone at 37°C (red triangles) and 25°C (blue inverted triangles). Each point represents an independent experiment.

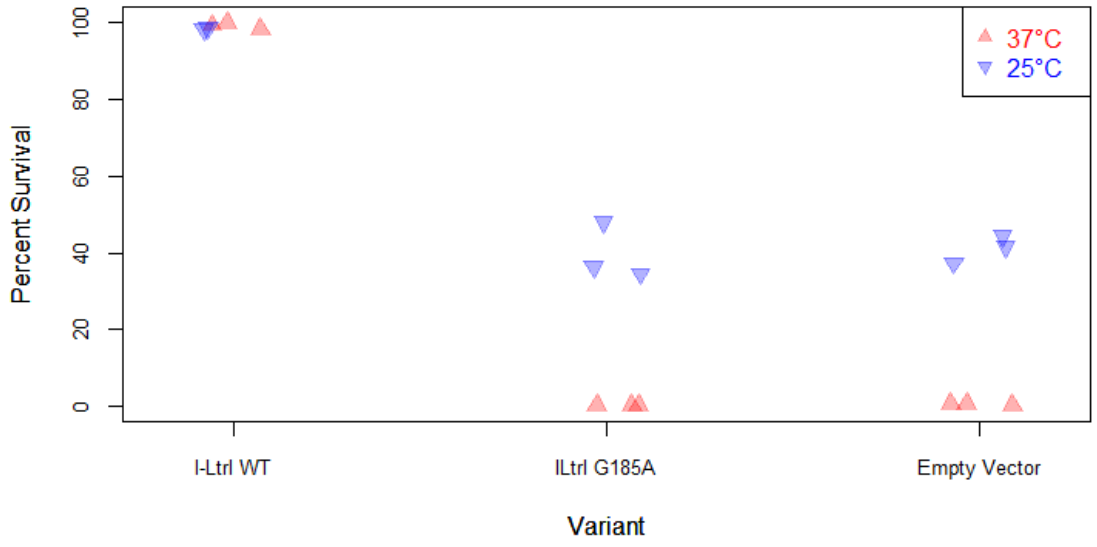


Figure 6. Two-plasmid selection using pCcdB (G229A) continues to display a problem with the screen at 25°C. Two-plasmid selection was performed using pCcdB Nova XGF' *E. coli* cells carrying pEndo I-LtrI WT, G185A, or an empty pEndo backbone at 37°C (red triangles) and 25°C (blue inverted triangles). Each point in a column represents an independent experiment.

3.1.2 Insertion in the RNAII region of the ColE1 promoter within pCcdB

Following the isolation of a version of pCcdB without point mutation in the *ccdB* gene, sequencing revealed a 24 base pair insertion into the RNAII region of the ColE1 origin of pCcdB. Removing the origin containing the 24 base pair insertion and replacing it with a pUC origin resolved the problem of the negative control having a non-zero survival rate at 25°C. Replacing the origin resulted in approximately 100% survival of the wild-type and approximately 0% survival of the slow (I-LtrI G185A) enzymes at both temperatures (Fig. 7). The replacement origin was intended to be a pBR322ΔROM origin, which has been noted to be stable by Kobayashi *et al.*, the key -444 base pair was a thymidine, not cytosine, resulting in a pUC origin[55]. The unintended base-pair difference resulted from an error in the reported sequence for pMSP3535 used to create the replacement origin of replication and not a mutation during Gibson Assembly.

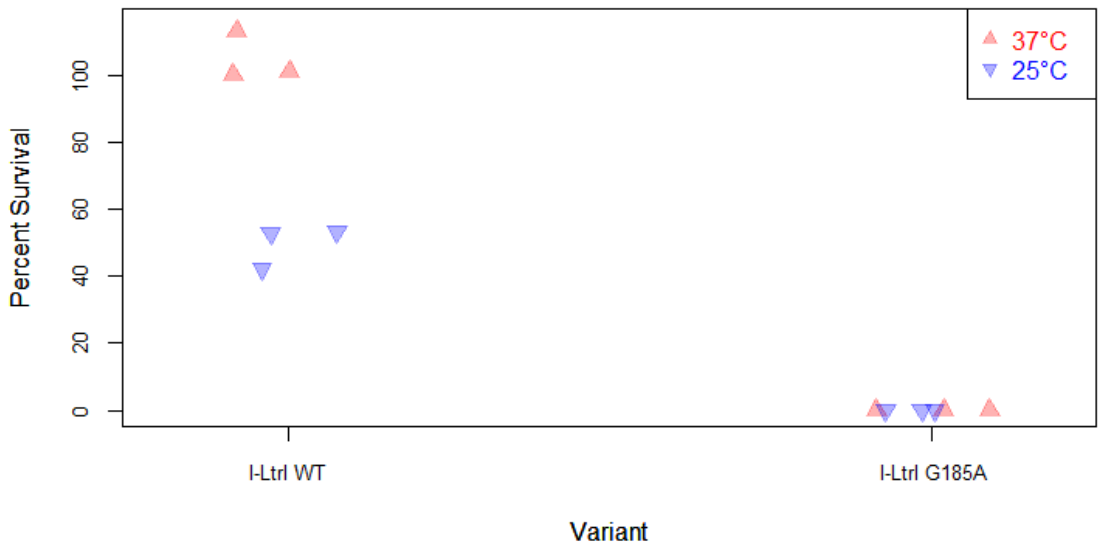


Figure 7. Two-plasmid selection using pCcdB with a pUC origin sequence devoid of an insertion. Two-plasmid selection was performed using pCcdB Nova XGF' *E. coli* cells carrying pEndo I-LtrI WT or G185A at 37°C (red triangles) and 25°C (blue inverted triangles). Each point in a column represents an independent experiment.

3.2 Creating and Screening LHE Mutagenic Libraries

3.2.1 LHE Mutagenic Library Complexity and Features

Libraries were created using mutagenic PCR to introduce random mutations in the I-OnuI, I-LtrI, and I-HjeMI coding sequences. The resultant libraries had theoretical complexities in the range of approximately 21,000-53,000 variants (Table 1). Four clones sequenced from each library displayed relatively balanced rates of transitions to transversions, except for the I-OnuI clones that have approximately twice as many transitions (Table 2). Most mutations result in non-synonymous mutations and low rates of non-sense mutations (Table 2). The I-LtrI library was combined with the I-LtrI FLL_E library and I-LtrI H1-IR library to create a library with a theoretical complexity of approximately 189,000.

Table 1. Library theoretical complexity based on CFUs after transformation of ligation mixtures. Library complexity was calculated as described in section 2.3.1.

Group	Complexity
l-OnuI randomized	21,088
I-LtrI randomized	52,759
l-HjeMI randomized	26,448

Table 2. Sanger sequencing of 4 clones from each library.

Group	Transversion:Transition	Synonymous	Non-synonymous	Non-sense
l-OnuI randomized	0.55:1	23.5%	76.5%	0%
I-LtrI randomized	1.09:1	12%	84%	4%
l-HjeMI randomized	1.08:1	19.2%	69.3%	11.5%

3.2.2 Identification of a Thermosensitive I-OnuI Variant

A total of 2,600 colonies were passaged through the initial screen; however, this only resulted in 2 potential hits from the I-OnuI library to later be passaged through the confirmatory screen (Table 3). These resulted in one low quality hit, and one high quality hit that warranted sub-cloning and further investigation (Table 4). Despite considerable effort, the I-LtrI and I-HjeMI libraries did not yield any potential hits. To screen for thermosensitive variants, I-OnuI, I-LtrI, and I-HjeMI libraries were independently transformed into *E. coli* harbouring pCcdB (fixed origin) and colonies were screened at 25°C and 37°C (Table 3). This initial screen of 608 independent I-OnuI library transformants resulted in 12 potential hits. Screening of 1308 and 684 independent colonies from the I-LtrI and I-HjeMI libraries resulted in no potential hits.

The 12 preliminary I-OnuI hits were screened again by isolating pEndo from each clone and transforming into naive *E. coli* pCcdB (fixed origin). This secondary screen revealed that 2 of 12 clones showed thermosensitive growth (Table 4). Sequencing of these clones revealed one with a P14Q substitution and the other with S42Y, F116S, and K150T substitutions (Table 4). The I-OnuI P14Q displayed better temperature dependence, with 0% survival at 37°C and 100% survival at 25°C, and was thereby selected for further study.

Table 2. Colonies screened in the initial and confirmatory screens.

Group	Colonies Screened	Potential Hits	Confirmed hits
I-OnuI randomized	608	12	2
I-LtrI combined library	1308	0	0
I-HjeMI randomized	684	0	0

Table 3. Survival rates of two I-OnuI hits in a confirmatory screen. This table is displaying data from 3 independent replicates. Note: Higher than 100% survival rates result from fewer colonies surviving on the non-selective M9 plates. This effect is likely partially due to leaky CcdB expression or the extra metabolic stress of carrying two plasmids. Also, the LHE would be repressed on these plates, and therefore less able to remove them compared to the selective condition.

Group	Survival %	
	25°C	37°C
Wild-type I-OnuI	108.7	82.3
I-OnuI P14Q	101.8	0
I-OnuI S42Y, F116S, K150T	29.2	0
Empty Vector	0	0

3.2.2.1 I-OnuI P14Q Appears to be a Thermosensitive Ideal Candidate *In Vivo* Based on the Two-Plasmid Screen.

I-OnuI P14Q proved to be an exciting prospect, displaying ideal properties (Fig. 8). This I-OnuI variant displays 0% survival at 37°C and approximately 100% survival at 25°C. I-OnuI P14Q behaved the same as the wild-type I-OnuI enzyme at 25°C ($p=0.325$, Fig. 8B), and the same as G177A slow mutant or empty vector at 37°C ($p=0.999$, Fig 8A). As a result, I-OnuI P14Q was prepared for further investigation *in vitro*.

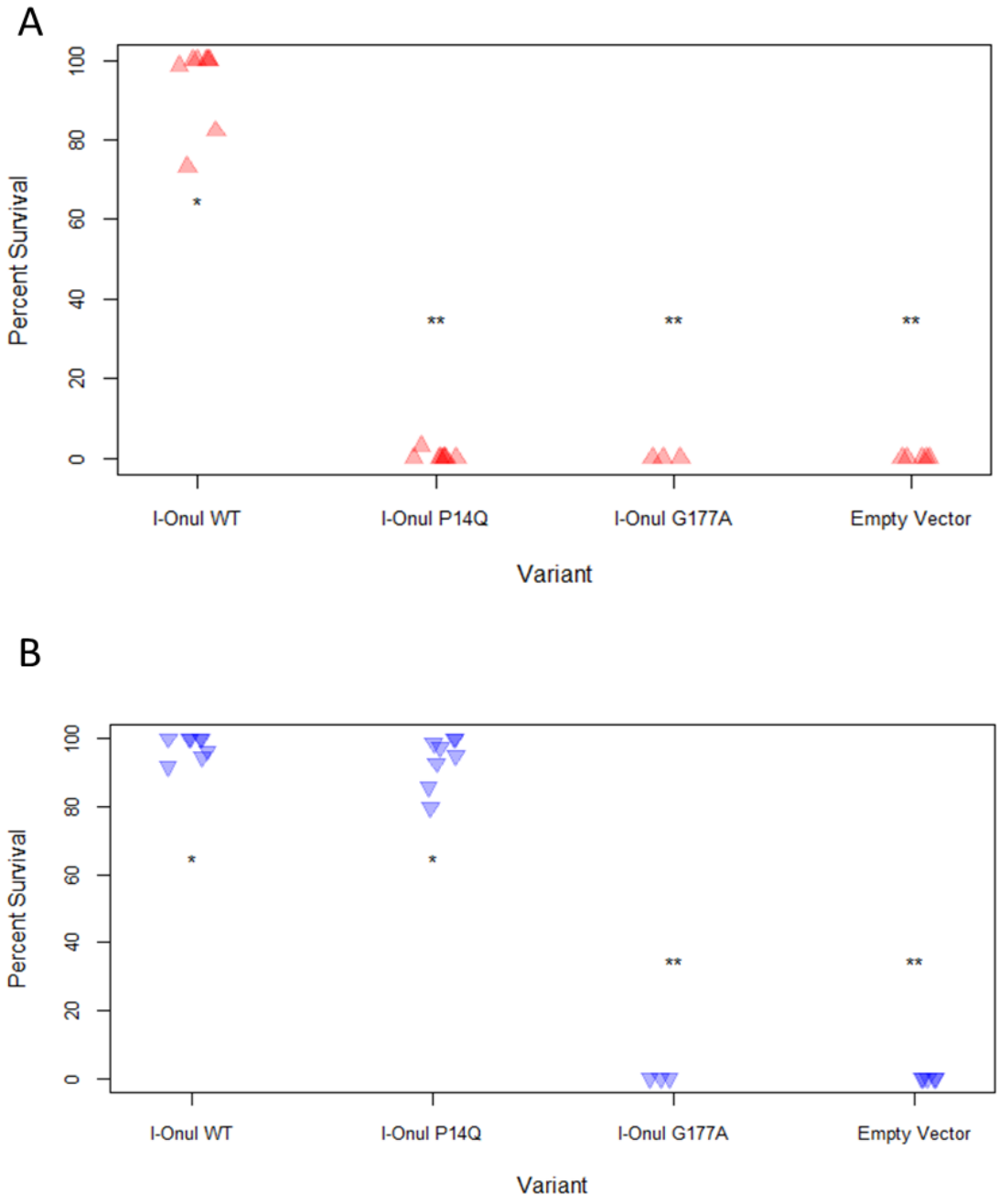


Figure 8. Two-plasmid selection using pCcdB with the corrected origin sequence identifies I-OnuI P14Q as a hit. Two-plasmid selection was performed using pEndo Nova XGF' *E. coli* cells carrying pEndo I-OnuI WT (N=8), P14Q (N=8), G177A (N=3), and the empty vector (N=5) at 37°C (panel A, red triangles). and 25°C (panel B, blue inverted triangles.) Significant differences ($p<0.05$, Tukey's post hoc test) between I-OnuI variant's percent survival are indicated by (*, **). Each point in a column represents an independent experiment

3.3 Purification of I-OnuI WT, I-OnuI G177A, and I-OnuI P14Q.

The I-OnuI P14Q mutation is located at the top of the first α-helix that forms the enzyme's structured core (Fig 9). Therefore, it was anticipated that the P14Q mutation would result in temperature-sensitive activity *in vitro* due to misfolding. The P14Q variant was sub-cloned from pEndo into a bacterial expression vector to test this supposition. The protein was purified using metal affinity and size-exclusion chromatography. The I-OnuI WT and G185A variants were also purified for comparative cleavage assays

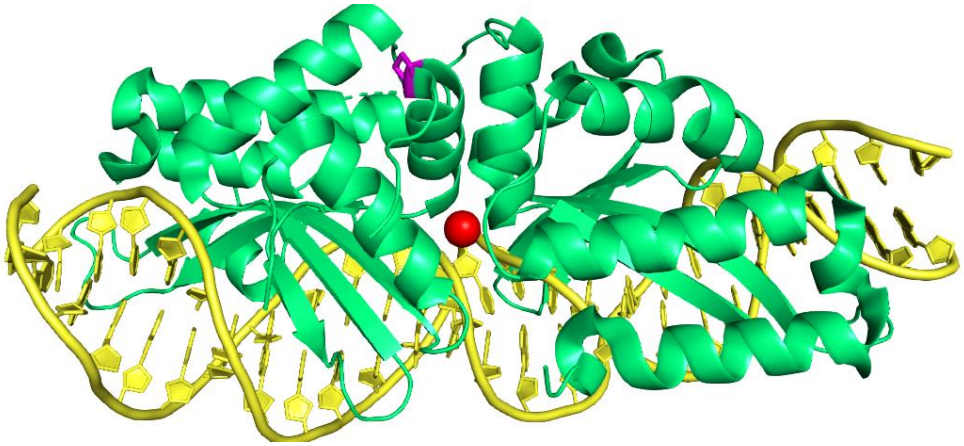


Figure 9. Structure of I-OnuI (green, PDB 3QQY) to DNA (yellow) in the presence of a divalent cation cofactor (red sphere). Residue P14's side chain is displayed and coloured magenta for emphasis.

3.3.1 Nickel-nitrilotriacetic Acid Immobilized Metal Affinity Chromatography based Purification of I-OnuI WT, I-OnuI G177A, and I-OnuI P14Q.

Following the identification of a putative thermosensitive I-OnuI variant, *in vitro* cleavage assays were performed. The coding sequence of the P14Q variant of I-OnuI was moved into a vector with a 6x his tag and purified. The 6x his tagged P14Q variant of I-OnuI was expected to be 38 kDa, and TEV was expected to be 27 kDa. The final product for each variant (35 kDa) was not considered pure enough for downstream work, and therefore another subsequent purification by size-exclusion chromatography was performed (Fig. 10).

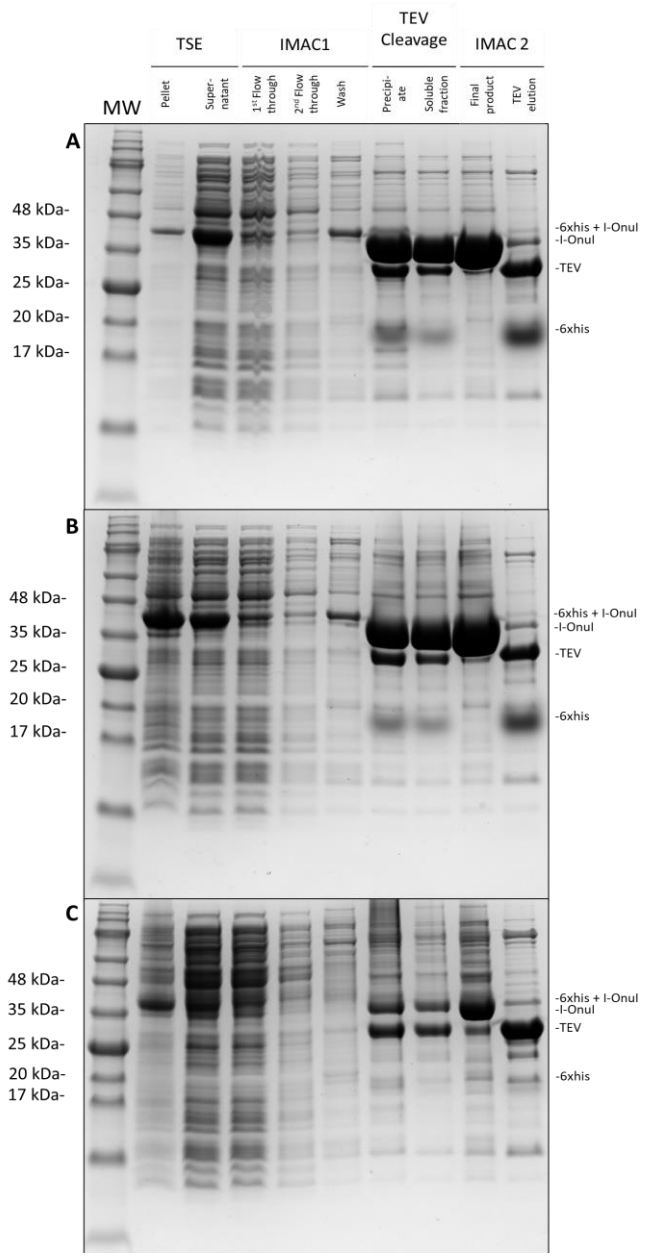


Figure 10. Nickel-nitrilotriacetic acid immobilized metal affinity chromatography-based protein purification of (A) I-OnuI wild-type, (B) I-OnuI G177A, and (C) I-OnuI P14Q. I-OnuI wild-type, I-OnuI G177A, and I-OnuI P14Q proteins were purified as per methods section 2.6 Protein Purification. Protein species were resolved on SDS-PAGE and visualized with Coomassie blue. A BLUelf pre-stained protein ladder was used (FroggaBio catalogue #PM808-0500G). The expected molecular weights of 6xhis + I-OnuI, I-OnuI, TEV, and 6xhis are 38 kDa, 35 kDa, 27 kDa and 3 kDa respectively. Abbreviations: TSE- total sample extract, IMAC- immobilized metal affinity chromatography, TEV- tobacco etch virus.

3.3.1.1 Size-Exclusion Chromatography based Purification of I-OnuI WT, I-OnuI G177A, and I-OnuI P14Q.

Size-exclusion fractions from wild-type I-OnuI protein purification elucidated which subsequent fractions of I-OnuI G177A and I-OnuI P14Q would be worth retaining (Fig. 11A). The fractions with the least contaminants based on banding patterns were retained for subsequent work (Fig. 11B). Fractions 18 and 19 were pooled, as were fractions 20 and 21 based on their similar appearance (Fig 11B). This was also done for fractions: 46 and 47.

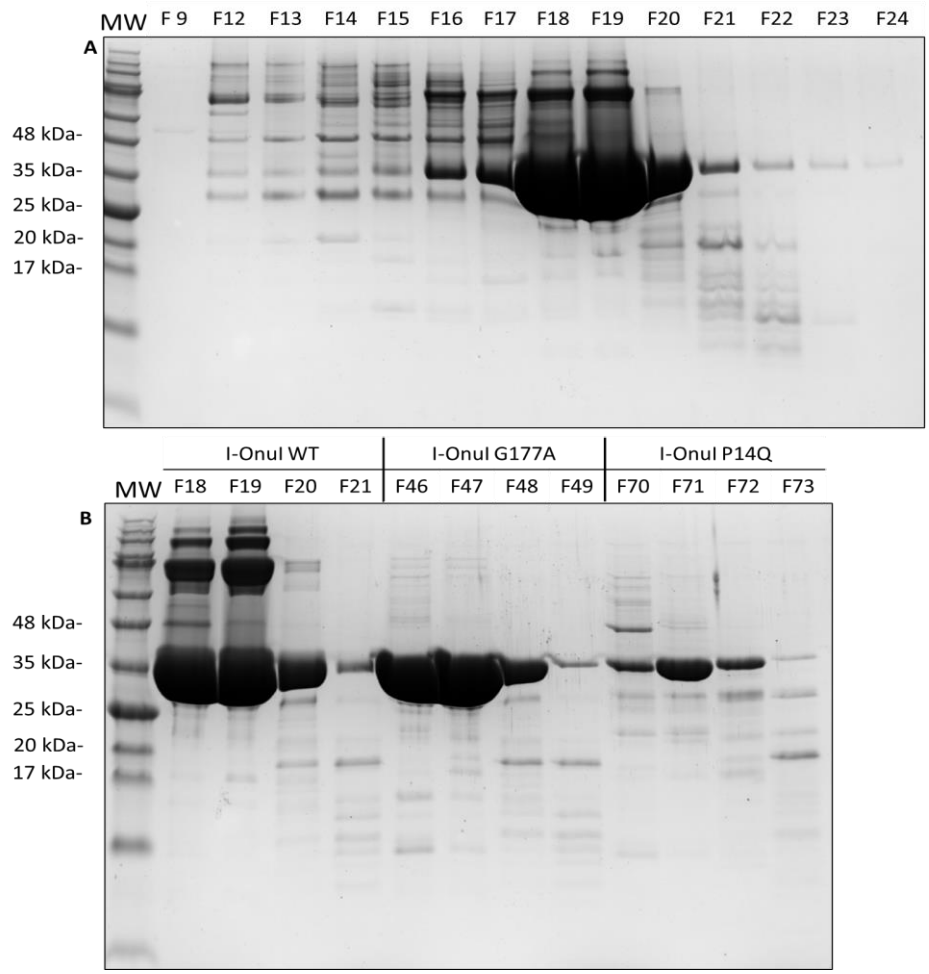


Figure 11. SDS-PAGE visualization of size exclusion chromatography (SEC) fractionation of (A) I-OnuI wild-type identified 18 mL retention volume of I-OnuI, which was (B) sampled in subsequent SEC of I-OnuI wild-type, I-OnuI G177A, and I-OnuI P14Q. After affinity chromatography, SEC was used to further separate larger molecular weight species. Fractions were collected as per methods section 2.6 Protein Purification. Protein samples were injected sequentially after allowing 0.5 column volumes of buffer to ensure the previous sample had eluted completely. Fraction numbers are indicated by the letter F at the top of the gels. For clarity, I-OnuI WT fractions correspond in panels (A) and (B). Protein species were resolved on SDS-PAGE, visualized with Coomassie blue staining, and a BLUelf pre-stained protein ladder was used (FroggaBio catalogue #PM808-0500G). The expected molecular weight of I-OnuI is 35 kDa.

3.3.2 Second Nickel-nitrilotriacetic Acid Immobilized Metal Affinity Chromatography based Purification of I-OnuI P14Q

I-OnuI P14Q was purified a second time to have another independent sample of this mutant protein. Using a high salt wash during this purification appears to have helped get the protein pure enough that size-exclusion chromatography was not required (Fig.10, Fig. 12). There was also less precipitation noticed during dialysis of this purification of I-OnuI P14Q.

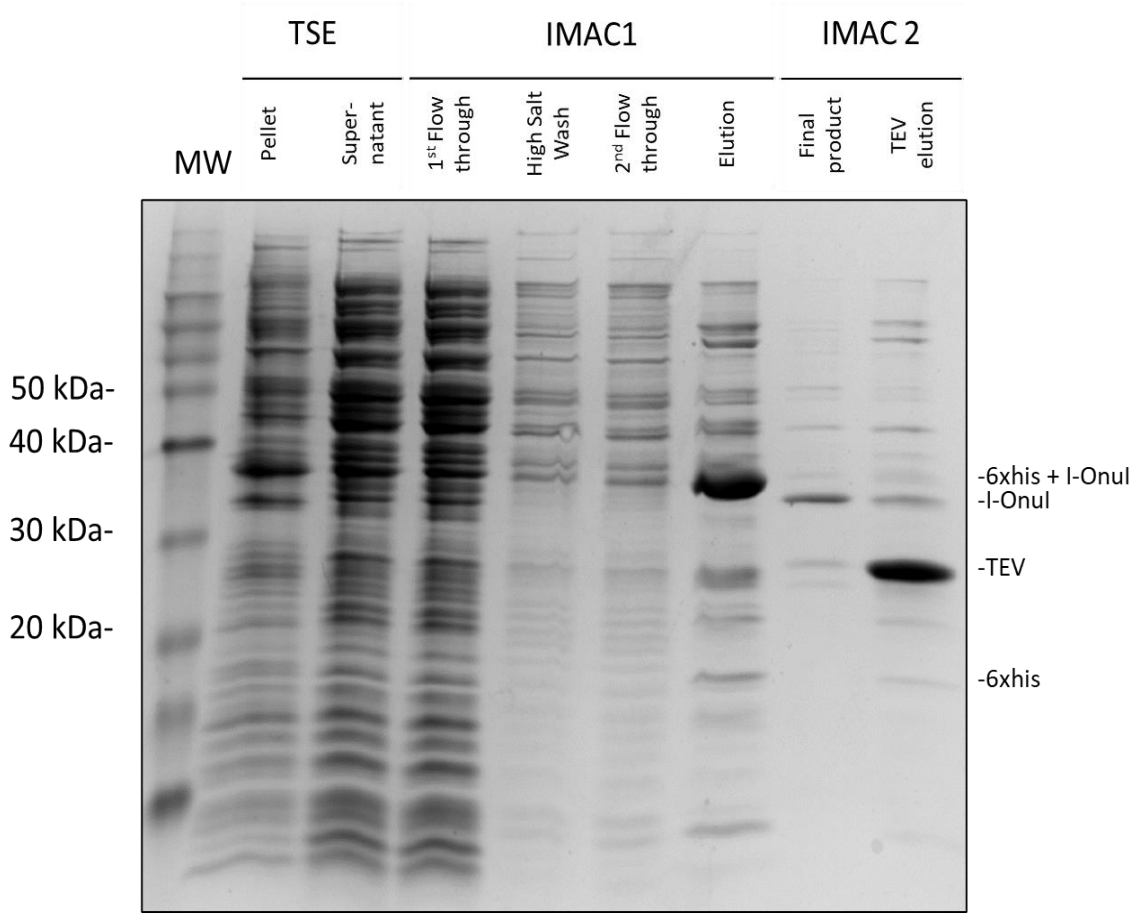


Figure 12. Second nickel-nitrilotriacetic acid IMAC based purification of I-OnuI P14Q. I-OnuI P14Q was purified as described in methods and was monitored by removing samples at the indicated points in the process. Protein species were resolved on SDS-PAGE, visualized with Coomassie blue staining, and a PiNK PLUS pre-stained protein ladder was used (GeneDireX catalogue #PM005-0500). Abbreviations: TSE- total sample extract, IMAC- immobilized metal affinity chromatography, TEV- tobacco etch virus

3.4 *In Vitro* Cleavage Assays Revealed No Temperature Dependence of I-OnuI P14Q Activity

After protein purification, wild-type I-OnuI and I-OnuI P14Q were assayed against their target, pCcdB, *in vitro* at 25°C and 37°C (Fig. 13). The cleavage reaction appears to proceed through a nicked intermediate before arriving at a linear product. The nicked intermediate is especially apparent for I-OnuI P14Q at time points under 30 minutes at 25°C and 37°C, causing a lower percentage of linear products (Fig. 13B, Appendix E). In contrast to what would be expected based on the *in vivo* data, there was no statistical difference in the rate of cleavage between wild-type I-OnuI and I-OnuI P14Q at 37°C *in vitro* ($p<0.05$, Fig. 19A). Purified wild-type I-OnuI and I-OnuI P14Q display rates that are statistically different from the purified slow mutant (I-OnuI G177A) at both temperatures but not significantly different from each other at 25°C or 37°C ($p<0.05$, Fig. 14). This suggests that the protein product of I-OnuI P14Q is not responsible for the thermosensitive properties it displays *in vivo*.

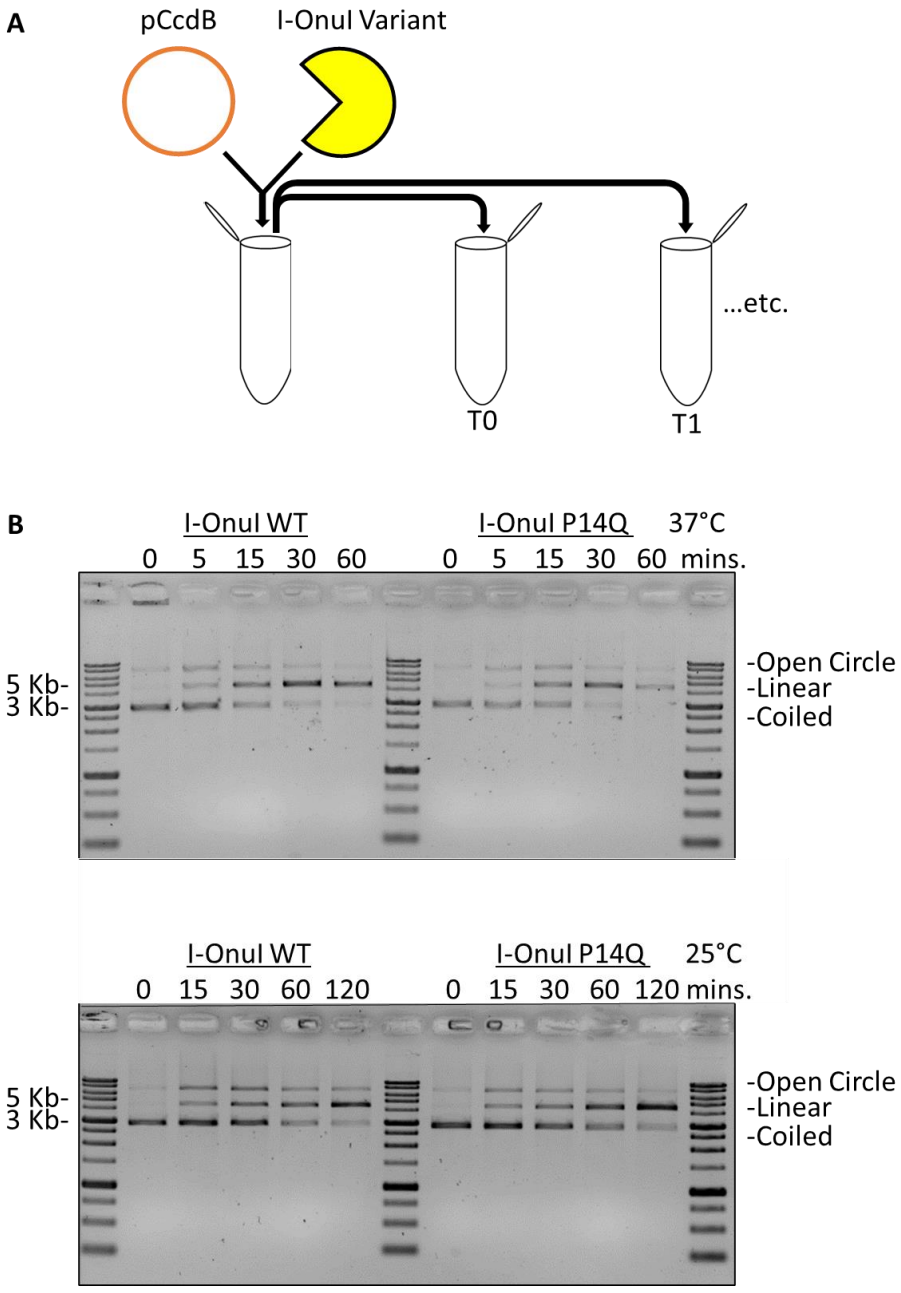


Figure 13. *In vitro* cleavage assay workflow (panel A) and sample gel (panel B). A) a cartoon representation of the *in vitro* cleavage assay workflow where pCcdB and an I-OnuI variant are mixed in a reaction solution and then stopped at various time points as described in the methods section. B) Sample *in vitro* cleavage assay agarose gels of I-OnuI WT and P14Q at 25°C and 37°C. These are 1% agarose gels, run at 60V until resolved, then stained in RedSafe Nucleic Acid Staining Solution (FroggaBio catalogue #21141), and destained in 1x TAE.

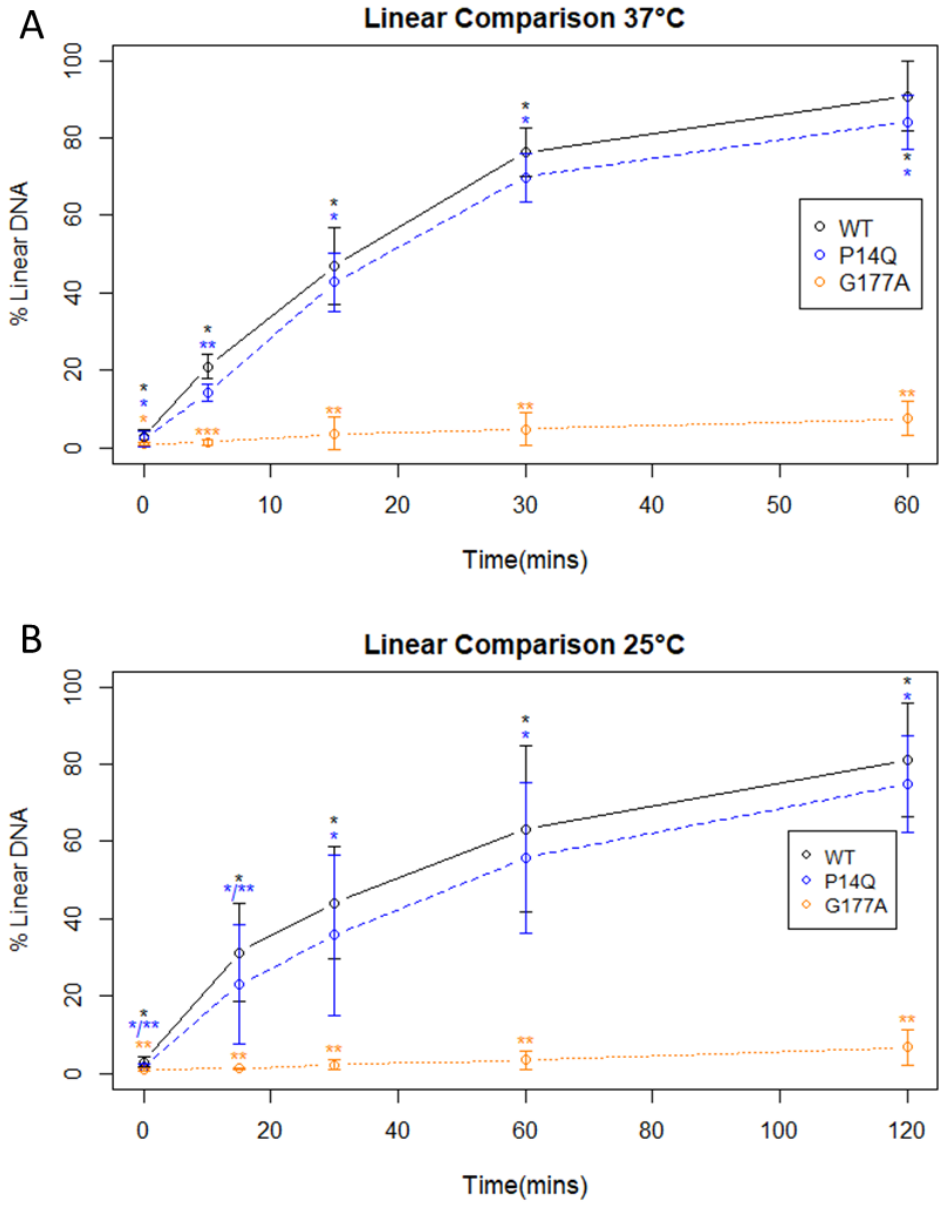


Figure 14. *In vitro* cleavage assay using purified I-OnuI variants targeting pCcdB showed few differences between I-OnuI and P14Q at both temperatures. *In vitro* cleavage assays were performed as described in methods using purified I-OnuI WT (N=4, black circles), P14Q (N=4, blue circles), and G177A (N=4, orange circles) at 37°C (panel A). and 25°C (panel B). Means are represented by the points on the graph, and error bars correspond to +/- 1 standard deviation. Significant differences ($p<0.05$, Tukey's post hoc test) between I-OnuI variant's generated linear DNA percent are indicated by (*, **, ***).

3.5 A Silent Nucleotide Substitution in Codon 2 of I-OnuI Determines Temperature Sensitivity

To further understand how I-OnuI P14Q displayed temperature sensitivity in the two-plasmid assay but not *in vitro*, the nucleotide sequence of P14Q was examined. In addition to the C→A transition that changes codon 14 from proline to glutamine (CCG→CAG), an additional A→C transition was found in the third position of the second codon (GGA→GGC) that specifies glycine. This nucleotide substitution does not change the codon identity. All three possible mutations of these two codons were constructed to test the impact on I-OnuI P14Q temperature sensitivity (Table 5).

The *in vitro* cleavage assay results indicated that the protein sequence is likely not what was causing the thermosensitive property of I-OnuI P14Q *in vivo*. All 3 permutations were explored in an *in vivo* survival assay to investigate whether it was one of the mutations themselves or their combination that was responsible for the thermosensitive property (Fig.15). The only variant that was significantly different from the wild-type at 37°C was the one identified initially using the two-plasmid assay that contained both mutations ($p=0.000$, GGA2GGC, CCG14CAG, Fig. 15A). This result shows that both point mutations are involved in the thermosensitive behaviour of I-OnuI P14Q.

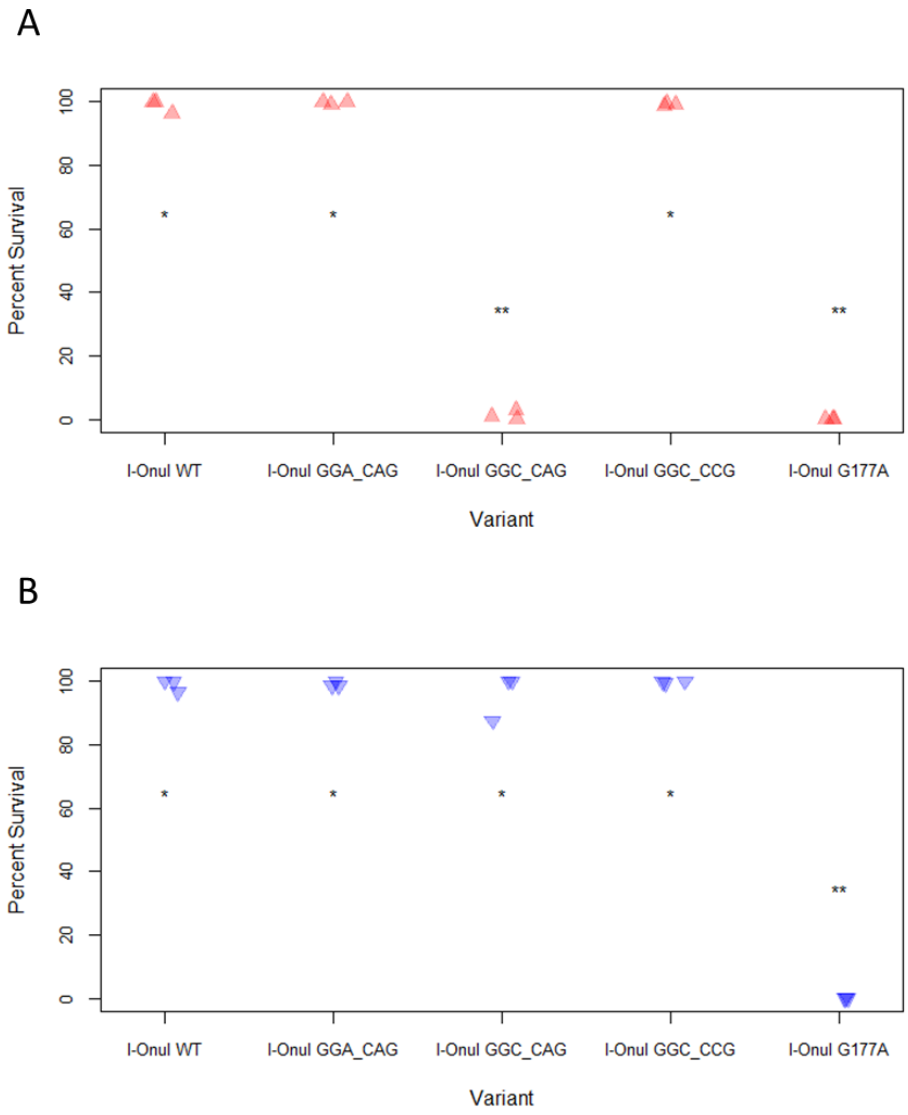


Figure 15. Two-plasmid selection showing that both mutations in I-OnuI P14Q (GGA2GGC, CCG14CAG) are required for its thermosensitive properties. The two-plasmid selection was performed using pEndo Nova XGF' *E. coli* cells carrying pEndo I-OnuI WT (N=3), I-OnuI P14Q minus the silent mutation (CCG14CAG, N=3), the I-OnuI P14Q variant originally identified (GGA2GGC, CCG14CAG, N=3), I-OnuI with only the silent mutation in codon 2 (GGA2GGC, N=3), and I-OnuI G177A (N=3) at 37°C (panel A, red triangles) and 25°C (panel B, blue inverted triangles). Significant differences ($p<0.05$, Tukey's post hoc test) between I-OnuI variant's percent survival are indicated by (*, **). Each point in a column represents an independent experiment.

Table 5. Changes in I-OnuI at codons 2 and 14 influence thermosensitivity in a survival assay. This table is displaying data from 3 independent replicates. Note: the first row corresponds to the wild-type enzyme.

		Mean Survival %	
Codon 2	Codon 14	25°C	37°C
GGA	CCG	98.8	98.8
GGA	CAG	99.1	99.7
GGC	CAG	95.8	1.2
GGC	CCG	99.7	99.2

Chapter 4

4 Discussion and Future Directions

4.1 Development of a Two-Plasmid Screen that Works at 25°C and 37°C

Elucidating why the two-plasmid screen behaved in a porous manner at 25°C proved difficult (Fig. 5, Fig. 6). Given that a G100E mutation in *ccdB* is a known inactivating mutation, it was a logical explanation[57]. When removing the mutation did not resolve the issue, the fact that the problem was temperature-dependent was considered. This led to the discovery of the insertion in the origin of pCcdB. Specifically, a 24 base pair insertion into the RNAII region of pCcdB's ColEI1 origin of replication proved to be the cause of the two-plasmid screen's disruption at 25°C (Fig. 7). RNAII initiates the replication of the leading strand on ColE1 origins, and it is plausible that an insertion here might disrupt this process in various ways, including the temperature of melting (Tm), secondary structure, or binding affinity to downstream enzymes[55,58,59].

However, the way it disrupted the screen hints at the possibility of using origins of replication in the ColE1 family with mutations to the RNAII regions as a different means of thermosensitive biocontainment. Plasmid copy number is inherently thermosensitive, which could suggest that alterations in that region amplify that property[55,59,60].

4.2 Creating and Screening LHE Mutagenic Libraries

Admittedly, the libraries could have had higher complexities, but the complexities generated have proved sufficient thus far (Table 1). The randomized libraries have a largely balanced transition to transversion rate based on the clones sequenced, allowing an unbiased sampling of the available protein space (Table 2). While the clones sampled from the I-OnuI library have approximately twice the rate of transitions as transversions; this is likely due to random error when compared to the other two libraries and the Stratagene manual[51].

After screening 2600 clones in the initial screen, only 12 candidates were found, and they were all I-OnuI variants (Table 3). This might be related to the I-OnuI library being smaller,

therefore making the variants easier to find, and some luck that the correct variant existed in the smaller library. Of those 12, only 2 proved to have a survival percent of 0 at 37°C (Table 4). Between those two candidate I-OnuI variants, the best one was selected for subcloning and further validation (Table 4). I-OnuI P14Q behaved superbly in the *in vivo* assay after being subcloned into a new pEndo backbone, providing precisely the parameters that one would hope for when creating a thermosensitive biocontainment LHE. It displayed 0% survival at 37°C and approximately 100% survival at 25°C (Fig. 8).

4.3 Purification of I-OnuI WT, I-OnuI G177A, and I-OnuI P14Q.

Purification of the wild-type I-OnuI enzyme proved to be difficult as it did not purify as well as the other two mutants during size exclusion chromatography (Fig 11). This may hint at the wild-type binding the DNA target more tightly than I-OnuI G177A and P14Q, although it is difficult to say without a controlled experiment with loading controls included. It is clear that the general problem in the first round of purifications was the lack of a high-salt wash to remove DNA, as I-OnuI is a DNA binding protein, and the addition of a high-salt wash removed a lot of contaminants (Fig 10., Fig. 11, Fig 12)[39,43,46].

4.4 *In Vitro* Cleavage Assay with WT I-OnuI, P14Q, and G177A.

The observation that the I-OnuI cleavage reaction appears to proceed through a nicked intermediate is in line with previous data on I-OnuI and other LHEs (Fig. 13B, Appendix E)[38,61–67]. A plausible explanation for I-OnuI P14Q's observed thermosensitivity *in vivo* would be the mutation in position 14, which destabilizes the N-terminal domain via the interfacial α-helix. Suppose the thermosensitive properties of I-OnuI P14Q were a result of changes in enzyme kinetics or folding at 37°C. In that case, these changes should be noticeable during an *in vitro* cleavage assay using the purified protein and its cognate target. In such a case, given the open circle intermediate transition to a final linear product, one would expect an accumulation of open circle product, and finally, less linear product. However, at both 25°C and 37°C, we fail to reject the null hypothesis that purified I-OnuI P14Q behaves differently than the wild-type enzyme (Fig. 13, Fig. 14). This would suggest that the thermosensitive property of I-OnuI is not a result of changes to the protein.

4.5 Two Codon Assay

Following the *in vitro* cleavage assays, it became clear that there must be another cause for the thermosensitive properties I-OnuI P14Q displayed *in vivo*. After re-examining the coding sequence of P14Q, it became apparent that there was a silent mutation added to codon 2's 3rd position (GGA2GGC). This mutation was added via the primer DE1268 used to create the mutant libraries. Both the synonymous GGA2GGC mutation and the non-synonymous P14Q mutation (CCG14CAG) were screened individually and compared to the mutant identified via the two-plasmid screen (GGA2GGC, CCG14CAG, Fig 15). Given that the *in vitro* cleavage assay showed that the protein itself was not responsible for the thermosensitivity of I-OnuI P14Q, it was unsurprising that both mutations were required for I-OnuI P14Q (GGA2GGC, CCG14CAG) to behave in a thermosensitive manner (Fig. 15, Table 5).

4.6 Suggestions for further studies

Future studies should attempt to elucidate the specific mechanism that gives I-OnuI P14Q its thermosensitive properties. To this end, the amount of transcript available to the cell at each temperature should be investigated using reverse transcriptase quantitative PCR. Additionally, an inquiry into the level of protein available using Western blot is required to compare them to the transcript levels between 25°C and 37°C. If the specific means by which thermosensitivity is conferred to I-OnuI P14Q becomes apparent after that, it should be possible to port this mechanism to I-LtrI and I-HjeMI. Next, it remains to be seen whether a thermosensitive LHE can be used in the context of other promoters or origins of replication. Furthermore, it is unclear whether thermosensitive LHEs can target the vector expressing them, and what the escape frequency would be under the context of self-targeting.

Finally, whether a self-targeting thermosensitive LHE could provide a useful means of containing a bacterial host to a living system remains to be seen as well. This final test could be done by gavaging mice with E. coli carrying a self-targeting (this would require moving the I-OnuI target sequence onto the same vector) version of pEndo-P14Q under control of a pBAD promoter grown up overnight in 2xYT and 0.2% glucose at 37°C. At various time points after gavaging, stool samples could be collected, serially diluted, and

quickly plated. Two types of plates would be needed: 100 µg/mL ampicillin and 0.2% arabinose, and 100 µg/mL ampicillin and 0.2% glucose. These plates would then be left at room temperature for 48 hours, and their survival rate could be calculated. This test would be similar to the one performed by Stirling *et al.* (2017)[23].

4.7 Conclusion

Before this line of inquiry, most attempts at biocontainment have followed the same previously existing conventions, and instead of getting simpler and more modular, attempts at reducing escape rates were becoming increasingly complex. Most attempts at biocontainment share the same fundamental flaws of their predecessors. Additionally, these increases in complexity also increased the size of the biocontainment module. An ideal biocontainment method would be more straightforward and controlled by an inherent indicator of environmental escape, such as how a drop in temperature would indicate an escape from containment for many bacteria relevant to health and industry. A pertinent example application in the short-term for I-OnuI P14Q is a thermosensitive containment module on Hamilton *et al.*'s (2019) conjugative *cas9* plasmid to eliminate bacterial infections in the gut[25]. Indeed, if this turns out to be a small temperature-sensitive module that is also reliable and resistant to inactivation, it would be an ideal candidate for many health-related and industrial applications of synthetic biology.

A thermosensitive variant of a LAGALIDADG Homing Endonuclease (LHE) and its cognate target site would meet these requirements. This research has identified a strong candidate for temperature-based biocontainment to prevent escape from a 37°C incubator (or a living organism) to an environment at room temperature[IV]. However, the exact mechanism responsible for I-OnuI P14Q's (GGA2GGC, CCG14CAG) thermosensitive property seen in an *in vivo* two-plasmid screen remains undetermined. The possible

[IV] Important note: during the *in vivo* two-plasmid screen the LHE is targeting a bacteriostatic toxin-producing plasmid. So, 100% survival at 25°C in the two-plasmid screen would translate to 100% cleavage at 25°C when being used as a biocontainment mechanism. The same would be true for 0% survival in the two-plasmid screen at 37°C, where that would translate to 0% cleavage at 37°C when the LHE is employed as a biocontainment mechanism.

explanations include changes to the rate of transcription, changes in transcript stability, or changes in the translation rate. It is a promising candidate that deserves further inquiry.

References

1. Sulkin, S. Edward; Pike, R. M. Survey of Laboratory-Acquired Infections. *Am. Public Heal.* **41**, 769–781 (1951).

2. Barbeito, M. S. & Kruse, R. H. A History of the American Biological Safety Association Part I: The First Ten Biological Safety Conferences 1955–1965. *J. Am. Biol. Saf. Assoc.* **2**, 7–19 (1997).

3. Wedum, A. G. Laboratory Saftey in Research With Infectious Aerosols. *Public Health Rep.* **79**, 619–633 (1964).

4. Berg, P., Baltimore, D., Brenner, S., Roblin, R. O. & Singer, M. F. Summary Statement of the Asilomar Conference on Recombinant DNA Molecules. *Proc. Natl. Acad. Sci. U. S. A.* **72**, 1981–1984 (1975).

5. Richardson, John H.; Emmett, B. W. . *Biosafety in Microbiological and Biomedical Laboratories*. (1984).

6. Wright, O., Delmans, M., Stan, G. B. & Ellis, T. GeneGuard: A modular plasmid system designed for biosafety. *ACS Synth. Biol.* **4**, 307–316 (2015).

7. Chan, C. T. Y., Lee, J. W., Cameron, D. E., Bashor, C. J. & Collins, J. J. 'Deadman' and 'Passcode' microbial kill switches for bacterial containment. *Nat. Chem. Biol.* **12**, 82–86 (2015).

8. Gallagher, R. R., Patel, J. R., Interiano, A. L., Rovner, A. J. & Isaacs, F. J. Multilayered genetic safeguards limit growth of microorganisms to defined environments. *Nucleic Acids Res.* **43**, 1945–1954 (2015).

9. Curtiss III, R. *et al.* Construction and Use of Safer Bacterial Host Strains for Recombinant DNA Research. in *Molecular of Cloning of Recombinant DNA* (ed. Scott, W. A.; Werner, R.) 99–114 (Academic Press, 1977). doi:10.1016/B978-0-12-634250-5.50011-2

10. Braat, H. *et al.* A Phase I Trial With Transgenic Bacteria Expressing Interleukin-

10 in Crohn's Disease. *Clin. Gastroenterol. Hepatol.* **4**, 754–759 (2006).

11. Steidler, L. *et al.* Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. *Nat. Biotechnol.* **21**, 785–789 (2003).

12. Molin, S. *et al.* Conditional Suicide System for Containment of Bacteria and Plasmids. *Nat. Biotechnol.* **5**, 328–330 (1987).

13. Anderson, E. S. Viability of, and transfer of a plasmid from, E. coli K12 in the human intestine. *Nature* **255**, 502–504 (1975).

14. Rovner, A. J. *et al.* Recoded organisms engineered to depend on synthetic amino acids. *Nature* **518**, 89–93 (2015).

15. Zhang, Y. *et al.* A semisynthetic organism engineered for the stable expansion of the genetic alphabet. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 1317–1322 (2017).

16. Mandell, D. J. *et al.* Biocontainment of genetically modified organisms by synthetic protein design. *Nature* **518**, 55–60 (2015).

17. Ravikumar, A. & Liu, C. C. Biocontainment through reengineered genetic codes. *ChemBioChem* **16**, 1149–1151 (2015).

18. Lopez, G. & Anderson, J. C. Synthetic Auxotrophs with Ligand-Dependent Essential Genes for a BL21(DE3) Biosafety Strain. *ACS Synth. Biol.* **4**, 1279–1286 (2015).

19. Schmidt, M. & Pei, L. Synthetic toxicology: Where engineering meets biology and toxicology. *Toxicol. Sci.* **120**, 204–224 (2011).

20. Citorik, R. J., Mimee, M. & Lu, T. K. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat. Biotechnol.* **32**, 1141–1145 (2014).

21. Molin, S., Boe, L.-J., Jensen, L. B., Kristensen, C. S. & Givskov, M. Suicidal

Genetic Elements and Their Use in Biological Containment of Bacteria. *Annu. Rev. Microbiol.* **47**, 139–166 (1993).

22. Knudsen, S. M. & Karlstrom, O. H. Development of efficient suicide mechanisms for biological containment of bacteria. *Appl. Environ. Microbiol.* **57**, 85–92 (1991).

23. Stirling, F., Bitzan, L., Oliver, J., Way, J. & Silver, P. A. Rational Design of Evolutionarily Stable Microbial Kill Switches. *Mol. Cell* **68**, 686–696 (2017).

24. Nielsen, K. M., Johnsen, P. J., Bensasson, D. & Daffonchio, D. Release and persistence of extracellular DNA in the environment. *Environ. Biosafety Res.* **6**, 37–53 (2007).

25. Hamilton, T. A. *et al.* Efficient inter-species conjugative transfer of a CRISPR nuclease for targeted bacterial killing. *Nat. Commun.* **10**, 1–9 (2019).

26. Ahrenholtz, I., Lorenz, M. G. & Wackernagel, W. A conditional suicide system in Escherichia coli based on the intracellular degradation of DNA. *Appl. Environ. Microbiol.* **60**, 3746–3751 (1994).

27. Caliando, B. J. & Voigt, C. A. Targeted DNA degradation using a CRISPR device stably carried in the host genome. *Nat. Commun.* **6**, 6989 (2015).

28. Richter, F. *et al.* Engineering of temperature- and light-switchable Cas9 variants. *Nucleic Acids Res.* **44**, 10003–10014 (2016).

29. Xiang, G., Zhang, X., An, C., Cheng, C. & Wang, H. Temperature effect on CRISPR-Cas9 mediated genome editing. *J. Genet. Genomics* **44**, 199–205 (2017).

30. Horton, J. R. *et al.* Structure of 5-hydroxymethylcytosine-specific restriction enzyme, AbaSI, in complex with DNA. *Nucleic Acids Res.* **42**, 7947–7959 (2014).

31. Fomenkov, A. & Xu, S. yong. Isolation of temperature-sensitive mutants of the BamHI restriction endonuclease. *Gene* **157**, 303–310 (1995).

32. Halford, S. E., Johnson, N. P. & Grinsted, J. The EcoRI restriction endonuclease with bacteriophage λ DNA. *Biochem. J.* **191**, 581–592 (1980).

33. Stoddard, B. L. Homing endonuclease structure and function. *Q. Rev. Biophys.* **38**, 49–95 (2005).

34. Jurica, M. S., Monnat, R. J. & Stoddard, B. L. DNA recognition and cleavage by the LAGLIDADG homing endonuclease I-Crel. *Mol. Cell* **2**, 469–476 (1998).

35. Takeuchi, R. *et al.* Tapping natural reservoirs of homing endonucleases for targeted gene modification. *Proc. Natl. Acad. Sci.* **108**, 13077–13082 (2011).

36. Grishin, A. *et al.* Identification of conserved features of laglidadg homing endonucleases. *J. Bioinform. Comput. Biol.* **8**, 453–469 (2010).

37. Dalgaard, J. Z. *et al.* Statistical modeling and analysis of the LAGLIDADG family of site-specific endonucleases and identification of an intein that encodes a site-specific endonuclease of the HNH family. *Nucleic Acids Res.* **25**, 4626–4638 (1997).

38. McMurrough, T. A. *et al.* Active site residue identity regulates cleavage preference of LAGLIDADG homing endonucleases. *Nucleic Acids Res.* **46**, 11990–12007 (2018).

39. Baxter, S. *et al.* Engineering domain fusion chimeras from I-OnuI family LAGLIDADG homing endonucleases. *Nucleic Acids Res.* **40**, 7985–8000 (2012).

40. Prieto, J. *et al.* Understanding the indirect DNA read-out specificity of I-CreI Meganuclease. *Sci. Rep.* **8**, 1–9 (2018).

41. Molina, R. *et al.* Non-specific protein-DNA interactions control I-CreI target binding and cleavage. *Nucleic Acids Res.* **40**, 6936–6945 (2012).

42. Chevalier, B. S. *et al.* Design, activity, and structure of a highly specific artificial endonuclease. *Mol. Cell* **10**, 895–905 (2002).

43. Sethuraman, J., Majer, A., Friedrich, N. C., Edgell, D. R. & Hausner, G. Genes within genes: Multiple LAGLIDADG homing endonucleases target the ribosomal protein S3 gene encoded within an rnl group i intron of ophiostoma and related taxa. *Mol. Biol. Evol.* **26**, 2299–2315 (2009).

44. Jacoby, K. *et al.* Expanding LAGLIDADG endonuclease scaffold diversity by rapidly surveying evolutionary sequence space. *Nucleic Acids Res.* **40**, 4954–4964 (2012).

45. Simmons, E. G. Classification of some cellulase-producingTrichodermaspecies. in *Proceedings of the Second International Mycological Congress, University of South Florida, Tampa, Florida, USA 27 August - 3 September 1977* (ed. Simmons, E. G.) 618 (University of South Florida, 1978).

46. McMurrough, T. A., Dickson, R. J., Thibert, S. M. F., Gloor, G. B. & Edgell, D. R. Control of catalytic efficiency by a coevolving network of catalytic and noncatalytic residues. *Proc. Natl. Acad. Sci.* **111**, E2376–E2383 (2014).

47. Bikard, D. *et al.* Exploiting CRISPR-cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.* **32**, 1146–1150 (2014).

48. Theuretzbacher, U. Antibiotic innovation for future public health needs. *Clin. Microbiol. Infect.* **23**, 713–717 (2017).

49. Lee Ventola, C. The antibiotic resistance crisis: Part 2: Management strategies and new agents. *P&T* **40**, 344–352 (2015).

50. Rossolini, G. M., Arena, F., Pecile, P. & Pollini, S. Update on the antibiotic resistance crisis. *Curr. Opin. Pharmacol.* **18**, 56–60 (2014).

51. Stratagene. GeneMorph II EZClone Domain Mutagenesis Kit. (2009).

52. New England Biolabs. Ligation Protocol with T4 DNA Ligase (M0202). 1 (2014). doi:10.17504/protocols.io.irscd6e

53. Bahassi, E., O'Dea, M., Allali, N., Messens, J. & Gellert, M. Interactions of CcdB

with DNA Gyrase. *J. Biol. Chem.* **274**, 10936–10944 (1999).

54. Chen, Z. & Zhao, H. A highly sensitive selection method for directed evolution of homing endonucleases. *Nucleic Acids Res.* **33**, 1–7 (2005).

55. Kobayashi, J. *et al.* Unique plasmids generated via pUC replicon mutagenesis in an error-prone thermophile derived from Geobacillus kaustophilus HTA426. *Appl. Environ. Microbiol.* **81**, 7625–7632 (2015).

56. Püllmann, P. *et al.* Golden Mutagenesis: An efficient multi-site-saturation mutagenesis approach by Golden Gate cloning with automated primer design. *Sci. Rep.* **9**, 1–11 (2019).

57. Bahassi, E. M., Salmon, M. A., van Melderen, L., Bernard, P. & Couturier, M. F plasmid CcdB killer protein ccdB gene mutants coding for non-cytotoxic proteins which retain their regulatory functions. *Mol. Microbiol.* **15**, 1031–1037 (1995).

58. Itoh, T. & Tomizawa, J. Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proc. Natl. Acad. Sci. U. S. A.* **77**, 2450–2454 (1980).

59. Lin-Chao, S., Chen, W. -T & Wong, T. -T. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Mol. Microbiol.* **6**, 3385–3393 (1992).

60. Wong, E. M., Muesing, M. A. & Polisky, B. Temperature-sensitive copy number mutants of ColE1 are located in an untranslated region of the plasmid genome. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 3570–3574 (1982).

61. Moure, C. M., Gimble, F. S. & Quiocho, F. A. Crystal structures of I-SceI complexed to nicked DNA substrates: Snapshots of intermediates along the DNA cleavage reaction pathway. *Nucleic Acids Res.* **36**, 3287–3296 (2008).

62. Wende, W., Grindl, W., Christ, F., Pingoud, A. & Pingoud, V. Binding, bending and cleavage of DNA substrates by the homing endonuclease PI-SceI. *Nucleic Acids Res.* **24**, 4123–4132 (1996).

63. Gimble, F. S. & Stephens, B. W. Substitutions in conserved dodecapeptide motifs that uncouple the DNA binding and DNA cleavage activities of PI-SceI endonuclease. *Journal of Biological Chemistry* **270**, 5849–5856 (1995).

64. Niu, Y., Tenney, K., Li, H. & Gimble, F. S. Engineering Variants of the I-SceI Homing Endonuclease with Strand-specific and Site-specific DNA-nicking Activity. *J. Mol. Biol.* **382**, 188–202 (2008).

65. Champer, J., Buchman, A. & Akbari, O. S. Cheating evolution: Engineering gene drives to manipulate the fate of wild populations. *Nat. Rev. Genet.* **17**, 146–159 (2016).

66. Chan, S. H., Stoddard, B. L. & Xu, S. Y. Natural and engineered nicking endonucleases - From cleavage mechanism to engineering of strand-specificity. *Nucleic Acids Res.* **39**, 1–18 (2011).

67. Silva, G. H. & Belfort, M. Analysis of the LAGLIDADG interface of the monomeric homing endonuclease I-DmoI. *Nucleic Acids Res.* **32**, 3156–3168 (2004).

68. Hawksworth, D. L. *et al.* The Amsterdam Declaration on Fungal Nomenclature. *IMA Fungus* **2**, 105–112 (2011).

69. Roberts, R. J. *et al.* A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res.* **31**, 1805–1812 (2003).

70. Esvelt, K. M., Smidler, A. L., Catteruccia, F. & Church, G. M. Concerning RNA-guided gene drives for the alteration of wild populations. *Elife* **3**, (2014).

Appendices

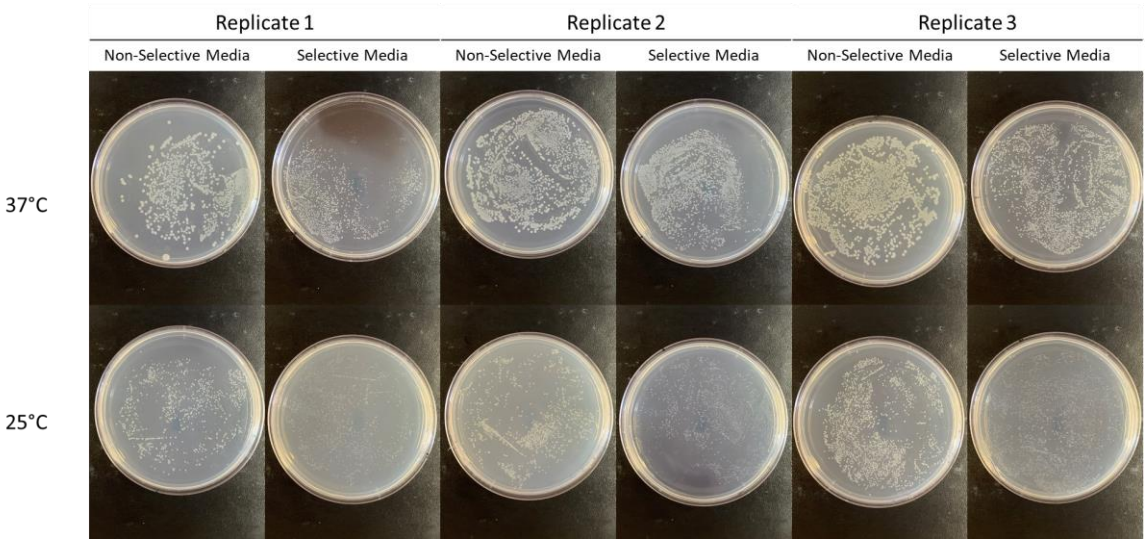
Appendix A: List of Primers

Primer Name	Sequence (5' to 3')	Description
DE 704	CCATAAGATTAGCGGATCCTACCTG	Forward sequencing primer for the multicloning region of pEndo
DE 705	CGGGAAGATGCGTGATCTGATCC	Reverse sequencing primer for the multicloning region of pEndo
DE 796	CACCGATATGGCCAGTGTG	Sequencing primer for the end of *CcdB*, the I-OnuI target site, and the I-LtrI target site.
DE 1268	TAAGAAGGAGATATACCCATGGGC	Forward mutagenesis primer for the multicloning region of pEndo
DE 1269	CCAATTCTGAGCGGCCGC	Reverse mutagenesis primer for the multicloning region of pEndo
DE 4282	CATTGGTAACTGTCAGACCA	Sequencing primer for the origin of replication on

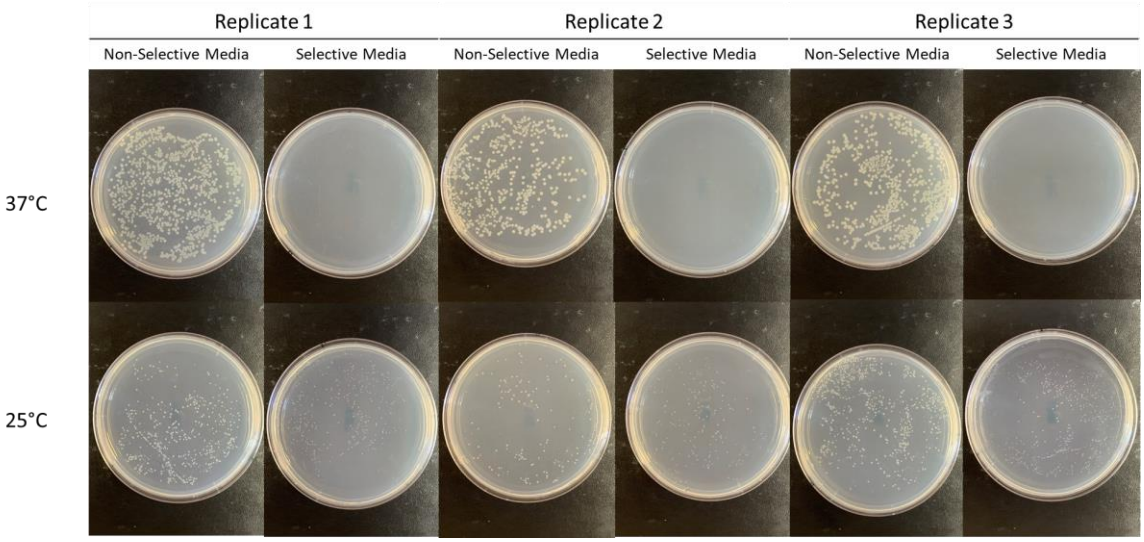
		pCcdB and pMSP3535
DE 4668	GCATTTCCGCGGAGACAGATCGCTGAGATAGG	Forward gibson assembly primer the new origin of replication
DE 4669	TTGCGCGACGTCAACCTGCATTAATGAATCGG	Reverse gibson assembly primer the new origin of replication
DE 5140	GGCGGCCGTCTCACCAGTGGATTCTCACCGGTT	Forward primer for I-OnuI (CCG14CAG) golden mutagenesis
DE 5141	GGCGGCCGTCTCACTGGTTAATGGACTCGCGAC	Reverse primer for I-OnuI (CCG14CAG) golden mutagenesis
DE 5158	GGCGGCCGTCTCACCGGTGGATTCTCACCGGTT	Forward primer for I-OnuI (CAG14CCG) golden mutagenesis
DE 5159	GGCGGCCGTCTCACGGGTTAATGGACTCGCGAC	Reverse primer for I-OnuI (CAG14CCG) golden mutagenesis
DE 5232	GGCGGCCGTCTCTGGGCTCCGCCTACATGTC	Forward primer for I-OnuI (GGA2GGC) golden mutagenesis

DE 5233	GGCGGCCGTCTCAGCCCATGGGTATATCTCCTTCT	Reverse primer for I-OnuI (GGA2GGC) golden mutagenesis

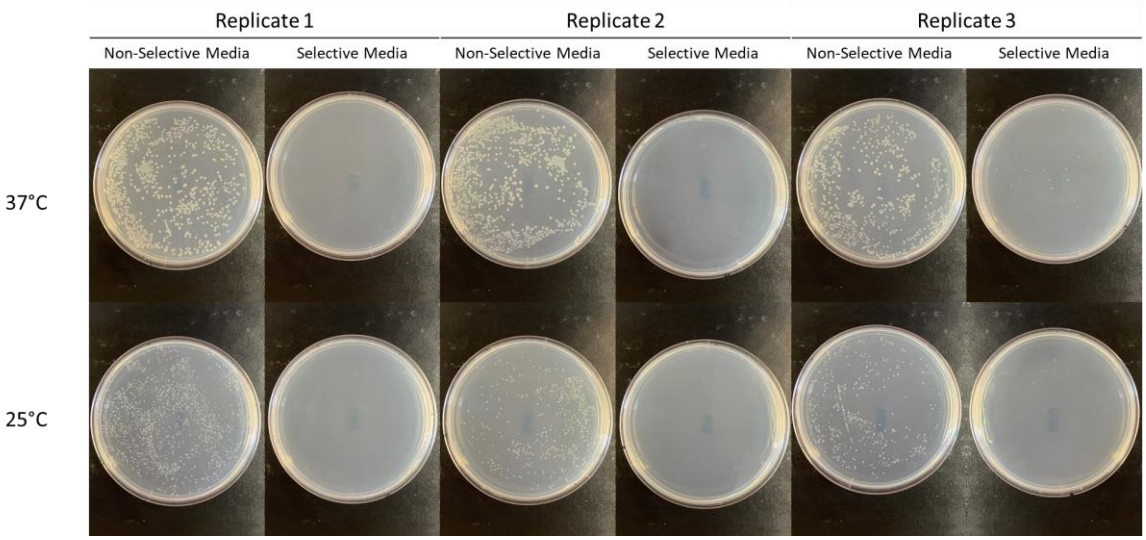
Appendix B. I-OnuI WT plates used to generate figure 12.



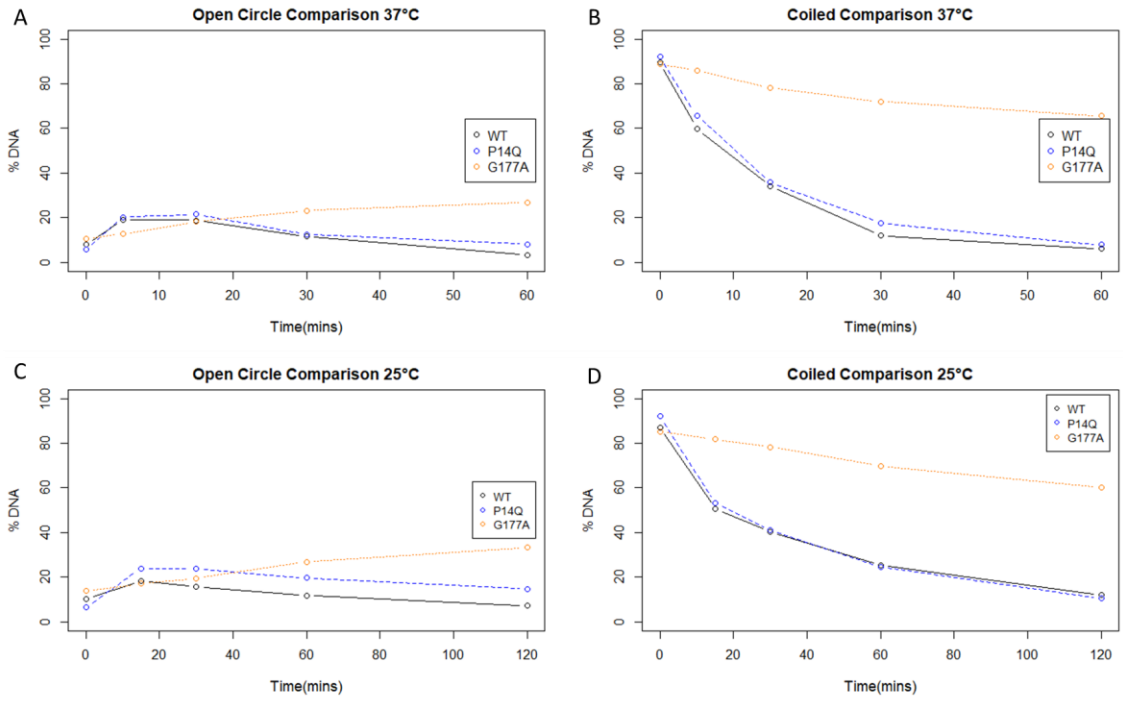
Appendix C. I-OnuI P14Q plates used to generate figure 12.



Appendix D. I-OnuI G177A plates used to generate figure 12. Note: the spots seen on 37°C selective plate (replicate 3) are air bubbles, not colonies.



Appendix E. I-OnuI P14Q and I-OnuI WT also display few differences across temperatures when looking at the percentage of open circle and closed products over time during an *in vitro* cleavage assay. Panel A, B, C, and D correspond to the charts of open circle at 37°C, coiled at 37°C, open circle at 25°C, and coiled at 25°C, respectively. *In vitro* cleavage assays were performed as described in methods using purified I-OnuI WT (N=4, black circles), P14Q (N=4, blue circles), and G177A (N=4, orange circles) at 37°C and 25°C. The points on the graph represent the mean.



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