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Leveraging Queueing Theory to Develop Advanced Synthetic Biological Circuits

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LEVERAGING QUEUEING THEORY TO DEVELOP ADVANCED SYNTHETIC BIOLOGICAL CIRCUITS

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

Abbreviation	Description
AAA+	ATPase Associated with diverse cellular Activities
Amp	Ampicillin
AHL	Acyl-homoserine lactone
ATP	Adenosine triphosphate
AU	Arbitrary Units
CFP	Cyan fluorescent protein
CFU	Colony forming units
ClpP	Caseinolytic protease P
Cm	Chloramphenicol
Dox	Doxycycline
DF	Dual feedback
FU	Fluorescence unit
GFP	Green fluorescent protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth-Miller's media
mRNA	Messenger Ribonucleic acid
MMB	Minimal medium A
Km	Kanamycin
OD	Optical density
PCR	Polymerase chain reaction
RBS	Ribosome binding site
SmpB	Small protein B
SsrA	Small stable RNA
TF	Transcription factor

tmRNA	Transfer-messenger RNA
YFP	Yellow fluorescent protein
σ	Sigma factor
Δ	Delta (generally associated with mutation)

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ABSTRACT

LEVERAGING QUEUEING THEORY TO DEVELOP ADVANCED SYNTHETIC BIOLOGICAL CIRCUITS

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The complexity and redundancy in the network topology of the cell hinder our understanding of the natural system, making it challenging to engineer living organisms with the desired functionality. The long-term goal of synthetic biology and bioengineering is to engineer cells to perform specific functions with increased robustness. The robust engineered systems can be easily applied to different species with the same outcome. An effective approach is a bottom-up approach where we tease apart the biological pathways and study them independently. Understanding the underlying mechanisms of biological pathways can be further applied to construct complex biological circuits that function similarly to natural systems. In this study, I have used the Design-Build-Test-Learn cyclic approach using a widely used organism, *E. coli*, to engineer synthetic circuits that relied on post-translational, degradation, and transcriptional control. This work is novel because traditional synthetic circuits often rely only on transcriptional control.

Synthetic circuits contain engineered genetic frameworks that rely on basic principles of the natural system. I took advantage of the vast knowledge of biological queueing theory to develop robust synthetic circuits. Queueing theory is the study of waiting lines caused due to the bottleneck in the system. It is classically applied to common human activities such as line form (queues) and dissipation at grocery stores, roadways, at the airport, the flow of traffic at traffic signals, etc. Our approach takes the available knowledge we have

gained in the macro-world and applies it to the micro (bacteria) and nano-world (proteins, RNA, etc.). The use of queueing theory is explained in detail in Section 1.4. In this study, I examined and utilized the queue formed with proteins at the proteolytic system. Proteases in the cell are limited in numbers (bottleneck) where the queue forms when proteins targeted to proteolysis are waiting in the line to be degraded. I used the understanding of proteolytic queueing and constructed circuits that uses proteolytic queues as a core mechanism.

In Chapter 2, I have reviewed the application of proteolytic queueing in synthetic biology, especially in dynamic synthetic oscillators (a review chapter with the most up-to-date information). ClpXP proteolytic system is the most explored proteolytic pathway and has been employed in synthetic biology using a well-studied SsrA degradation tag from *E. coli*. The SsrA tag is a known substrate for ClpXP, and the affinity of the SsrA tag is relatively constant for the ClpXP complex. To acquire a faster degradation rate, I deconstructed and modified the SsrA tag to develop an ultra-high affinity degradation tag (Chapter 3). Faster degradation of tagged protein increases the turnover rate; thus, the processing at the proteolytic queue is faster. However, the specific component of the SspB-ClpXP proteolytic system causing the queue was unknown, limiting our understanding of the queue complexity. But here, I provide substantial evidence that the ClpX chaperone likely causes queue formation (Chapter 3). I engineered a new synthetic dual-feedback oscillator using this information and the ultra-high affinity tag. This new oscillator dynamic output is unique from previously developed ones (Chapter 4). The understanding of ClpXP proteolytic queueing and synthetic circuits developed in this study can be used to engineer complex circuits for future biotechnology applications.

1. CHAPTER 1: INTRODUCTION.

1.1. Synthetic biology and Synthetic circuits.

Synthetic biology is an emerging field used to develop innovative technologies and products for medical, agriculture, and industrial applications[1-3]. The impact of synthetic biology is expanding to many areas of science, engineering, medicine as well as the food industry. Few examples include the development of the impossible burger[4], engineered CAR-T cells for battles against cancer that save lives[5], and others. The most influential synthetic biology products are the COVID-19 mRNA vaccines that billions have used[2, 3, 6-12]. These vaccines currently control the pandemic spread, reducing deaths and illness substantially. Synthetic biology combines multiple disciplines that allow engineering well-controlled synthetic circuits for organisms as a tool. As mentioned in the abstract, I used the Design-Build-Test-Learn cyclic approach, where first-generation circuits evolved over many iterations to improve circuit topology (Fig. 1.1). Testing the design, we gained a valuable understanding of the current system, and then we applied this new knowledge to make future designs.

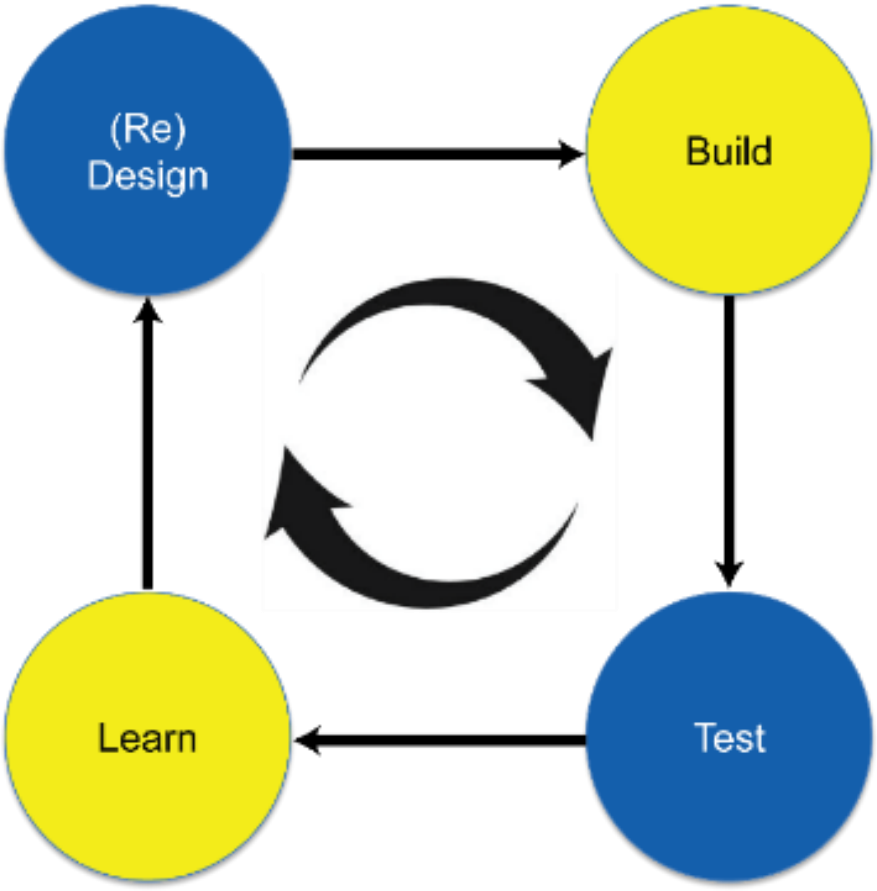


Fig. 1.1. The design-build-test-learn cyclic approach of synthetic biology.

Synthetic circuits primarily use principles underlying natural systems such as the "Central Dogma of Life" (Fig. 1.2). Synthetic circuits are designed to perform logical functions similar to electrical circuits. Instead of wires and switches, we develop biological circuits that utilize gene networks regulated transcriptionally, translationally, and post-translationally. Transcriptional regulation (DNA-RNA) involves the expression of RNA from DNA under inducible or constitutive (uncontrollable and tends to produce RNA at a consistent amount) promoters. Translational control regulates production of proteins. Typically, controlling protein levels with different translational rates depends on how the

mRNA is used as a template for protein synthesis. For example, the Ribosomal Binding Site (RBS) controls how well the ribosome binds to the mRNA, and thus weak RBS leads to low protein levels, and strong RBS leads to high protein production. Protein modification and degradation are a type of post-translational control.

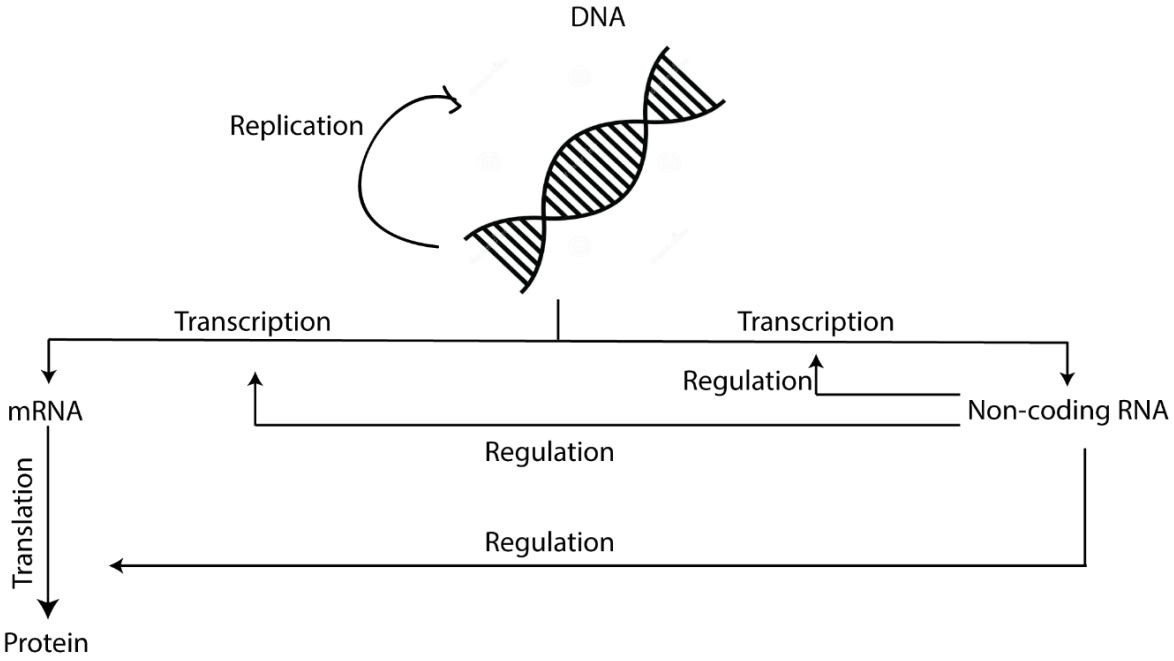


Fig. 1.2. Central Dogma of Life.

Several circuit topologies have been explored using these regulators, such as binary logical gates, toggle switches, and self-sustaining oscillatory networks[13-18]. During the past 20 years, synthetic systems have been developed to mimic the functionality of natural systems[19-23]. Through these systems' development and output, we often gain a new understanding of the natural systems. This research also improves our ability to reprogram biological networks to engineer organisms.

1.2. Protein regulation and proteolytic systems.

Cells function through the action of transcription factors, enzymes, polymerases, chaperones, proteases, and other important proteins. The correct balance of these proteins enables the proper operation of pathways in the cell. Hence, protein regulation is important to maintain cell function. Many diseases are caused due to the imbalance of some of the proteins responsible for regulating pathways. For example, insulin imbalance in the body causes diabetes[24]. Knowledge of protein regulations has been advantageous not only for tackling diseases, but also for biotechnology applications. It can be used to engineer bacterial strains to mass-produce many medically relevant products (e.g., insulin)[25].

Both protein production and turnover rates maintain the protein homeostasis in a cell. The protein production rate involves the rate of transcription (mRNA levels) and translation (protein biosynthesis)[26, 27]. The turnover rate of proteins is determined by exportation[28, 29], cell division, and degradation rate via proteases[30]. The turnover rate is the rate at which a protein is recycled and is known as the 'half-life' of a protein. It ensures the availability of a protein in the cell for definite periods to perform assigned functions to maintain cell function[31]. The cell requires proteins with different stability (varying half-life) to perform assigned roles, and proteases regulate their stability based on the amino acid sequence of proteins. Some proteins are required for short durations or only during specific environmental conditions. Many stresses (e.g., temperature) or errors in protein production can cause misfolding of proteins. Chaperones/proteases can also identify misfolded proteins. Misfolded proteins have altered shapes, which exposes amino acid recognition site to proteases and chaperones[32], and this increasing their degradation. Cells utilize different measures to avoid unnecessary degradation of regulatory proteins. Limited

numbers of chaperones and proteases allow for tight regulation of overall protein levels[33] and minimize degradation of functional proteins. Overexpression of some proteases can affect cell growth since it degrades regulatory proteins that are not supposed to be degraded[34, 35].

1.2.1. ATP-independent and ATP-dependent proteases.

There are two types of proteases present in bacterial cells, 1) ATP-independent proteases (no energy required, passive degradation), and 2) ATP-dependent proteases (required energy in ATP form, active degradation). Some examples of ATP-independent proteases in *E. coli* are HtrA[36, 37], HtpX[38], and Lon-like protease[39]. ATP-independent proteases are primarily responsible for degrading linearized or small peptides that do not require unfolding (thus, ATP is not required)[39]. Nevertheless, larger peptides/proteins are typically present in a folded configuration and thus resistant to passive degradation. Such polypeptides need to be linearized before degradation, which is a limiting step in the proteolytic pathway. Linearization requires energy and is accomplished by ATP-dependent proteases, which rely on an ATPase chaperone or domain. However, some ATP-dependent proteases (ClpP, Lon) sometimes can degrade in an ATP-independent manner if they encounter small or linearized peptides[40, 41].

Most proteases in the cell are ATP-dependent proteases, which can be further divided into subtypes depending on the architecture, location, and proteolytic active sites[42-45]. In the cytoplasm, ATP-dependent serine proteases (Ser-based proteolytic active site) degrade more than 90% of the proteins[46]. These proteases consist of two important modules, 1) an ATPase module of the AAA+ family (ATPase Associated with diverse cellular Activities) uses ATP to unfold and translocate the polypeptide into the 2) proteolytic module, which

breaks polypeptide bonds to amino acid monomers without the use of ATP. Lon, ClpXP, and ClpAP are major cytoplasmic serine proteases. Lon is a single component, a multi-domain (includes ATPase domain and proteolytic domain), and was the first serine protease to be studied[47, 48]. All three domains of life (bacteria, archaea, and eukaryotes -in the mitochondria) contain homologs of this protease, and it regulates many cellular processes[49-51]. For example, in *E. coli,* Lon is important to the heat-shock response[52], nucleotide biosynthesis[53], energy metabolisms[53], and surviving amino acid starvation[54]. Unlike Lon, ClpP (caseinolytic protease P) proteases form a two-component system where ATPase activity resides in a separate chaperone protein. ClpP is the heptameric protein[55] that functions in dimeric form along with a hexameric chaperone (ClpX[56, 57] or ClpA[58]). These chaperones are responsible for translocating and unfolding polypeptide chain (ATP-dependent) to carry out proteolytic degradation by ClpP (ATP-independent). The chaperones ClpX and ClpA harbor ATPase[56-60] activity to form ClpXP and ClpAP proteolytic systems, respectively. Furthermore, substrate selection, especially in *E. coli* can be enhanced by binding accessory chaperones (called adaptor proteins) like SspB for ClpXP[61-65] and ClpS for ClpAP[66, 67]. Currently, most synthetic circuits with degradation tags have relied on the ClpXP as a major protease but Lon and ClpAP have been explored to some extent[68, 69]. Another cytoplasmic protease, ClpYQ, similar to ClpP proteases (ClpXP, ClpAP), is a two-component system with ATPase (ClpY) and proteolytic (ClpQ) chaperone[70, 71]. ClpYQ along with other ATP-dependent proteases regulates the levels of σ^{32} (RpoH), the master regulator for heat stress[72, 73]. A few examples of ClpYQ substrates in *E. coli* include SulA, RscA, and Arc repressor[74-77]. It commonly shows redundancy in the substrate pool for degradation with other proteolytic systems[72, 73] and is not ideal for

synthetic biology applications. In addition to cytoplasmic proteases, membrane-bound proteases, such as FtsH is responsible for degrading both cytoplasmic and membrane proteins[78, 79] and periplasmic protease, Prc that typically degrades proteins with non-polar C-termini (tail-specific protease)[80]. FtsH are ATP-dependent metalloproteases, which require Zn^{2+} divalent metal ions for peptidase activity[81]. FtsH and Prc has not been leveraged in the synthetic biology field, likely because in *E. coli*, it can also recognize and degrade the commonly used ClpXP degradation tag (SsrA tag)[82-84]. However, they have a potential to use in spatiotemporal studies of membrane bound proteins.

1.3. Trans-translational mechanism.

Cellular processes can be error-prone. Sometimes, cells make mistakes, particularly in stressful environments, but they also have mechanisms to correct those mistakes. Pathways responsible for correcting mistakes are important for cell survival. The translation process in central dogma is regulated stringently, but it is error-prone. Bacteria have developed multiple means to tackle such errors and ensure fidelity in protein production[85-88] (e.g., trans-translation mechanism). If the error-correction mechanism is faulty, it leads to cell death. The trans-translational (also called SsrA-mediated system) mechanism is predominant and a critical quality control mechanism where bacteria add the SsrA-tag at the C-terminus of a compromised peptide[85]. In 1979, SsrA was first characterized as 10S stable RNA in *E. coli*[89]. It was named SsrA (small stable RNA) after the identification of the 10Sa gene[90]. The Simpson's group was the first to discover that SsrA adds 11 amino acid (AANDENYALAA) residues in *E. coli* at the C-terminal end of truncated protein[91]. This mechanism is critical for cell function, as evident by growth defects in SsrA gene mutants[92-94]. Further, peptides with SsrA tags are targeted for

proteolytic degradation for recycling amino acids. Trans-translation is also referred to as ribosome rescue since translational errors consist of non-active ribosomes, and SsrA-mediated rescue is employed to recycle ribosomes to maintain cellular functionality.

1.3.1. Mechanism of tmRNA (SsrA).

Translation errors occur because of ribosomal stalling or non-stop translation, a missing stop codon, and if the ribosome continues past the protein-coding region. Ribosomes can stall for several reasons such as a lack of a stop codon (premature RNA)[95] and/or a lack of nutrients (low nutrients, insufficient amino acids, uncharged tRNA, etc.)[96-98]. In some cases, ribosomal stalling is programmed using rare codons and utilized to regulate specific translation rates in the cell[99-102]. For example, translation of MifM (controls YidC membrane protein integration/folding pathway) in *Bacillus subtilis* and SecM (monitors Sec protein export pathway) in *E. coli* is regulated by ribosomal stalling[102]. Most translation errors lead to non-active ribosomes that are still bound to mRNA. The SsrA system is a recycling mechanism. Non-active ribosomes are freed by completing the translation process and adding the SsrA-tag at the C-terminus of the growing polypeptide. The SsrA mediated rescue is accomplished by tmRNA. It is comprised of two domains: the tRNA and the mRNA domain[94]. In *E. coli*, the tRNA domain is charged with the amino acid alanine, and the mRNA domain codes for the 11 amino acid residue AANDENYALAA. The mRNA domain of tmRNA acts as a template for the non-active ribosome while releasing the mRNA template. The first alanine incorporated in the 11 amino acid residues is added from the tRNA domain. The chaperones SmpB (small protein B) and EF-Tu (elongation factor thermo unstable) facilitate the tmRNA rescue mechanism[103-107]. The SsrA system is highly effective in dealing with compromised

peptides. Cells often possess redundant ribosome-rescue mechanisms[85-87]. The other ribosome-rescue mechanisms ArfA and YaeJ still hold potential for synthetic biology. Recently an alternate C-terminal tagging mechanism was identified in *B. subtilis* that adds a poly-alanine tail using an RqcH mediated mechanism that shows homology to eukaryotic and archaeal proteins[88].

1.4. Queueing theory.

Queueing theory is the study of waiting lines. It has traditionally been applied to traffic engineering, telecommunication, and computer networking, where customers compete for processing by the limited servers. Recently, studies of limited resources (queueing theory) have been applied to biological systems, especially biological pathways that contain a bottleneck due to limited resources in the cell. Cellular bottlenecks are important regulating approaches cells use to manage different pathways and hence are key for cell survival. Biological pathways can be regulated through queues (e.g., regulation of sigma factors)[108]. When proteins compete for the enzymatic processing, it forms a queue where an enzyme is limited in numbers. This has been studied mathematically and experimentally[109]. Mathematical models for queueing can further predict the functionality of the biological pathways related to bottleneck[110]. The proteolytic pathway is one of the examples where proteases bottlenecks lead to proteolytic queueing[110-112]. Proteins targeted to proteolysis form a queue when the system becomes overloaded, and they must wait to be degraded because of the protease being limited in numbers. The turnover rate of proteins depends on the effective degradation by proteases, and proteolytic queueing increases the turnover rate. The underloaded regime is when few proteins are targeted to proteases such as ClpXP; they are degraded faster. However, the overproduction of tagged proteins

targeted to ClpXP proteolytic system further overload the system. This results in queue formation. Multiple synthetic biological circuits have exploited proteolytic queueing, particularly biological oscillators[110, 113]. The dual-feedback oscillator[114] relies on queue formation at ClpXP to function. However, the oscillator output can be altered by controlling the proteolytic queue through unrelated proteins tagged with the same degradation tag (SsrA tag)[112].

2. CHAPTER 2: BACTERIAL PROTEASES AND THE THEORY OF PROTEOLYTIC QUEUEING FOR THE DESIGN OF SYNTHETIC CIRCUITS. (REVIEW-PAPER)

2.1. Introduction.

Synthetic biology applies knowledge from multiple disciplines to construct different network topologies, such as toggle switches[13, 15, 115, 116], binary logic circuits[18, 117], or dynamic circuits with positive and negative feedback loops[114, 118-120] to mimic natural system network topologies. Most circuits rely on transcriptional regulation, even though natural systems apply transcriptional, translational, and post-translational regulation to fine-tune protein levels. Only recently, synthetic circuits relying on translational and post-translational regulations have been developed, particularly regulation at the protease level in bacteria[121] as well as eukaryotes[18, 122, 123]. Proteases are important to maintain protein homeostasis in the cell[44, 124, 125] and recognize specific amino acid sequences in the protein, a degradation tag or degrons[30, 126], for active degradation. The degradation tag (amino acids) varies within proteins. The tags are either intrinsically present in protein sequence or can be added post-translationally at the N- or C-terminus of the protein. Such degradation tags, when fused to a protein of interest, result in a fast and tunable degradation by a protease[121, 125, 127, 128].

In the early 1960s, the first theoretical model highlighted the importance of controlling protein degradation rates to obtain robust synthetic circuits, particularly in synthetic oscillators (Goodwin 1965)[129, 130]. The discovery of the degradation tag sequences in bacteria[91, 125] allow us to exploit them as a research tool in synthetic biology. The target proteins in synthetic circuits are typically non-native proteins and are often

expressed with strong, inducible promoters present on a high copy number plasmid leading to a high level of protein output, which can cause a cellular burden on host cells[131-133]. Under this scenario, protein levels are usually diluted with cell division, but this process can be slow and highly dependent on the strain and environment. It further leads to the coupling of the circuit output with cell division[69, 134]. This limits the complexity, temporal resolution, scalability, and ultimately the ability to design more complex circuits. Hence, degradation tags add an extra layer of circuit regulation and provide a handy post-translational method to uncouple the growth rate from a circuit's output. Another important characteristic of proteases is that they are naturally maintained in limited numbers for tight regulation of cellular proteins, hence acting as a bottleneck. Overproduction of tagged proteins saturates these proteases, and due to protease bottlenecks, a proteolytic queue forms when proteins wait to be degraded[110]. The queue formation has been classically applied to computer systems and call centers, and has a significant impact on the output of synthetic circuits that rely on the proteases. Its importance is often underappreciated in the design. Nevertheless, some have intentionally leveraged proteolytic queuing in synthetic circuits to synchronize circuit outputs[112, 135].

The saturation of proteolytic pathways leads to cellular coping mechanisms and portrays redundancy in the degradation pathways. Because of redundancy, when one protease is saturated, a fraction of proteins targeted to the corresponding protease are recognized and degraded by other proteolytic systems[136]. Multi-recognition of tagged proteins for proteases can be another nuisance in building robust synthetic circuits with a limited set of degrons. Furthermore, using limited degradation tags hinders our ability to precisely control degradation and limits our understanding of proteolytic queueing

mechanisms. In the last few years, promising advances have been made to alter the current degradation tags with enhanced specificity and search for new degradation tags with varying protease affinities[137-139].

The advancement in queueing study in biological system have allowed researchers to understand how finite resources (e.g., proteases) can enhance or limit the design of more complex circuits[112, 135, 136]. In particular, the connection between processing pathways in biological contexts and queuing theory has improved our understanding of queueing competition that can be applied as an intentional design attribute in synthetic circuits. Here we will review the use of degradation tags in synthetic biology focusing on two main aspects. First, we discuss recent advances in synthetic biology with available degrons employed in circuit design and alternate degrons with the potential to be used in future designs. Next, we reviewed proteolytic queues and synthetic circuits, particularly in synthetic oscillators where the proteolytic queue has been extensively studied. Finally, we introduce mathematical aspects of queuing theory and available theoretical models to build an intuitive modeling framework to understand synthetic networks in the context of finite resources (bottleneck).

2.2. Bacterial degrons in circuit engineering.

A recent review has highlighted different types of proteases and their application in targeted degradation[121]. This section will shortly summarize the bacterial degradation signals and their application in biological circuits. In bacteria, three types of degradation signals exist: C-terminal[127] (Fig. 2.1A), N-degrons[125, 140, 141] (Fig. 2.1B) and intrinsic or constitutive degrons[128] (Fig. 2.1C). The most extensively studied degradation tag is C-terminal SsrA-tag[142, 143]. It is naturally added to proteins after translational error by the

trans-translational rescue system[144, 145] (Fig. 2.1A). This mechanism frees stalled ribosomes, thus maintaining the ribosomal pools by adding a degradation tag (SsrA-tag) to the C-terminal of unfinished polypeptides to undergo proteolysis[84, 93, 95, 97, 99, 146]. The SsrA tag sequence is conserved in most bacterial species that ends with a common C-terminal motif (LAA) asides from a class of *Mollicutes* (including *Mycoplasma* species), which display a conserved NYAFA motif and longer sequences[147]. The SsrA tag from *E. coli* (*ec*-SsrA) consists of an 11 amino acid sequence (AANDENYALAA) that has binding sites for three chaperones: ClpX, ClpA, and SspB[148]. Chaperons ClpX and ClpA harbor ATPase activity (member of AAA+ family) that unfolds polypeptide in an ATP-dependent manner and transfers to the caseinolytic protease ClpP, which breaks polypeptide bonds to amino acid monomers without the use of ATP[55, 149]. The ClpXP is the major proteolytic system for ec-SsrA tagged proteins, and its proteolytic activity is boosted with the binding of the adapter proteins, SspB[61, 64, 65]. SspB is unessential for ClpXP degradation but increases the substrate specificity to ClpXP[150], which is important to avoid interference of other proteolytic systems. Conversely, ClpS binding to ClpA reduces ClpA interactions with the SsrA tag[67] and is available in the cell to degrade proteins with specific N-degrons targeted to ClpSAP degradation[151, 152] (Fig. 2.2A).

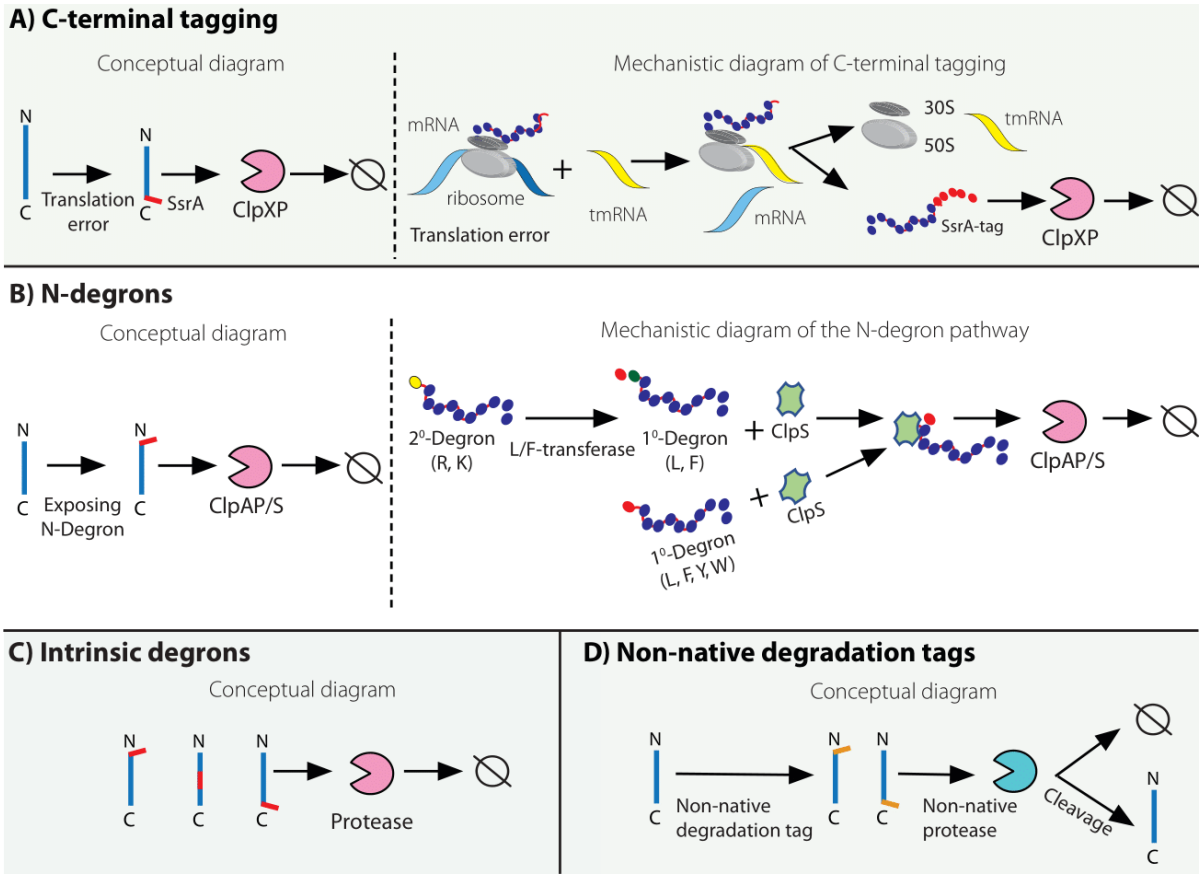


Fig. 2.1. Example of native (A, B, C) and non-native (D) degradation tagging mechanisms. **A)** The post-translational C-terminal tagging and ClpXP degradation system (left). The mechanism of C-terminal tagging (right). Detailed description: tmRNA recruitment during translational error e.g., stalled ribosome and release of mRNA. tmRNA acts as a template by adding the SsrA tag to the end of a polypeptide. Ribosomes are released at the end of the process, and tagged proteins are targeted to ClpXP for degradation**. B)** The post-translational N-degron exposure and ClpAP degradation system (left). The mechanism of N- degron recognition (right). Detailed description: The 1°-degron (L, F, Y, W) is recognized by ClpS. The 2°-degron (R, K) is first converted to 1°-degron state (L or F) by enzyme L/F-transferase. The ClpS bound protein is targeted to ClpAP for degradation. **C)** Intrinsic degrons are amino acid sequence recognized by the protease anywhere in the protein sequence: N- or C-terminus, or internally. Intrinsic degrons are recognized by the corresponding native proteases for degradation. **D)** Non-native degradation tags can be added to proteins, and tagged proteins are degraded or cleaved if the non-native proteases are produced.

Although *ec*-SsrA tagged proteins are primarily recognized for degradation by ClpXP and ClpAP, a small fraction is processed by other proteases, such as Lon, FtsH, and Prc[82, 83, 153, 154]. Protein recognition and degradation by multiple proteases of the same tagged proteins is known as proteolytic crosstalk[136], which hinders the ability to control degradation levels in synthetic circuits tightly, and reduces the ability to construct orthogonal and uncoupled circuits from one another[112, 135, 136].

The application of synthetic circuits is expanding beyond the C-terminal SsrA tags to the use of N-degron. Similar to SsrA tagging system, N-degron pathways are universal, where the set of N-terminal amino acids (N-degrons) in a protein affects its half-life[31, 141, 155] (Fig. 2.1B). ClpS recognizes N-terminal residues in bacterial proteins for ClpAP degradation[141, 152]. The N-terminal destabilizing amino acids have been divided into primary degrons (*E. coli* specific 1°-degrons: Leu, Phe, Tyr and Trp) and secondary degrons (*E. coli* specific2°-degrons: Arg and Lys)[156]. Primary degrons can be directly recognized by the adaptor protein ClpS. In contrast, secondary degrons require the action of an amino acid transferase to facilitate ClpS binding by the addition of a primary degron sequence to the N-terminus[151, 152, 157, 158]. For example, the L/F-transferase (LFTR) enzyme can modify the N-terminus of proteins by adding a Leu or Phe (primary degrons) to a secondary degron[159]. Other degradation signals include constitutive or intrinsic degrons naturally present in the coding sequences of proteins and can be located at any position along their sequence (Fig. 2.1C). The proteins with intrinsic degrons are constantly being monitored by proteases[53, 160-164], although they are often protected from proteolytic action with amino acid scaffolds, and only exposed by external signals such as stress or the action of chaperones[165].

2.3. The application of degradation signals in circuit topologies.

2.3.1. The application of SsrA tag in synthetic circuits.

The *E. coli's* SsrA-tag (ec-SsrA) was first discovered in 1995[91] and soon after applied to study promoter kinetics using fluorescent proteins fused with SsrA to their C-terminus[84, 166]. Hence, the application of SsrA tag widely used in experimental designs from the beginning in synthetic biology. To date, most synthetic circuits, especially dynamic circuits, utilize the ec-SsrA tag for targeted degradation across many bacterial species. Apart from *E. coli*, few other species are tested for SsrA tagged based synthetic circuits includes *bacillus*[167, 168], *salmonella*[169], *mycobacteria*[170], and *cyanobacteria*[171] . The most common design incorporates fusing the ec-SsrA tag to the C-terminus of the proteins in the circuits. However, there is a limitation of using the ec-SsrA tag especially in *E. coli*. Since, SsrA tag has a conserved sequence in most bacteria, *ec*-SsrA tag is recognized by other proteases in the cell and lack exclusive specificity towards ClpXP. Advances has been made towards identifying the molecular players involved in the recognition of the SsrA tag[137] and researchers have found three chaperone binding sequences for ClpX, ClpA and SspB[148]. As discussed in previous section, SspB improves the substrate specificity to ClpXP, hence many synthetic circuits are developed that harnesses SspB's specificity towards ClpXP using SsrA variant, DAS+4[137]. The DAS+4 variant consists of a modified tag in which the ClpX binding sequence (LAA) is mutated to DAS (Asp-Ala-Ser)[137] and further improved by linking 4 additional amino acids (SENY) between SspB and ClpX binding sequences for better binding of chaperones (DAS+4 tag: AANDENY-SENY-ADAS, Fig. 2.2A). It is experimentally validated that DAS+4 variant degrades effectively only in the presence of SspB by targeting it to ClpXP degradation in a ΔSspB strain[137] (Fig. 2.2B). Its potential has also been tested in other species such as *B. subtilis*[168] and

mycobacteria[170]. The additional complexity is added with the DAS+4 tag circuits with a split adaptor system[172] where SspB protein is split into two domains: $SspB_{CORE}$ and $SspB_{XB}$ that can only form a functional SspB in the presence of the antibiotic rapamycin[173-175] (Fig. 2.2C).

Another approach to build complex circuits is by using hybrid tags that depends on two or more distinct proteases. One such hybrid tag where the *ec*-SsrA tag is combined with viral cleavage sites for viral proteases[18, 176] (Fig. 2.1D). The FENIX system (functional engineering of SsrA/NIa-based flux control) is an excellent design example that utilizes a hybrid of *ec*-SsrA tag and NIa (viral nuclear inclusion protein A)[177] tag from the turnip mosaic potyvirus[178]. In the FENIX system (Fig. 2.2D), the absence of NIa proteases in the host allows for active degradation of the protein by ClpXP, but expression of the NIa protease cleaves the NIa-tag, removing the SsrA-tag from the target protein, and stabilizing its levels[177]. So far, FENIX system has been tested in bacterial metabolic engineering to uncouple the production of biopolymers to growth rate[177]. Although the SsrA tag sequence is conserved in most bacterial species, *mycoplasma* species (*Mollicutes*) show large variations in the amino acid sequence of their SsrA tag due to a lack of a ClpXP proteolytic system[143, 179]. These variations have been explored as potential alternatives for synthetic biology applications, in particular SsrA sequences from the species *Mesoplasma florum* (*mf*-SsrA)[139]. The *mf*-SsrA sequence is longer than its analogous in *E. coli* and ends with a conserved NYAFA motif that is recognized by a *M. florum*'s specific Lon (*mf*-lon) protease[147]. The Collins's group explored and built a library of *mf*-SsrA variants[139] and few synthetic circuits are developed based on two variants (pdt#1 and pdt#2) [139, 180, 181]. In *mf*-SsrA tag based circuits, degradation of tagged proteins can only occur if *mf*-Lon is co-

expressed in host cells. However, recent study has shown that *mf*-SsrA is being degraded to some extend by proteases in *E. coli*[182]. Nevertheless, using non-native proteases has some important limitations: the burden of expressing non-native proteases (e.g., *mf*-lon, viral proteases) on the bacterial host is currently unknown especially proteolysis action on the host's proteome. Sauer's group explored application of *mf*-Lon in *E. coli* where they have shown that *mf*-Lon is not able to degrade RscA, a known *E. coli* Lon substrate[147]. But limited study has done to check the effect of *mf*-Lon on *E. coli's* native proteins. Since SsrA tagging system is universal, the scalability of the circuit topologies can be enhanced if there is a diverse library of degradation tags are present. Hence, increasing the number of available degron sequences, especially those that are recognized by proteases other than ClpXP, is necessary in order to provide researchers to mix & match orthogonal degradation tags as needed.

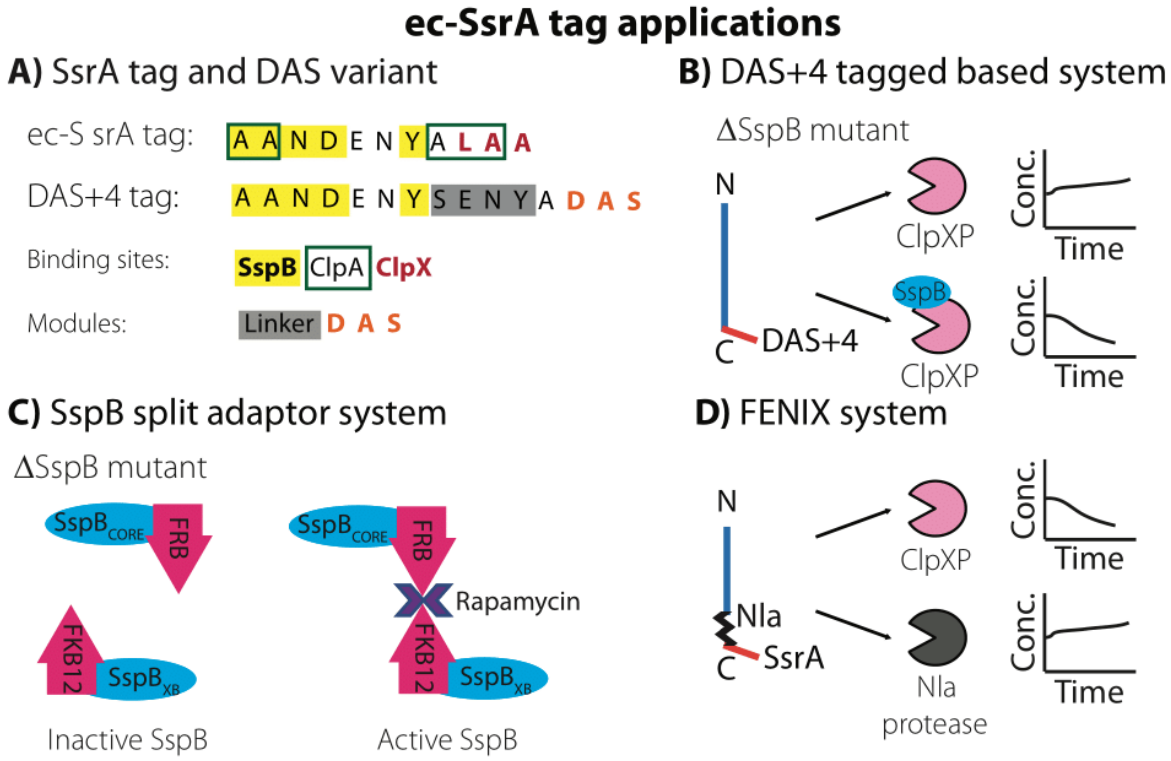


Fig. 2.2. A) The *E. coli's* SsrA tag and variant DAS+4 tag. The ec-SsrA tag with SspB, ClpA, and ClpX binding sites. The DAS+4 tag with retained SspB site, modified ClpX binding site to DAS, and a SENY linker. **B)** DAS+4 tagged based system. In ΔSspB, DAS+4 tagged protein is stable and targets to ClpXP degradation when SspB is produced. **C)** The SspB split adapter system. The SspB is divided into two halfs, $SspB_{CORE}$ (interacts with DAS+4 tag) and $SspB_{XB}$ (interacts with ClpX chaperon). $SspB_{CORE}$ and $SspB_{XB}$ are attached to FRB and FKB12 which interact and form active the SspB protein in the presence of rapamycin antibiotic. This system works with DAS+4 tagged system described in B. **D)** FENlX (functional engineering of SsrA/NIa-based flux control) system uses SsrA/NIa hybrid tag. The SsrA tagged protein is actively degraded by ClpXP. The non-native Nla protease production cleaves the SsrA tag and produces stable protein.

2.3.2. Synthetic circuits based on N-degrons.

The N-terminal pathway has been explored minimally in synthetic biology but holds great potential[126, 183]. Only a few synthetic circuits have been constructed with these degrons so far[184]. The available degradation strategy consists of a hybrid tag containing a stable amino acid sequence followed by a destabilizing N-degron sequence. The stable protein and N-degron sequences are separated by the cleavage site of a viral protease. A stable target protein is produced in the absence of the viral protease as the N-degron is protected from ClpS recognition. Production of the viral protease results in the cleavage of the viral tag, exposing the N-degron to the ClpSAP complex leading to active degradation. A variety of Boolean gates use this concept with three orthogonal viral proteases from Potyvirus (TEVp, TVMVp, and SuMMVp) along with N-degron sequences: Y-Degron (YLFVQ) and F-Degron (FLFVQ)[185]. Hence, using N-degron we can employ ClpAP proteases instead of ClpXP for targeted protein degradation in synthetic circuits.

2.3.3. Application of intrinsic degron sequences in synthetic circuits.

Currently, only a few synthetic circuits have successfully used intrinsic degrons. The first two relied on degradation tags MarAn20 and RepAn70[134, 136]. MarA is a transcription factor responsible for regulating multiple genes involved in several antibiotic resistance pathways[186] and is degraded by the Lon protease[187]. The MarAn20 tag was developed by cloning the last 20 amino acids from the N-terminus of MarA at the end of fluorescent protein encoding gene, and then comparing fluorescent levels with and without the tag. The MarAn20 tag allowed for quick degradation of the fluorescent protein, and was used in synthetic circuits[134, 136]. It also produced a proteolytic queue, which will be discussed later.

RepA is a dimer that binds to DNA as a monomer by the proteolytic action of ClpAP. The N-terminal 70 amino acid sequence (RepAn70) of RepA is degraded by ClpAP[188, 189]. This degradation tag has been construct in a similar manner as MarAn20, shown to lead to quick degradation the fluorescent protein, and was used in synthetic circuits[134, 136].

Though we know of only two intrinsic degrons have been exploited in a meaningful synthetic circuit, other intrinsic degradation sequences hold potential. For example, the Lon proteases specifically targets UmuD (15-29 amino acids)[190], SulA (150-169 amino acids)[191], HipB (HipBc20: 20 amino acids from the C-terminus) [136, 192], SoxS (1- 21 amino acids)[193], and in a 20 amino acid sequence of the β-galactosidase protein (49-68 amino acids)[194]. From these, UmuD, SulA, HipBc20 and SoxS have been studied for potential alternative degradation tags for synthetic biology applications by fusing them to fluorescent proteins and monitoring fluorescence levels over time[136]. Currently, no advanced circuits have been developed though UmuD and SulA tags lead to rapid degradation (suggesting high infinity degradation tags), while HipBc20 and SoxS tagged proteins degraded slowly.

2.3.4. The application in spatiotemporal studies.

The cellular protein distribution is typically non-uniform; hence, spatiotemporal protein expression is gaining importance in synthetic biology to build circuit topologies using native and non-native proteases. Recently, a well-characterized design was implemented to obtain intracellular asymmetries in *E. coli* using a hybrid tag[195]. The circuit uses a split TEV protease bound to the membrane by a PopZ-based polarity system from *Caulobacter crescentus*[196]. A protein of interest is fused with a C-terminal SsrA tag with TEV cleavage site between the protein and degradation tag. Due to PopZ-based polarity, TEV cleavage appears only at the poles. In other cellular vicinity, protein is actively

degraded due to SsrA tag but at the cellular poles TEV protease cleaves the SsrA tag. This results in the protein levels stabilization[195], creating a pattern of expression between the poles and the cytoplasm. In the last few years, there has been an increased interest in developing synthetic spatial patterning strategies in bacteria[22, 197, 198]. This development would allow researchers to better understand fundamental biological processes from developmental biology to tissue engineering[199]. Two membrane proteases, FtsH[81] and Prc (Tsp)[80], hold the potential for application in the design of membrane-localized circuits.

2.4. The basic principle of oscillators.

This section reviews the self-sustaining, dynamic circuit (specifically synthetic oscillator), which improves our understanding of natural dynamic systems using a bottom-up approach. To obtain dynamic activity, a synthetic circuit could express proteins periodically such as wave dynamics. In this case, the protein levels should increase and decrease in periodic format over time (representing sine waves). The output of the synthetic oscillator is typically quantified by its amplitude and period (Fig. 2.3A). Many natural biological systems behave periodically. Some natural examples include the cyanobacterial circadian clock[200], the bacterial cell cycle (Min oscillations)[201], the tumor suppressor p53 protein[202], and the firing of a particular subset of neurons[203]. However, natural systems are complex as they rely on many interconnected pathways that have evolved over many years and are hard to detangle from one another[204]. Despite their complexity, natural oscillators harbor three important qualities: they are robust (maintain their behavior independently of external disturbances), coherent (display a consistent response), and tunable (easily manipulated)[205]. One of the long-term goals in synthetic biology is to build oscillators that

exhibit these properties for industrial and medical applications[206-213], but this endeavor has proven to be challenging.

So far, research has advanced to acquire robust, coherent, and tunable synthetic oscillators to some extent using three core design principles: a delayed negative feedback loop, a delayed positive feedback loop, and, more importantly, in the context of this review, rapid protein turnover[205, 214, 215] (Fig. 2.3B). Other than protein turnover through cell division, the most efficient way to achieve rapid protein turnover is by active degradation. Thus, use of degrons have enabled the uncoupling of the synthetic circuit dynamics from the bacterial growth rate. In other words, degradation tags and protease activity have allowed the development of synthetic oscillators that are independent of the bacterial cell cycle. This allows for shorter or longer oscillations than the cell's division time. Hence, understanding oscillatory dynamics that rely on proteases for protein turnover is closely linked to understanding protein degradation dynamics in the cell. This is confirmed by extensive theoretical work demonstrating that oscillations are critically affected by the speed of degradation of circuit regulators[216-218]. To date, most designed bacterial oscillators are based on the *ec*-SsrA tag (targeted to *E. coli*'s ClpXP) for targeted degradation of both the transcription factors (TFs) controlling circuit dynamics and the output fluorescent reporters[110, 114, 118, 119, 219-221]. The variation in degradation rate changes the output of the synthetic oscillator with variable periods and amplitudes over time.

2.5. The importance of proteases in dynamic systems.

Dynamic circuits often rely on the proteolytic system and proteolytic queues (bottleneck). Proteases are kept at relatively low numbers in the cell (Table 1), and are a limiting resource for protein degradation. Before we talk about the importance of proteases

in synthetic circuits, it is important to understand how proteases regulate natural bacterial pathways and form proteolytic queues. Because of the low number of proteases in bacteria, cells are accustomed to proteases becoming saturated with targeted proteins. This saturation (bottleneck) leads to the formation of a proteolytic queue (a line of proteins waiting to be degraded by a finite number of proteases) within the cell. Queueing theory is employed to understand and predict the effect of the buildup of tagged proteins. A good example of the application of queueing theory in the macro world is at a restaurant: Customers wait to be served and start accumulating if the server is overloaded. The goal of a restaurant owner is to have the proper number of servers at a given time to accommodate the customers but too many servers will drive up cost. The customers in the cells are the proteins, and the server are the proteases. Customers (proteins) wait to be served and start accumulating if the servers (proteases) are overloaded, proteolytic queue forms due to saturation of proteases with proteins targeted for degradation.

Proteolytic queues can be an important regulatory signal, especially during stress. Sigma factors[108, 222-225] such as σ^S and σ^{32} levels are, in part, regulated during stress such as antibiotic survival[225, 226] through proteolytic queues. σ^S controls genes related to stationary phase and is actively degraded by ClpXP[222]. The number of unfolded proteins increases during the stationary phase, but ClpXP cannot keep up with the influx of demands resulting in a proteolytic queue building. In an underloaded state, σ^S is quickly degraded by ClpXP. However, as ClpXP becomes saturated, σ^S levels increase in the cell because it must wait in the queue to be degraded. The higher level σ^S allows it to activate the expression of stationary phase-related genes including the expression of the σ^S gene (self-expression)[223, 224, 227]. *In vivo* ClpXP is speculated to work near saturation[111]. Hence when

circuit regulatory elements are fused with SsrA tags and overproduced, they often have minimum effect on the cell's growth rate. However, the specific impact synthetic circuits have on cell physiology has not been explored meaningfully. In general, our understanding of the burden that synthetic systems have on the cell's physiology is lacking. However, there is a growing push in the community to understand this better. Nevertheless, the advantage of utilizing degradation tags and proteases in the dynamic system circuits is they can be engineered into the system without causing effects on growth rates (at least in lab settings).

The synthetic oscillator output requires rapid turnover of regulatory elements, which can be achieved by tagging proteins with degradation tags for active degradation without affecting cell physiology. When the turnover of regulatory elements relies on proteases, the formation of a proteolytic queue becomes particularly relevant in the context of synthetic oscillators. The formation of proteolytic queues with regulatory elements also adds time delay in the circuit to acquire improved output (see next section). Synthetic oscillator based on proteases for turnover rates and applying proteolytic queue for better functioning has another advantage to controlling oscillatory output. It was previously demonstrated that queueing leads to coupling mechanisms of two unrelated proteins targeted to the same proteolytic system via the use of the same degradation tag[110]. Hence, the oscillatory output can be altered by changing the proteolytic queue size [105, 211, 219]. From a theoretical perspective, the theory of oscillations has been extensively modeled and characterized over the years, even long before the first experimentally built oscillators appeared. The modelers to understand oscillatory dynamics typically use ODEs; however, an important limitation is these models very rarely consider queueing effects. In next

section we discuss the design of oscillators that depend on proteolytic queues, and few exceptions.

Table 2.1. The average number of known proteolytic units in *E. coli* during exponential and stationary phase. The underscore indicates the number of units that build a functional enzyme (e.g., $ClpX_6$ indicates the number of ClpX hexamers per cell). Note that in bacteria, highly expressed proteins (e.g., ribosomes) are about 27,000 molecules/cell[228]. The numbers described here may vary by strain and environmental factors.

	Molecules/cell	
Protease	**Exponential**	**Stationary**
Lon[229]	500	unknown
$ClpX_{\mathbf{6}}$[230]	75-100	75-100
$SspB_{\mathbf{2}}$[230]	140-160	140-160
$ClpA_{\mathbf{6}}$[230]	40-50	150
$ClpP_{\mathbf{14}}$[230]	100	250-300
ClpS[230]	250-300	250-300
FtsH[231]	400	unknown

2.6. The working of synthetic oscillators.

Synthetic oscillators' core design principles include delayed negative feedback loops and a rapid turnover rate. The rapid turnover rate is achieved with targeted degradation via degron sequences for synthetic oscillators that rely on proteases[205]. The negative feedback loop is delayed in part due to the time it naturally takes for transcription, translation, and protein maturation processes, but it can also be enhanced with the formation of proteolytic queueing. As queues build up, the amount and availability of tagged regulatory elements in the oscillatory circuits increase, this enhances time delays in expression[109-111]. A

negative feedback loop (e.g., an action of a repressor) allows periodic protein expression because it represses the expression of circuit regulators (repressors and/or activators). Though delayed negative feedback loops are sufficient to achieve oscillations, as of yet, no robust biological oscillator has been produced that solely relies on negative feedback alone[112]. The addition of positive feedback loops is a key feature that can increase the robustness of oscillations[114]. The addition of positive feedback loops (action of an activator) can indirectly affect queueing dynamics. The presence of an activator allows higher production of circuit regulators. Since they all are typically targeted for proteolysis via the same degron sequence (e.g., SsrA), proteolytic queues build up faster, delaying both the positive and negative feedback loops. The potential importance of proteolytic queueing in synthetic oscillators was first discussed in the dual-feedback oscillator (DF)[114], although the output of the first experimental oscillator built, the repressilator[118] also relies on proteolytic queueing to some extent, as all proteins are tagged to be degraded by ClpXP (Fig. 3C). The repressilator operates on the most basic design principle, where only negative feedback loops are involved with three mutually repressing genes (lacI, tetR, cI), each tagged with an ec-SsrA tag (Fig. 2.3C). The reporter fluorescence gene (GFP), placed on a different plasmid, works as an indicator of the dynamics of the repressor cI. GFP, however, is tagged with the SsrA variant AAV (Ala-Ala-Val), which shows a slightly slower degradation rate compared to the original ec-SsrA tag[166]. The repressilator is a first functional oscillator but lacks in robustness and coherence, as only a small percentage of cells exhibit oscillations, and these are not synchronized[118]. The dual-feedback oscillator (DF)[114] appeared as a second-generation oscillator 8 years later to incorporate a positive-feedback loop along with a negative-feedback loop in its design (Fig. 2.3D). Instead of

three promoters, the DF contains the hybrid promoter $P_{lac/ara}$[232] that negatively controlled by LacI and positively controlled by AraC. The circuit design includes AraC, LacI and fluorescent reporter GFP (yemGFP) under the same $P_{lac/ara}$ promoter, which repress and activate themselves respectively. AraC and LacI activities can be turned on by the addition of arabinose and IPTG, where arabinose enhances AraC binding and IPTG inhibits LacI binding in a dose dependent manner. Furthermore, oscillations are retained at different temperatures (37°C or 25°C), in different media, and across organisms[114, 169]. To reduce the cell-to-cell variability due to the intrinsic stochastic nature of gene expression in the dual-feedback oscillator, a modified version, the synchronized or quorum oscillator (AaiA-SsrA, LuxI-SsrA, CFP-SsrA), was designed exploiting quorum sensing mechanism and achieve entrainment via cell-to-cell communication[119]. Entrainment is the phenomenon to synchronize circuit output in a definite period and amplitude using an external signal.

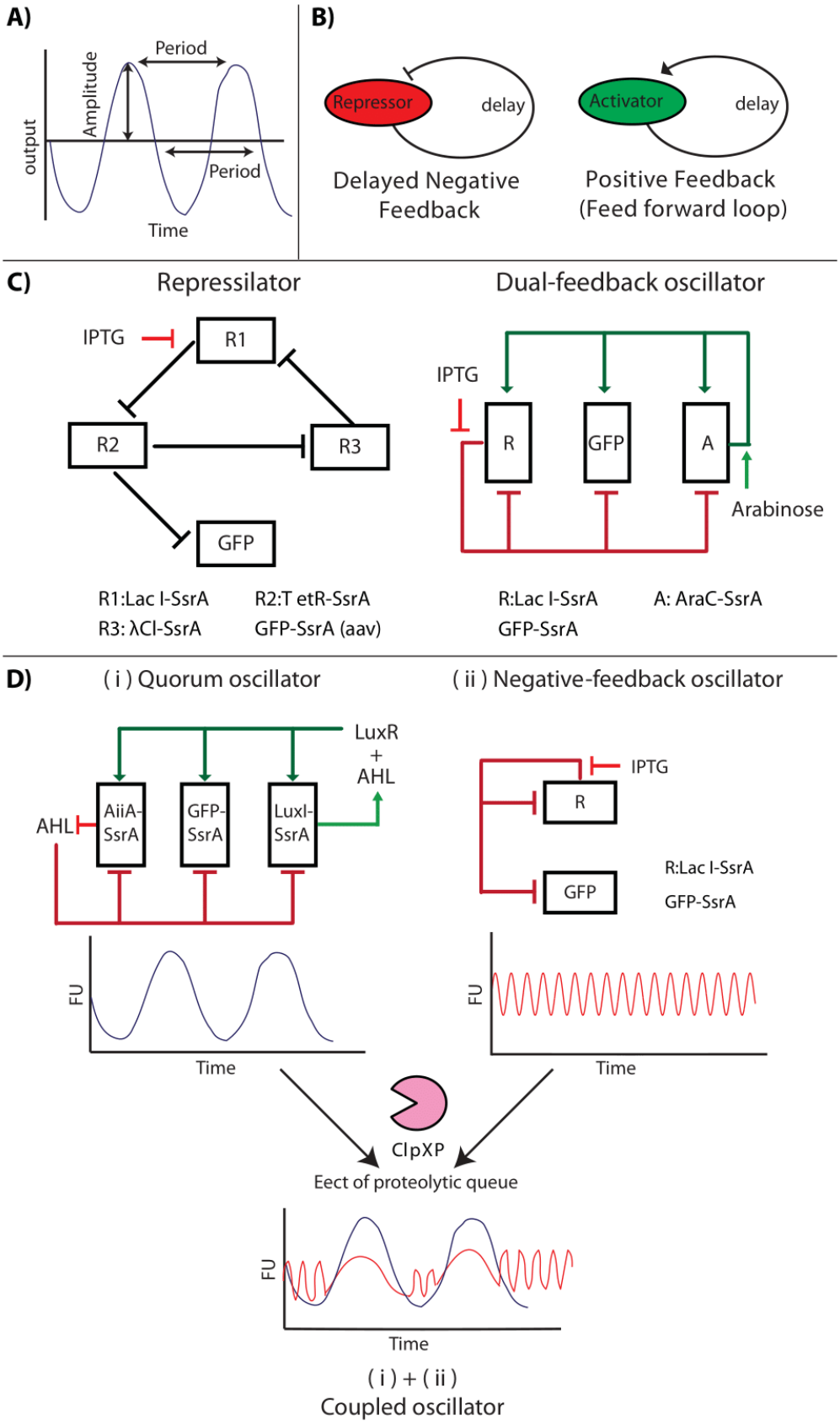

A)
Period
Amplitude
output
Period
Time
B)
Repressor
delay
Delayed Negative Feedback
Activator
delay
Positive Feedback (Feed forward loop)
C)
Repressilator
IPTG
R1
R2
R3
GFP
R1:Lac I-SsrA
R2:T etR-SsrA
R3: λCI-SsrA
GFP-SsrA (aav)
Dual-feedback oscillator
IPTG
R
GFP
A
Arabinose
R:Lac I-SsrA
A: AraC-SsrA
GFP-SsrA
D)
(i) Quorum oscillator
(ii) Negative-feedback oscillator
LuxR + AHL
AHL
AiiA-SsrA
GFP-SsrA
LuxI-SsrA
FU
Time
IPTG
R
GFP
R:Lac I-SsrA
GFP-SsrA
FU
Time
ClpXP
Eect of proteolytic queue
FU
Time
(i) + (ii)
Coupled oscillator

Fig. 2.3. A) Period and amplitude of sine wave. **B)** Two important regulations of the oscillatory system. Delayed negative feedback where repressor in the synthetic oscillator represses regulatory elements in a delayed manner. Positive feedback where activator in synthetic oscillator activates regulatory elements. **C)** Basic design of repressilator and dual feedback oscillator. In repressilator, three repressors R1 (LacI), R2(TetR) and R3(λCl) regulate each other's expression. One node R1(LacI) is externally controlled with an IPTG inducer. Repressors are tagged with original SsrA-tag and GFP with SsrA-variant (aav) which is actively degraded by ClpXP. In dual-feedback oscillator design, repressor (LacI) represses regulatory elements (delayed negative feedback), and activator (AraC) activates regulatory elements (positive feedback loop). Both nodes are controlled with inducers (IPTG and arabinose) because of the same promoter. Regulatory elements (R-LacI and A-AraC) and reporter (GFP) are tagged with SsrA-tag for rapid protein turnover. **D)** Two oscillator- quorum and negative feedback oscillator outputs (left) when they are independently present in the bacteria that depend on the same proteases (ClpXP) for rapid protein turnover and output (right) when they are present together in the bacteria. Coupling (synchronized output) of oscillators occurs due to the effect of the proteolytic queue (proteolytic bottleneck).

2.7. The proteolytic queue controls the output of several synthetic oscillators.

The change in oscillator output is possible by changing the proteolytic queue as oscillations depends on the queue size. In 2017, another potential effect of queueing was explored, entrainment of the oscillatory output in *E. coli* population[112, 233]. Entrainment is a process in which an external signal synchronizes the oscillations in a population onto a common period and amplitude. Hence, if the queue is actively modified with an external signal in a controlled manner by producing an unrelated fluorescent protein with the same degradation tag, the oscillator will start following the pulse signal. As such, the external signal is causing the oscillator to follow the signal because it has been entraining the oscillator. Proteolytic queue dependent entrainment of oscillator has been studied mathematically[218] and validated experimentally using microfluidics and microscopy, where the authors confirmed that queueing can be employed as an entrainment signal to achieve coherent oscillatory responses across a population of single cells[112, 135]. The dual-

feedback oscillator (DF) and quorum oscillator are shown effective entrainment with pulsing inducers for constitutive module tagged with the same degron sequence (CFP-SsrA) [112, 135].

As we can entrain the oscillator output using proteolytic queue, it can also be used in coupling of two independent oscillator outputs[135]. Prindle et al., demonstrated that the period and amplitude of two independent oscillators could be synchronized via queueing effects to form a synchronized hybrid-oscillator[135]. They used a version of the dual-feedback oscillator (LacI-SsrA and YFP-SsrA) that lacks the positive feedback loop (AraC-SsrA) and synchronized its output with the quorum oscillator (AaiA-SsrA, LuxI-SsrA, CFP-SsrA)[135] (Fig. 3E). Each circuit oscillates independently with a specific period and amplitude when they are present in the cell on their own, but the combination of both in the same host results in a strong coupling and oscillatory synchronization[135]. This coupling was argued to being associated to the same proteolytic queue, as all proteins in both oscillators are tagged with the same SsrA-tag and share the ClpXP protease for degradation. The coupled genetic oscillator output is more complex than individual oscillators. As proteolytic queues are susceptible to changes in protein levels, the coupled oscillator can detect a small change in the input signal increasing its sensitivity. It also shows higher robustness and possess greater coherence than the individual oscillators[135].

As proteolytic queue is valuable to obtain entrainment and synchronization of the oscillators, oscillator based on proteases for turnover rate typically shows period-amplitude dependency[234]. Mostly due to the degradation rate of the tag is relatively constant. Building oscillators with tunable and independent amplitude and period is a sought-after property as this is useful across a range of applications especially in biosensors. Tomazou et al.

identified that proteolytic bottlenecks are a major impediment to achieve independent period modulation and proposed new designs based on a theoretical framework in which the removal of queues either by tweaking the expression levels of proteases or by the use of orthogonal degradation tags enabled such modulation[234].

2.8. Protease independent synthetic oscillators.

Most synthetic oscillators used degradation tags and proteolytic queue to achieve output, but few exceptions are present that do not depend on proteases for rapid turnover and exhibit relatively longer period. The repressilator was modified to remove all SsrA tags and, used sponge elements (additional TF binding sites) to reduce the availability of free transcription factors[235]. Sponge elements is an alternative for increasing turnover rates of regulators and can be used to decouple the oscillatory output from cell division in the absence of degradation tags[236]. However, the protease independent repressilator oscillates with increased period (10-14 generations) compared to original repressilator (avg period 160±40 min)[118]. Recently a new family of synthetic circuits based on CRISPR interference has emerged, where oscillator design uses the action of dCas9 protein with specific guide RNA (sgRNA) as a negative feedback loop instead of repressor transcription factor[134, 237]. This design has an advantage over the use of repressor (TF), as the action of dCas9-sgRNA is very specific and sgRNA can be developed for any promoter sequence. The dCas9-sgRNA system can be applied for multiple promoters simultaneously in the same circuit. In the dCas9-repressilator design, only one promoter is controlled by action of dCas9 system[237]. The function of LacI is performed by the dCas9 protein and a promoter specific guide RNA (sgRNA) that binds to the P_{lac} promoter imitating LacI's role in the original repressilator. The change in the design still sustains oscillations over many generations. In

the dCas9-repressilator design, the turnover of TetR and λCI are still dependent on the ClpXP proteolytic system as they harbor a SsrA-tag. However, dCas9 is untagged and the sgRNA turnover rate depends on RNases instead of proteases, as well as on dilution due to cell division[237]. The dCas9-repressilator outputs longer periods (~12 hr)[237] as compared to the original repressilator (avg period 160±40 min)[118]. The authors successfully reduced the oscillatory period by adding "sponge elements" for extra binding sites of sgRNA and dCas9[237], highlighting the importance of rapid turnover rate of regulators in the design. Another group re-designed repressilator, CRISPRlator, where all modules are dependent on CRISPRi system[134]. The CRISPRlator utilizes three sgRNAs with different binding sites for three constitutive promoters controlling the expression of corresponding sgRNAs with fluorescent proteins. The fluorescent proteins (mCherry, mCitrine and Cerulean) report the behavior of each sgRNA. The fluorescent reporters are tagged with the degron sequences MarA, MarAn20 and RepAn70, respectively. CRISPRlator is the first and only dynamic circuit that rely on ClpAP and Lon proteases for turnover of fluorescent proteins. Since tagged fluorescent markers are targeted to two different proteases and are not involved in the operation of oscillators, proteolytic queue has minimum effect on the CRISPRlator output. The CRIPRlator works by the successive repression of sgRNAs, and the turnover rate depends on the action of RNase in the cell and cell division instead of protease degradation. Hence, CRISPRlator outputs longer periods (10-12 hrs) and are coupled to the growth rate to some extent similar to dCas9-repressilator.

From these studies, it is evident that the protease independent oscillators can output oscillations, typically with longer period. However, oscillators that utilizes degradation tag and active degradation for rapid turnover of regulators are more robust with shorter

periods and easy to manipulate their output by changing proteolytic queue. Hence, to help understand queuing effects better we will next introduce theoretical approximations that could assist in better characterizing synthetic systems.

2.9. Conclusion.

The use of degradation tags in the synthetic circuits has significantly improved our ability to control circuit outputs especially, to achieve spatiotemporal patterning in the cell. As most circuits exploit ClpXP proteases through the use of SsrA tag, it is important to expand the degradation tag library that rely on other proteases for active degradation. Recently, alternative degradation sequences are used in building synthetic circuits that includes N-degrons[141], or intrinsic degrons[136, 164]. They are tested in different synthetic circuits[69, 136, 185] but the use can be applied in combination with other tags. Few viral proteases have already used in combination to create a hybrid tags with native bacterial proteases[176 139].

Novel degradation tags that are specific to one protease can be beneficial to build more complex and orthogonal synthetic gene networks. The orthogonal degradation tag-protease system can be used to avoid the coupling of genetic networks and two or more circuit topologies can be tested in a same host that function independently. Non-orthogonal degradation tags are prone to proteolytic crosstalk due to proteolytic queuing[110, 238] and can be a nuisance for scaleup. However, some have intentionally used proteolytic queueing for coupling of genetic networks particularly synthetic oscillators via synchronization and entrainment[112, 135]. The proteolytic queue in the synthetic oscillator adds time delay for regulators which is important to build robust circuit[218] and can be used to alter oscillatory output by changing the queue size. Since the degradation rate of tagged proteins are

relatively constant, oscillators relying on proteases often exhibit period-amplitude dependency[234]. That means, increasing amplitude, increases period. But developing oscillator that can be modified period and amplitude independently is the goal in synthetic biology. As a result, researchers have started to engineer circuits away from degradation tags[134, 235, 237]. This approach comes with a price as the oscillators built are slower and less tunable. Hence, understanding queuing phenomenon will help us to develop robust and tunable synthetic circuits, particularly by altering period and amplitude independently with controlled manner. For that, deconstructing protease pathway to identify molecular player responsible for queueing can be helpful to fine tune proteolytic queue as needed. Another approach is to exploit the use of orthogonal degradation tags for different circuits to reduce queuing coupling and build scalable circuits.

3. CHAPTER 3: DECONSTRUCTING CLPXP PATHWAY TO UNDERSTAND PROTEOLYTIC QUEUEING MODEL.

3.1. Abstract.

In bacteria, proteolytic queue forms due to saturation of proteases with substrates. Several synthetic circuits rely on proteolytic queues at the ClpXP proteases, and they are used in fundamental research and biosensors. The processing rate of ClpXP is relatively constant and depends on the affinity of the degradation tag (SsrA tag). To improve the current designs of the synthetic circuits, we aim to change the affinity of the SsrA tag by deconstructing the SsrA sequence to be processed faster. Our goal to make fast-acting dynamic circuits with rapid degradation by increasing the affinity of the degradation tag. We successfully developed an ultra-high affinity SsrA variant, LAA-LAA, that degrades significantly faster than the SsrA tag. Most dynamic circuits are based on ClpXP proteolytic queueing, but the queueing mechanism is still explorative. To understand the cause of ClpXP queueing, we examined the three major proteins of the proteolytic system: ClpP (protease), ClpX (ATP-dependent chaperone), and SspB (adapter ATP-independent chaperone). We increased each component and monitored alterations in the established queue, and the results support ClpX being responsible for queue formation. The knowledge gained in this study can be further applied to develop advanced genetic circuits.

3.2. Introduction.

The overall protein levels in the cell are maintained by energy-dependent proteolytic systems, primarily including ClpXP, ClpAP, and Lon[124, 239]. They recognize specific amino acid sequences, called degradation tags, to target proteins for degradation. In *E. coli*, ClpXP is a well-studied proteolytic system, and the primary protease to degrade SsrA tagged proteins. This system has been successfully exploited in several synthetic circuits[114, 240, 241]. The SsrA tagged protein is also degraded at low levels by other proteolytic systems such as ClpAP, Lon FtsH, and Prc[82-84, 153, 154, 242]. Only a fraction of the SsrA tagged protein are targeted to these other proteolytic systems, and the protein affinity depends on the SsrA tag sequence[30]. Most synthetic circuits currently rely on the ClpXP proteolytic system that utilizes the SsrA tag because it is a high-affinity degradation tag for ClpXP. This means that the proteins will be degraded relatively fast by ClpXP protease allowing for fast-acting dynamic circuits. But interference from other proteolytic systems hinders the full potential of the ClpXP system. We aim to develop a SsrA tag variant with ultra-high affinity towards the ClpXP proteolytic system to improve circuit design. We would also like to make this variant more specific to ClpXP to minimize crosstalk with other cellular proteases. We hypothesize that increasing the SsrA tag affinity towards ClpXP can decrease the fraction of the SsrA tagged proteins recognized and degraded by other proteolytic systems.

When SsrA tagged proteins are overproduced (overloaded regime), they form a queue because proteases are limited in numbers i.e., proteins are waiting to be degraded[110]. The cell uses proteolytic queues to monitor many intracellular pathways such as sigma factors[108, 222-224]. Sigma S regulates stationary phase related gene, but proteases are responsible for sigma S regulation, particularly ClpXP. During exponential phase, the

sigma S is degraded quickly by ClpXP. As nutrients become low or the cell enters stationary phase, number of misfolded protein increases, readily saturating ClpXP. As ClpXP saturates, proteins targeted to ClpXP including sigma S wait in line to be degraded and the availability of sigma S in the cell increases due to formation of proteolytic queue. The build-up of Sigma S can then activate genes required for stationary phase. Proteolytic queues have been studied extensively over the years theoretically and experimentally[110, 111, 238] and have been applied in synthetic circuits, particularly dynamic self-sustaining synthetic oscillators[114]. Nevertheless, the underlying mechanism of proteolytic queueing is still exploratory.

The proteolytic queue occurs at the ClpXP proteolytic system, but we do know which component of the system that causes the waiting line (bottleneck). Based on the current state of knowledge, the queue could form at ClpP (protease), ClpX (ATP-dependent chaperone), or SspB (ATP-independent adapter chaperone that binds the SsrA tag and brings it to the ClpX chaperone[243]). The ClpX chaperone is a hexameric protein with an ATPase module of the AAA+ family (ATPase Associated with diverse cellular Activities) that uses energy from ATP to unfold and translocate the polypeptide into ClpP[244]. ClpP breaks polypeptide bonds to amino acid monomers without the use of ATP. The SspB adapter protein is not required for the degradation, but it enhances substrate specificity for ClpXP degradation[65, 243]. We hypothesize that the queue could form during the transportation of the protein (SspB, ClpX), the unfolding of the protein (ClpX), or the degradation of the protein (ClpP).

In this study, we propose to identify the key components involved in the process of ClpXP queueing, which will lead to a deeper understanding of ClpXP proteolytic systems.

We also modified the SsrA tag according to the chaperone binding sites to develop ultra-high affinity SsrA variants towards the ClpXP proteolytic system to decrease the turnover rate of the SsrA tagged protein. This study aims to use queueing theory to deconstruct ClpXP proteolytic pathways to design a synthetic circuit with increased robustness. As mentioned in earlier chapters, ClpXP is often used in synthetic systems, but it also has importance in the medical field. ClpXP is found in most bacteria but only found in human mitochondria, making it a potential drug target. A newly discovered ADEP4 antimicrobial drug has been developed that hyperactivates protease (ClpP)[245, 246]. This drug can be an alternative approach to battle against multi-drug resistant gram-positive bacterial infection[245, 247, 248] that will have minimal effect on human cells. Hence, a better understanding of the ClpXP proteolytic system may lead to the discovery of new drug targets.

3.3. Results.

The degradation rate of the SsrA tagged protein is relatively constant. Many researchers have attempted to change the degradation rate, but most SsrA tag variants constructed display lower affinity than the wild type tag[137, 166]. The most widely used *E. coli* SsrA tag is well conserved, has 11 amino acids (AANDENYALAA) with known binding sites for three chaperones, ClpX, ClpA, and SspB (Fig. 3.1A). We hypothesize that we can improve ClpXP affinity if we avoid the competition of multiple chaperones binding to the SsrA tag. The chaperon binding sequence is depicted in fig. 3.1A: ClpX (LAA), SspB ($AAND_{XX}Y$) and ClpA (AAxxxxxALA). Hence, the SsrA tag is known to process by ClpXP and ClpAP proteolytic systems (Fig. 3.1B). To make ClpXP specific SsrA variant, we modified ClpA binding sequence. We also tested ClpX and SspB binding sequences in this study. We further added multiple copies of binding sequence to allow better chaperone interaction.

3.3.1. The SsrA variant, LAA-LAA display ultra-high affinity towards ClpXP.

We constructed the library of SsrA tag variants shown in table 3.1 with corresponding chaperone binding sites. We included previously modified variants for comparison mostly where ClpX binding sites are mutated to LDD and DAS[137]. We eliminated ClpA competition by modifying ClpA binding sequence[148]. Each tag variant was constructed downstream of a fluorescent protein CFP with an inducible promoter. We used $P_{lac/ara-1}$ hybrid promoter[232] of P_{BAD} and $P_{Llac01.}$ AraC activates P_{BAD} promoter and LacI represses P_{Llac01} promoter. Hence, $P_{lac/ara-1}$ is controlled by AraC and LacI. Use of this hybrid promoter is advantageous as it stringently controls gene expression and further reduces noise in the design (less background expression). The expression of $P_{lac/ara-1}$

promoter is controlled by two inducers, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and arabinose. The IPTG inhibits repressor LacI binding to the promoter and arabinose binding to AraC facilitate AraC binding to the promoter. Hence, the concentrations of IPTG and arabinose facilitate CFP expression. The CFP-tagged levels are normalized with CFP-untagged levels at a given concentration of inducers, and calculated % CFP fluorescence unit (FU) using the formula, 100 X ($CFP_{Tag}/CFP_{Untagged}$). The CFP levels were measured at population levels with plate-based assays (batch detection) in a 96-well plate reader. First, we tested all the tags at constant inducer levels, 100 μM IPTG and 0.2 % Ara, to identify the tags that exhibit higher affinity than the SsrA tag (Fig. 3.2). Two tags $SsrA_{2X}$ tag and LAA-LAA tag displayed lower % CFP and were consider for further analysis. However, we also tested few interested candidates.

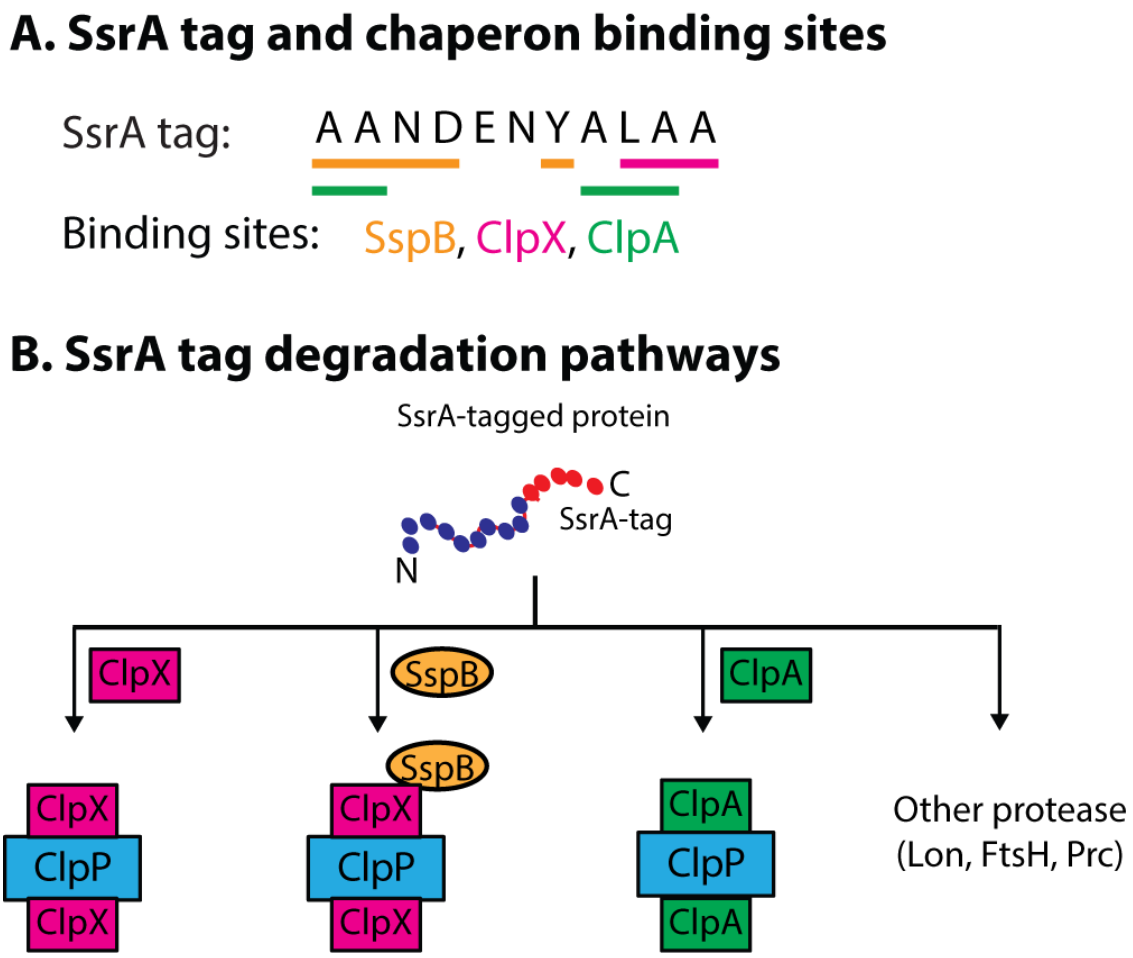


Fig. 3.1. A The Original *E. coli* SsrA tag contained 11 amino acid residues added to the C-terminus of a polypeptide. Color-coded ClpX (pink), SspB (orange), or ClpA (green) binding sites. **B.** SsrA tagged protein is recognized by either ClpX (pink), SspB (orange), or ClpA (green) chaperones to target it to the respective proteolytic system. Other proteases can also degrade a fraction of SsrA tagged protein.

Table 3.1. SsrA tag variants.

SsrA tag amino acid sequence	**Number of chaperone binding sites**
SsrA: AANDENYALAA	1 SspB, 1 ClpX, 1 ClpA
$SsrA_{2X}$: AANDENYALAA-AANDENYALAA	2 SspB, 2 ClpX, 2ClpA
AANDENYDLAA	1 SspB, 1 ClpX
LAA	1 ClpX
LAA-LAA	2 ClpX
LAA-LAA-LAA	3 ClpX
AANDENY	1 SspB
AANDENY-AANDENY	2 SspB
AANDENYADAS	1 SspB
AANDENYALDD	1 SspB

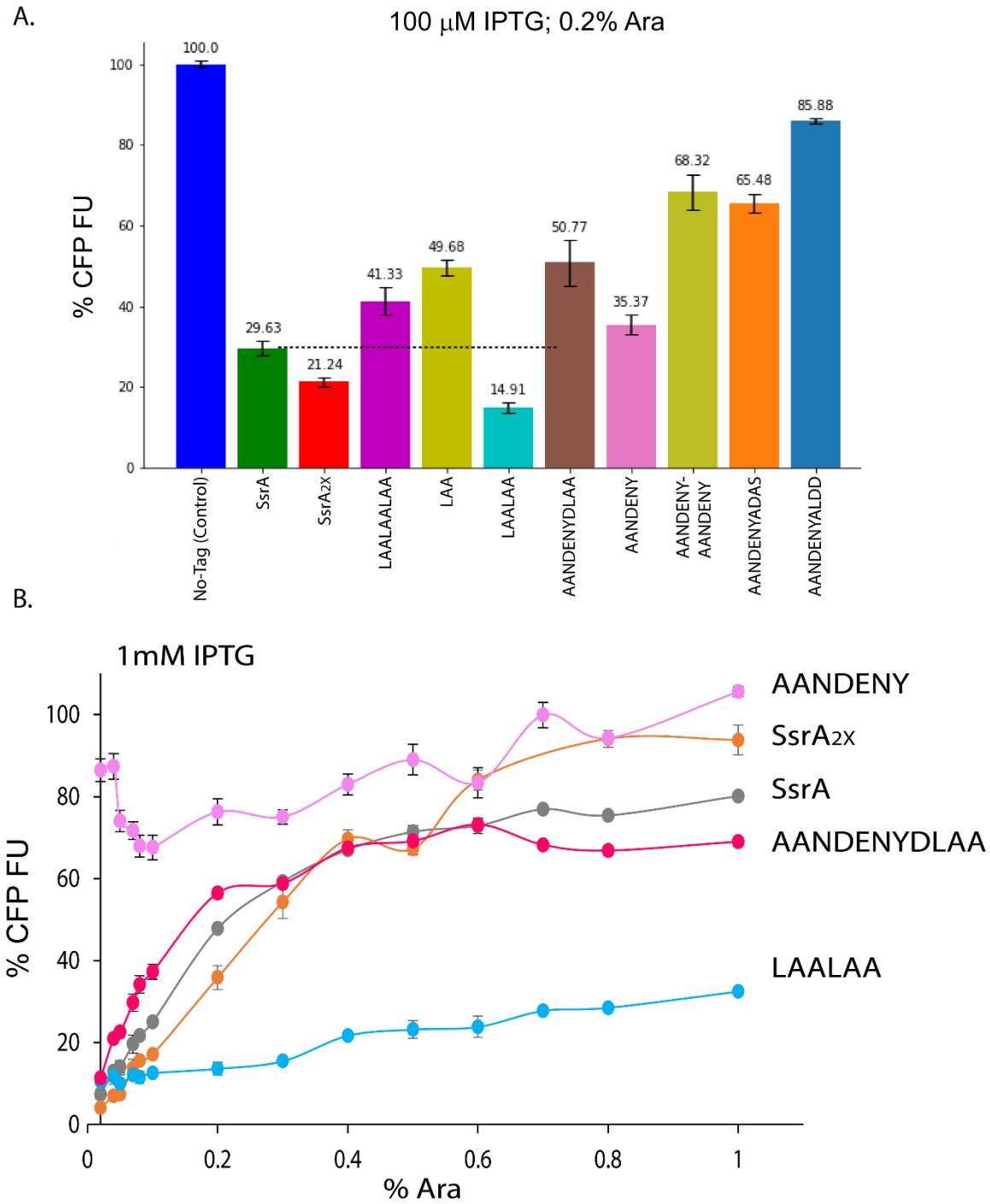


Fig. 3.2. Percent CFP levels of degradation tags. The CFP is expressed from the $P_{lac/Ara}$ promoter. **A.** % CFP levels of degradation tags at constant inducer levels, 100 μM IPTG, and 0.2% arabinose (Ara). **B.** % CFP levels of degradation tags with increasing concentration of inducer, Ara. Inducer IPTG concentration was kept constant at 1 mM, and Ara concentration was titrated from 0.02% to 1%. The CFP-tag levels are normalized with untagged CFP to get % CFP using formula, 100X ($CFP_{Tag}/CFP_{Untagged}$).

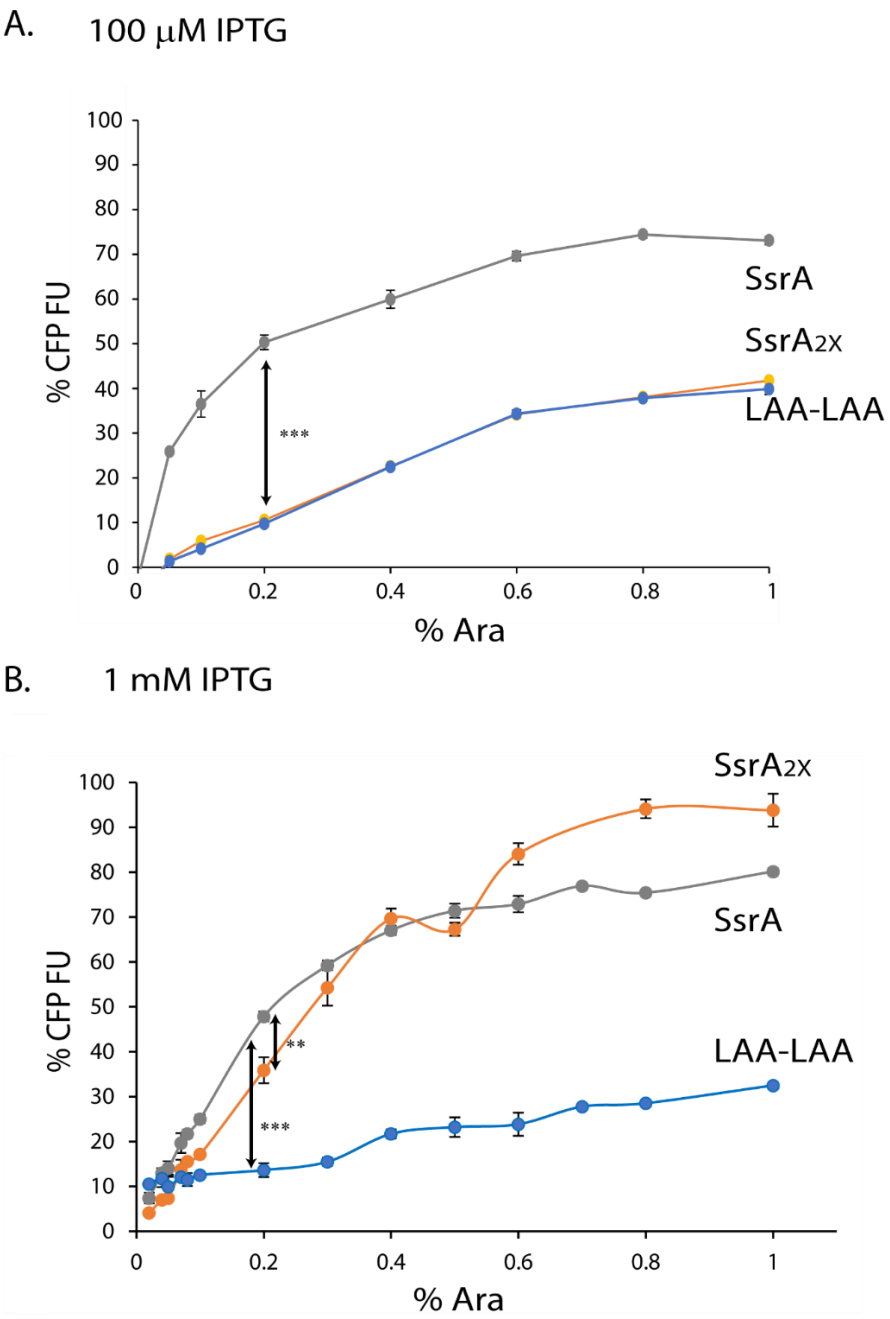


Fig. 3.3. Percent CFP levels of degradation tags. Promoter $P_{lac/Ara}$ controls $CFP_{(Tag/Untag)}$ expression. Inducer IPTG and arabinose (Ara) are added to express CFP. CFP untagged were considered producing maximum CFP levels with no degradation. % CFP was calculated using the formula: 100X ($CFP_{Tag}/CFP_{Untagged}$). **A.** % CFP at inducer 100 μM IPTG and 0.2% Ara. The $SsrA_{2X}$ and LAA-LAA variant shows a significant decrease in % CFP making them faster degrading tags. **B**. Inducer IPTG concentration is kept constant at 1 mM and arabinose concentration is altered from 0.02% to 1%. The LAA-LAA variant shows lower % CFP levels confirming it higher affinity tag. The $SsrA_{2X}$ tag shows faster and higher build-up than ec-SsrA. (p values: *<0.05; **<0.01; ***<0.001).

When we titrated arabinose concentrations from 0% to 1%, keeping IPTG at 100 μM (Fig. 3.3A) and 1 mM (Fig. 3.3B), we recorded the % CFP FU level increasing with the increase in arabinose concentration in a 96-well plate reader (Fig. 3.3). The SsrA tag is a high affinity tag that gets processed faster by ClpXP, hence at lower concentration of inducers, % CFP for SsrA tagged protein is low. But as we increased arabinose concentration, CFP level increases, mainly due to accumulation of proteins due to saturation of ClpXP protease. If all the SsrA tagged proteins are being degraded quickly, we would not detect any CFP levels. Hence, the titration curve is the result of active degradation of tagged protein and protein build-up due to proteolytic queue. When the SsrA tag titration curve is compared with LAA-LAA tags, the LAA-LAA tag display lower % CFP levels as compared to SsrA tag at 100 μM and 1 mM IPTG concentration. Yet, the SsrA tag and LAA-LAA tag displayed similar dynamic and saturation levels regardless of IPTG levels. But when compared with $SsrA_{2X,}$ the dynamic changes between 100 μM and 1 mM IPTG concentration. $SsrA_{2X}$ degrades faster at 100 μM IPTG and shows same saturation levels as LAA-LAA tagged protein and at 1 mM IPTG it appears to form a queue faster displaying saturation level similar to SsrA tag (Fig. 3.3).

Both tags contain two copies of ClpX binding sequence, we hypothesize that it may be responsible to regulate the queue at ClpXP proteolytic system and addition of extra copy (LAA sequence) increases the affinity towards proteases. Since many have constructed circuits with functional SspB sequence, we were interested in looking at queue formation with SspB tagged protein. We examined previously studied sequences where the last three amino acid sequence (LAA) was changed to LDD and DAS[137] to mutate ClpX binding sites but retain SspB binding site and likewise we observed poor degradation of CFP. It

was previously speculated that the simultaneous binding of SspB and ClpX can cause clashing of proteins that can be improved by adding a spacer between two binding sites[249]. To avoid SspB and ClpX chaperone clashing, we tested only SspB binding sequence, AANDENY. Even though it displayed no significant difference in degradation at low levels as compared to SsrA, when titrated, the CFP levels tagged with AANDENY remains constant (Fig. 3.2B). Addition of two SspB binding sequence (AANDENY-AANDENY) did not improve the affinity to ClpXP either. We also tested the variant with mutated ClpA binding sequence but retain SspB and ClpX binding sequence, AANDENYDLAA. We thought, if we eliminate ClpA competition, the degradation rate of tag variant will improve but it displayed higher levels of CFP (Fig. 3.2B).

3.3.2. Self-crosstalk to study proteolytic queueing phenomenon.

The queue formation is difficult to measure with only one protein is being produced where two factors are involved, queueing and degradation of tagged protein. So, we took a different approach to examine dynamics of the queue formation for selected tags. We produce two different fluorescent proteins with the same tag under different inducible promoters and tested at the batch culture in the plate reader assay. We produced YFP-tag with P_{TetR} promoter at constant inducer levels, 0, 10, 20, 75, and 200 ng/ml Dox. Then we produced CFP-tag with $P_{lac/ara-1}$ promoter at high levels, with 1 mM IPTG and 1% Ara, to saturate ClpXP proteolytic system with CFP-tagged protein and check the effect on the YFP-tag protein. When the queue is formed with CFP protein, YFP levels will go up as it will be a part of a queue to be degraded by the same proteases. This allows us to understand how fast queue can form with the same tag at the ClpXP proteolytic system without interference of degradation rates.

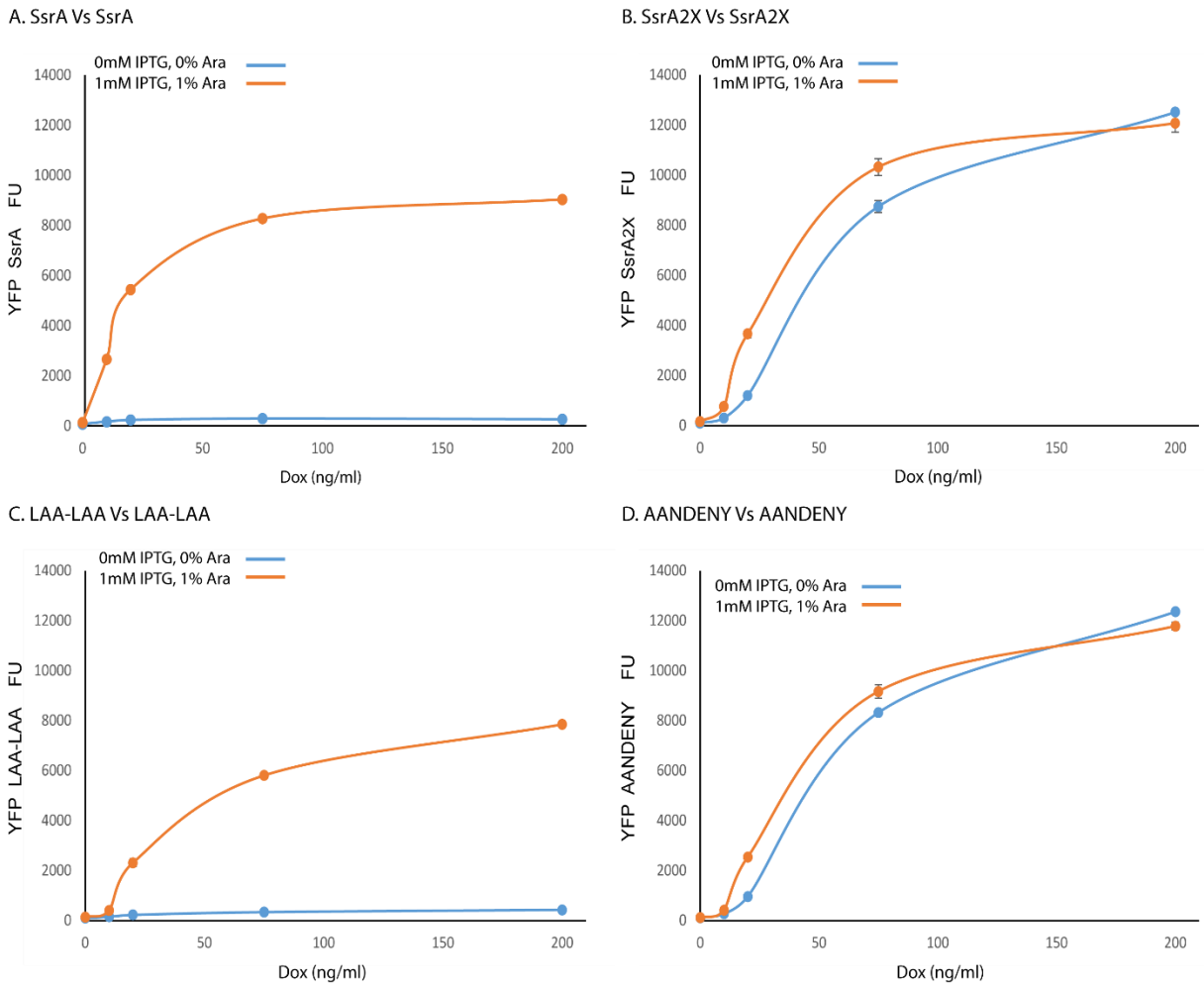


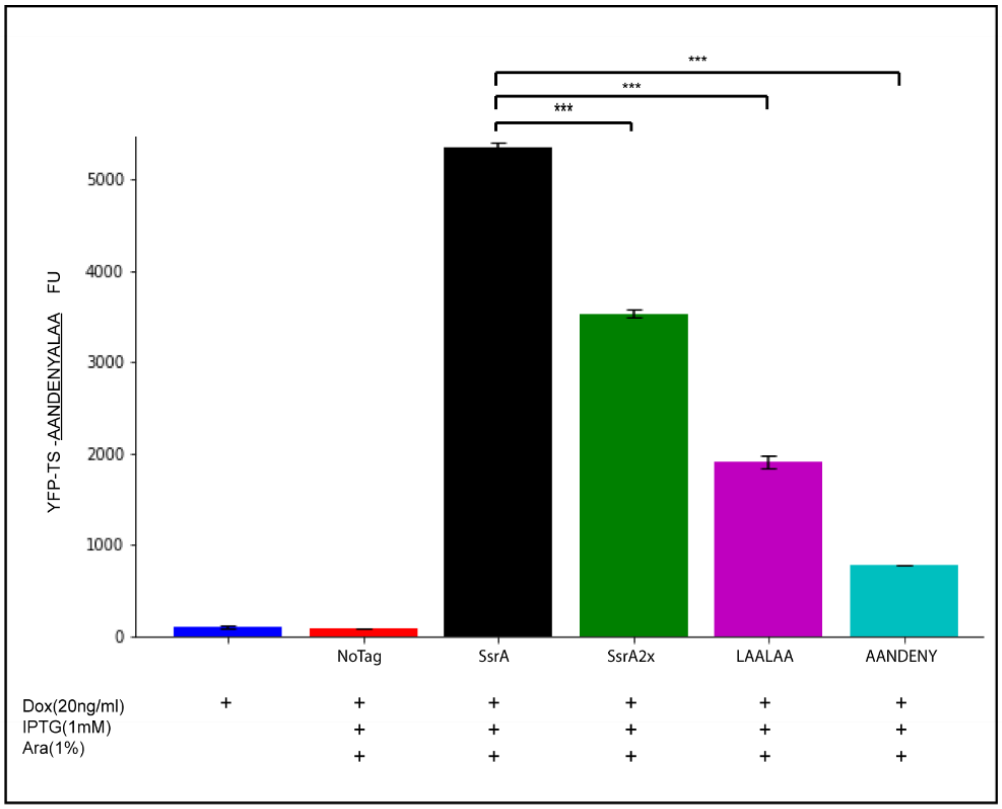
Fig. 3.4. Self-crosstalk. Proteolytic queue form when proteins with the same degradation tags are over-produced to target to ClpXP proteolytic pathway. YFP-tag is constantly produced with Dox inducer, 0, 10, 20, 50, 75, 200 ng/ml. CFP-tag is produced with 1 mM IPTG and 1% arabinose (Ara) inducers to saturate ClpXP. YFP levels were compared between YFP-tag (Dox induction alone) is produced to YFP-tag and CFP-tag were produced (Dox, IPTG, Ara induction). Queueing effect for **A**. SsrA tag**. B**. $SsrA_{2X}$ tag. **C.** LAA-LAA tag**. D**. SspB sequence, AANDENY tag. FU: arbitrary fluorescence unit.

Previously we have used this method to calculate queueing with the same tagged proteins[136]. Parallel to previous studies, SsrA tag forms a high queue as shown in Fig. 3.4A. When we compared the queue of SsrA to $SsrA_{2X}$ and AANDENY tag, the YFP levels increases without production of CFP-tagged protein (Fig. 3.4B, D). That means, the ClpXP system is saturated with only YFP-tagged protein probably due to their low degradation rates. However, when compared at lower Dox levels, 20 ng/ml, ClpXP saturation with CFP-tag ($SsrA_{2X}$ or AANDENY) protein further increases YFP level in both tags (Fig. 3.4B, D). Hence, the lower queue forms with $SsrA_{2X}$ and AANDENY tag, which is not ideal for building synthetic circuits. However, the ultra-high affinity LAA-LAA tag, results in high queue formation similar to the SsrA tagged proteins. For SsrA and LAA-LAA tags, the change in the queue is significant when ClpXP is saturated with CFP-tag proteins. Upon overloading ClpXP system with CFP-tagged proteins, The queueing effect plateau for YFP-SsrA at 75 ng/ml Dox, but LAA-LAA tagged YFP requires higher levels of Dox, 200 ng/ml, one reason is because of their faster degradation rate and higher affinity queue.

3.3.3. SsrA tag variants show different degree of crosstalk with the SsrA tagged YFP-protein.

Another question arises, how these variants form the queue with wild type SsrA tagged protein. We carried out similar experiment of crosstalk. We produced YFP-SsrA tagged protein at constant level with 20 ng/ml and 75 ng/ml Dox. Then we produced CFP-tag variants with 1 mM IPTG and 1 % Ara to saturate the ClpXP. We examined the changes occurred in YFP fluorescent level tagged with SsrA tag after overloading ClpXP system with CFP-tag variants. As a control, we monitored YFP-SsrA levels CFP no tag is produced. The over-production of CFP-no tag does not increase YFP levels as CFP-no tag is not targeted to ClpXP (Fig. 3.5).

The SsrA tag and SsrA tag variants all are targeted to same ClpXP proteases, hence, it is expected to observe the increase in YFP levels (crosstalk phenomenon). However, the degree of crosstalk will be different according to their degradation rates. When we saturated ClpXP system with CFP-SsrA tag, it forms a highest level of queue with YFP-SsrA tag and YFP level increased substantially. Self-crosstalk with SsrA tag of two protein is highest as the degradation rate for both tags are same. However, if the tagged protein is degrading faster, the queue level will decrease. When $SsrA_{2X}$ and LAA-LAA tagged CFP is overproduced, YFP level was higher with $SsrA_{2X}$ than LAA-LAA but lower in both cases than SsrA wild type tag. This confirms that LAA-LAA particularly is degrading faster. From previous experiments, we determined that AANDENY tags concentration remains constant throughout, (Fig. 3.2), we expected to have higher effect on queue formed with YFP-SsrA tag but surprisingly, the overproduction of AANDENY tagged protein, did not increase SsrA queue considerably. These results suggest that the queue is not affected by SspB binding sequence but affected by ClpX binding sequence, where we speculate the queue is most likely building up.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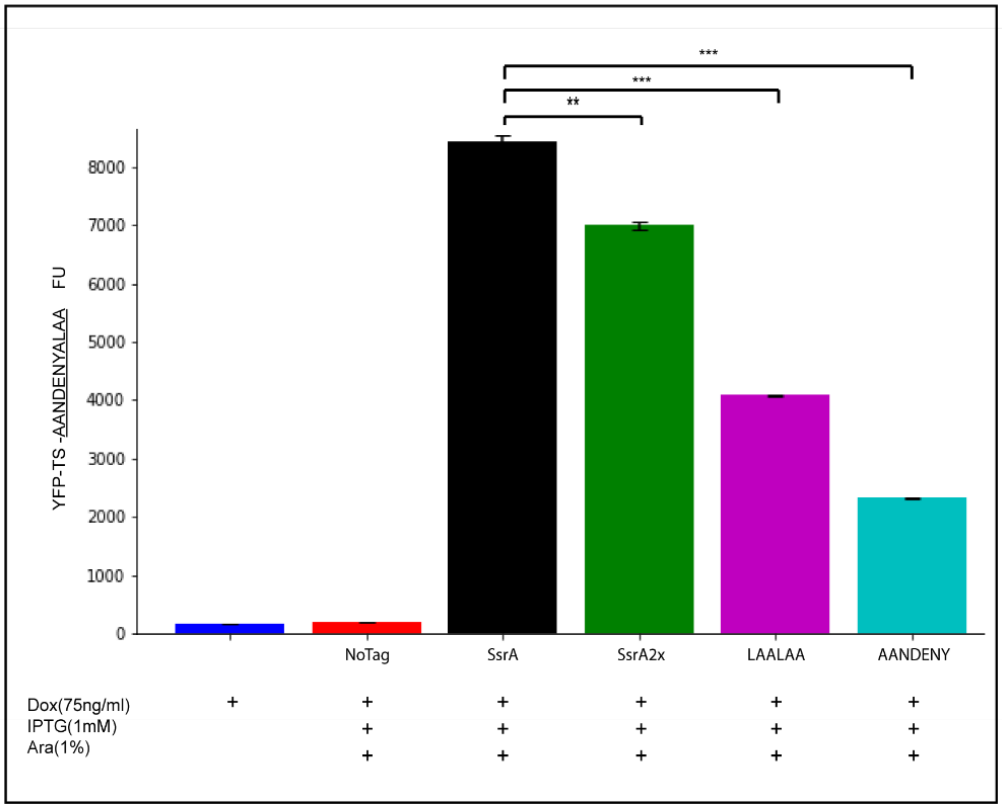
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LAALAA
AANDENY
Dox(75ng/ml)
IPTG(1mM)
Ara(1%)

Fig. 3.5. Crosstalk of SsrA tag with SsrA variants. The effect of YFP-SsrA tag when No-tag, SsrA, $SsrA_{2X,}$ LAA-LAA and AANDENY degradation tags are over-produced to saturate ClpXP proteolytic pathway. YFP-SsrA is produced with Dox inducer, 20 (Top) and 75 ng/ml (Bottom). CFP-tag protein is produced with 1 mM IPTG and 1% Ara inducers. YFP levels are compared between no saturation of ClpXP (Dox induction alone) to ClpXP saturation (Dox, IPTG, Ara induction). FU: arbitrary fluorescence unit. (p values: *<0.05; **<0.01; ***<0.001).

3.3.4. ClpX overexpression reduces queue with tagged CFP.

We seek to understand the queueing phenomenon at ClpXP especially at each chaperone levels. We were interested to examine the effect of each chaperone towards the overloaded ClpXP system. Since we do not know where the queue forms at ClpXP system, we overexpressed SspB, ClpX or ClpP proteins to check the effect on the queue speculating the component involved in a queue will display major effect. We hypothesize, if we overproduced chaperone/s involved in the queue, proteins (here tagged CFP) in the established queue will be processed faster due to increased numbers of limited chaperones. Hence, overproduction of limited resourced (Chaperone) will decrease the CFP fluorescent levels (Fig. 3.6A). We tested CFP levels tagged with SsrA, LAA-LAA and AANDENY tags after overproduction of chaperones. The CFP-tagged proteins were constantly produced with 1 mM IPTG and 1 % Ara to overload the ClpXP system. As the CFP levels are different for each tag because of their different degradation rates and they form different degrees of the queue, we considered the CFP levels with 1 mM IPTG and 1 % Ara as 100% for each CFP-tag construct.

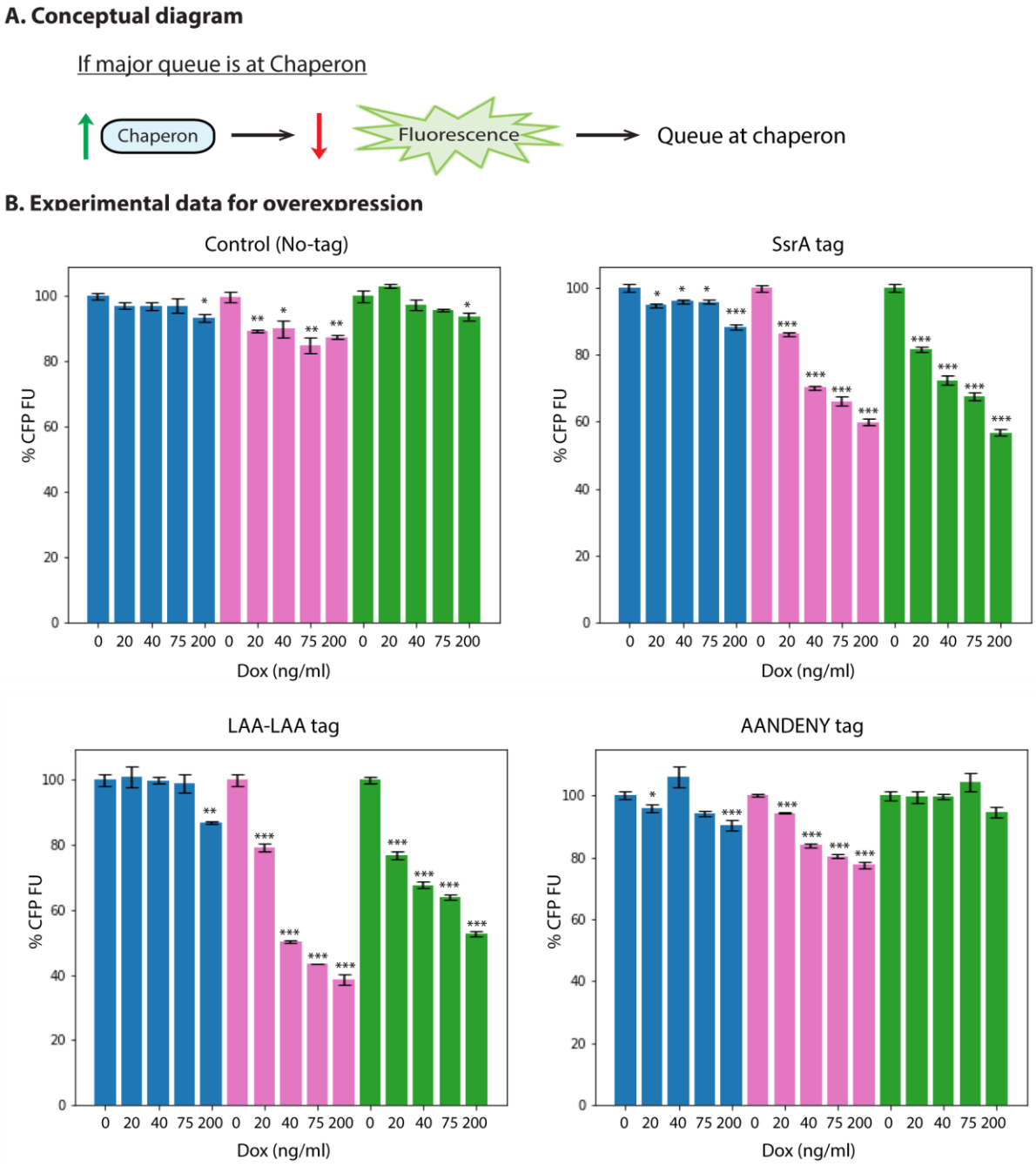


Fig. 3.6. The effect of overexpression of SspB, ClpX and ClpP proteins on the queue formed with **A.** No-tag CFP (Control), **B.** SsrA tagged CFP, **C.** LAA-LAA tagged CFP, and **D.** SspB binding sequence, AANDENY. The queue is formed with CFP tagged protein using 1 mM IPTG and 1 % Ara. The SspB, ClpX and ClpP proteins are expressed using Dox inducers. The maximum CFP levels with 1 mM IPTG and 1 % Ara are considered to be 100%. The over production of ClpX chaperone (pink) decreases the % CFP levels for all the tagged proteins. FU: arbitrary fluorescence unit. (p values: *<0.05; **<0.01; ***<0.001).

The chaperones SspB, ClpX or ClpP are under P_{tetR} inducible promoter and expressed with varying concentrations of Dox inducers. We used CFP-no tag as a control (Fig. 3.6B). CFP-no tag should have no effect due to the overexpression of ClpXP chaperones since it is not targeted to degradation. However, overexpression of ClpX chaperone, had an effect on CFP levels (<10% change) (Fig. 3.6B). The ClpXP proteolytic system is one of the major proteases and regulates most of the proteins in the cell. The over production of components will have some minor effect on the control. Hence, we considered any change withing 10% is normal for all the tags. The CFP- SsrA tag shows major decrease in fluorescence with ClpX and ClpP overproduction (Fig. 3.6B). To distinguish between SspB and ClpX, we overexpressed components with queue formed with only ClpX binding sequence, LAA-LAA and SspB binding sequence, AANDENY. Similar to CFP-SsrA, ClpX and ClpP both chaperone decreases the CFP-LAA-LAA fluorescence (Fig. 3.6B). But when we examine the change in the CFP levels tagged with SspB binding sequence, AANDENY, it only diminishes as we increase ClpX levels (Fig. 3.6B). As AANDENY tag does not directly interact with ClpP but only interact with SspB and then ClpX, the overexpression of ClpP does not have an effect. However, overexpression of SspB, did not have a major effect on CFP proteins tagged with LAA-LAA or AANDENY tags. The evidence from this study support that ClpX is responsible for the queue at ClpXP proteolytic system.

3.3.5. The ClpX binding sequence, not SspB binding sequence is responsible for crosstalk with ClpAP and Lon proteases.

From our previous results, SsrA tag crosstalk with MarAn20 and RepAn70 tags[136], i.e., SsrA tag are being degraded by Lon and ClpAP respectively. As SsrA tag degrades by Lon or ClpAP, the tags primarily targeting to those proteases backed up due to formation of queue. MarA and RepA are known substrates for Lon and ClpAP, respectively. Here, we tagged YFP with MarAn20 for Lon and RepAn70 for ClpAP to check the queueing phenomenon with respective tags. We continuously produced YFP-MarAn20 or YFP-RepAn70 proteins and checked the queueing effect when we saturate the protease system with SsrA tag variants. Firstly, we increased the concentrations of CFP with no tag, it has minimum effect on the degradation of YFP-MarAn20 and YFP-RepAn70 fluorescence. But when we increase the CFP proteins with SsrA tag and its variants, it may interfere with the degradation of YFP tagged proteins. If Lon or ClpAP recognizes SsrA tag variants, it will increase YFP (tagged with MarAn20 or RepAn70) levels as we increase CFP-tag proteins, depending on the degree of crosstalk. Higher YFP level indicates higher level of crosstalk due to higher queue build-up. The SsrA tag variants containing ClpX binding sequence, $SsrA_{2X}$ and LAA-LAA display same or higher levels of crosstalk as compared to SsrA tag (Fig. 3.7). But SspB binding sequences, AANDENY and DAS tag display reduced crosstalk (Fig. 3.7). Since SspB binding sequence is not a part of ClpXP proteolytic queue, from this result it suggests that it has minimum effect on queues formed with other proteolytic system (Lon and ClpAP).

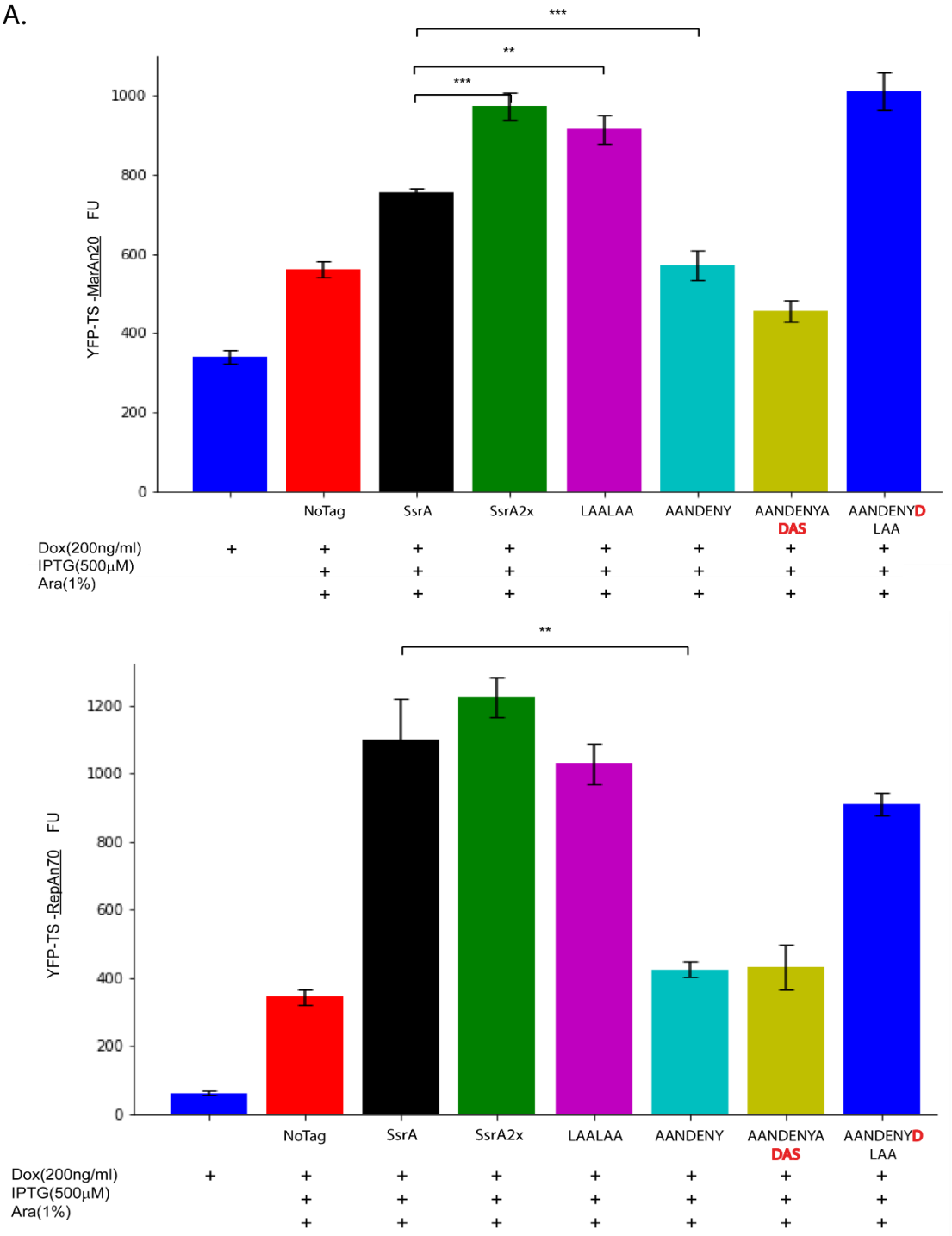


Fig. 3.7. Crosstalk between **A.** MarAn20 tag with SsrA tag variants. **B.** RepAn70 tag with SsrA tag variants. YFP with MarAn20 tag or RepAn70 tag produced with 200 ng/ml Dox. SsrA tag variants are produced with 500 µM IPTG and 1% arabinose (Ara). The change in MarAn20, RepAn70 queue was analyzed by monitoring YFP levels. FU: arbitrary fluorescence unit. (p values: *<0.05; **<0.01; ***<0.001).

3.4. Discussion.

ClpXP is a well-studied proteases and applied extensively in synthetic biology. We already covered the importance of SsrA degradation tags and ClpXP proteolytic queueing in synthetic circuits in chapter 2 (Review chapter). In this chapter, we deconstructed SsrA degradation tag in order to increase the affinity towards ClpXP to reduce crosstalk phenomenon with other proteolytic system[136] which can be a nuisance in building robust synthetic circuits. We constructed an ultra-high affinity tag, LAA-LAA that shown to degrade faster than SsrA tag but show similar self-crosstalk dynamic (high queue formation) similar to SsrA tag. Another SsrA tag variant, $SsrA_{2X}$ shows to be faster degrading at lower induction but as we increase the production of $SsrA_{2X}$ tagged proteins, it starts accumulating faster in the cell and degradation rate slows down. Since $SsrA_{2X}$ variant that contains 2 copies of each binding sequences, the protease bottleneck has a huge effect on the $SsrA_{2X}$ tagged protein. In contrast, LAA-LAA variant constantly shows higher degradation throughout. Saur's lab discovered that SspB and ClpX chaperones causes clashing due to their close binding sequences and able to improve tag's affinity by adding 4 amino acid spacer[137, 249]. Here we eliminated SspB binding sequence but added an extra ClpX binding sequence that shown to improve ClpX affinity significantly. Eliminating interaction with SspB protein, we reduce the time delay due to enzymatic interaction.

When we examined the crosstalk phenomenon with SsrA tag, LAA-LAA shows lower degree of crosstalk than $SsrA_{2X}$ tagged protein. Which means that queue formed with LAA-LAA gets processed faster and takes time to overload the ClpXP system. However, SspB biding sequence, AANDENY tag has lowest effect ClpXP system during crosstalk. SspB tagged protein display constant levels of fluorescence over the different inducer

levels, which suggest that it is getting degraded slower. We thought, increasing SspB would cause major effect on ClpXP system and affect YFP levels tagged with SsrA tag but surprisingly, it displayed lowest crosstalk with SsrA tag. This suggest that AANDENY tagged proteins are not a part of the ClpXP proteolytic queue. This makes us wonder, where exactly the queue is happening. To find the queueing point, we increased chaperones involved in ClpXP system and monitored the queue formed with different variants. In all cases, increasing levels of ClpX enhances protein degradation even for AANDENY tagged proteins. When we examined crosstalk between other proteolytic system using MarAn20 for Lon and RepAn70 for ClpAP, we discovered the least crosstalk happened with AANDENY, SspB binding sequence which is mostly not a part of ClpXP proteolytic queue. However, LAA-LAA still display the crosstalk with Lon and ClpAP proteolytic systems. Yet, the higher affinity of LAA-LAA can be beneficial in attaining faster turnover rate of tagged proteins, in case of dynamic circuits.

3.5. Materials and Methods.

3.5.1. Strains and Plasmids.

E. coli strains, DH5αZ1 was used in this study[250]. Plasmid with CFP downstream of the $P_{lac/ara}$ promoter of p24Km (kanamycin 25 μg/mL) and YFP downstream of the P_{tetR} promoter of p31Cm (chloramphenicol 10 μg/mL)[250]. The SsrA-tag and variants are oligo cloned to p24KmNB83 and p31CmNB02 using BsrGI and MluI sites. The ClpXP chaperones were cloned under P_{tetR} promoter of p31Cm (chloramphenicol 10 μg/mL)[250] and double transformed with CFP (p24KmNB83), CFP-SsrA tag (p24KmPJ254), CFP-LAA-LAA (p24KmPJ153) and CFP-AANDENY (p24KmPJ154).

All the experiments were carried out in MMB+ media which is a modified MMA media. The composition of MMB+ is as follows: K_2HPO_4 (10.5 mg/ml), KH_2PO_4 (4.5 mg/ml), $(NH_4)_2SO_4$ (2.0 mg/ml), $C_6H_5Na_3O_7$ (0.5 mg/ml) and NaCl (1.0 mg/ml), 2 mM $MgSO_4$ x $7H_2O$, 100 μM $CaCl_2$, thiamine (10 μg/ml), 0.5% glucose and amino acids (40 μg/ml). Corresponding antibiotics are used depending on the strain and plasmids. All cultures were incubated at 37ºC unless specified, and broth cultures were shaken at 250 rpm.

3.5.2. Batch culture experiment- 96-well plate reader assay.

Cells were diluted 1:100 from overnight cultures and was grown to OD 0.2-0.3 in MMB+ media. Then they are diluted to ~0.1 OD before loading to individual wells in 96-Well Optical-Bottom Plate with Polymer Base (*ThermoFisher*) for fluorescence measurement using *FLUOstar Omega* microplate reader. Different combinations of inducers applied to each well, IPTG (100 μM, 1 mM) and/or Ara (0.1%-1%) and/or Dox (10 ng/ml- 200 ng/ml) depending on experiment. The ODs and fluorescence for CFP

(440/480) and/or YFP (500/540) were measured every 8 minutes intervals with intermediate shaking to decrease variability between wells. Minimum of 3 replicates for each treatment were measured to determine Mean, SD and SEM for fluorescence.

4. CHAPTER 4: MODIFYING OSCILLATORY OUTPUT OF DUAL-FEEDBACK OSCILLATOR.

4.1. Abstract.

The synthetic oscillators are self-sustaining dynamic process with periodic change in the regulatory elements, especially transcription factor repressors. The oscillator output is measured with period (time for one complete cycle) and amplitude (the highest fluorescent output). However, they are often dependent on each other. Increasing the amplitude typically increases the period. The goal is to reduce this dependency and construct oscillator with higher amplitude and shorter periods that is ideal for biosensors. The dual-feedback oscillator is a robust oscillator that was eloquently designed to have few parts and relies on proteolytic queueing at ClpXP. But retain the period-amplitude dependency. The output of the dual-feedback oscillator depends on the proteolytic queue because the regulatory elements, LacI and AraC (and output marker, GFP) are tagged with SsrA tag that are targeted to ClpXP proteases. In this study, we successfully increase the amplitude of dual feedback oscillator when we replaced LacI with ultra-high affinity tag, LAA-LAA without changing the period of the oscillation. Further, we accomplished to change the period of the oscillator with increasing the concentration of a key component, ClpX and retaining the similar amplitude. The use of the SsrA tag and a variant with different affinity enable us to break the period-amplitude dependency. Additionally, we added extra regulation when we controlled chaperone ClpX levels that can help to further fine-tune the oscillator primarily the period of the oscillations.

4.2. Introduction.

Synthetic oscillators are a dynamic process where the concentrations of the regulatory elements (repressors and/or activators) change periodically. The periodic change is due to successive repression of the gene expression of the regulatory elements, called the negative feedback loop (Fig. 4.1A). Most regulatory elements in the cell are non-native proteins and are not actively degraded by native proteases. To acquire faster turnover rate, the regulatory elements are fused with a degradation tag that results in proteolysis. Naturally, protease numbers in the cell are maintained at relatively low quantities to ensure tight regulation of intracellular protein levels[230]. That makes proteases a limited resource, and when too many proteins are sent to the proteases for degradation, a cellular bottleneck called queues can form (a waiting line at the protease)[110] (Fig. 4.1B).

In the oscillators that rely on proteases, negative feedback (gene repression) is delayed due to the formation of the proteolytic queue (time-delay, half-life of target protein increases)[109-111]. The production rate and the degradation rate of the regulatory elements determine the proteolytic system overload to form a queue[110] (Fig. 4.1B). Overproduction of regulatory elements in synthetic circuits can build up the queue due to protease bottleneck in the cell and the half-life of tagged regulatory elements increases. As the proteolytic queue builds up, the amount and the availability of the repressor increases in the cell to repress the gene expression in the oscillator.

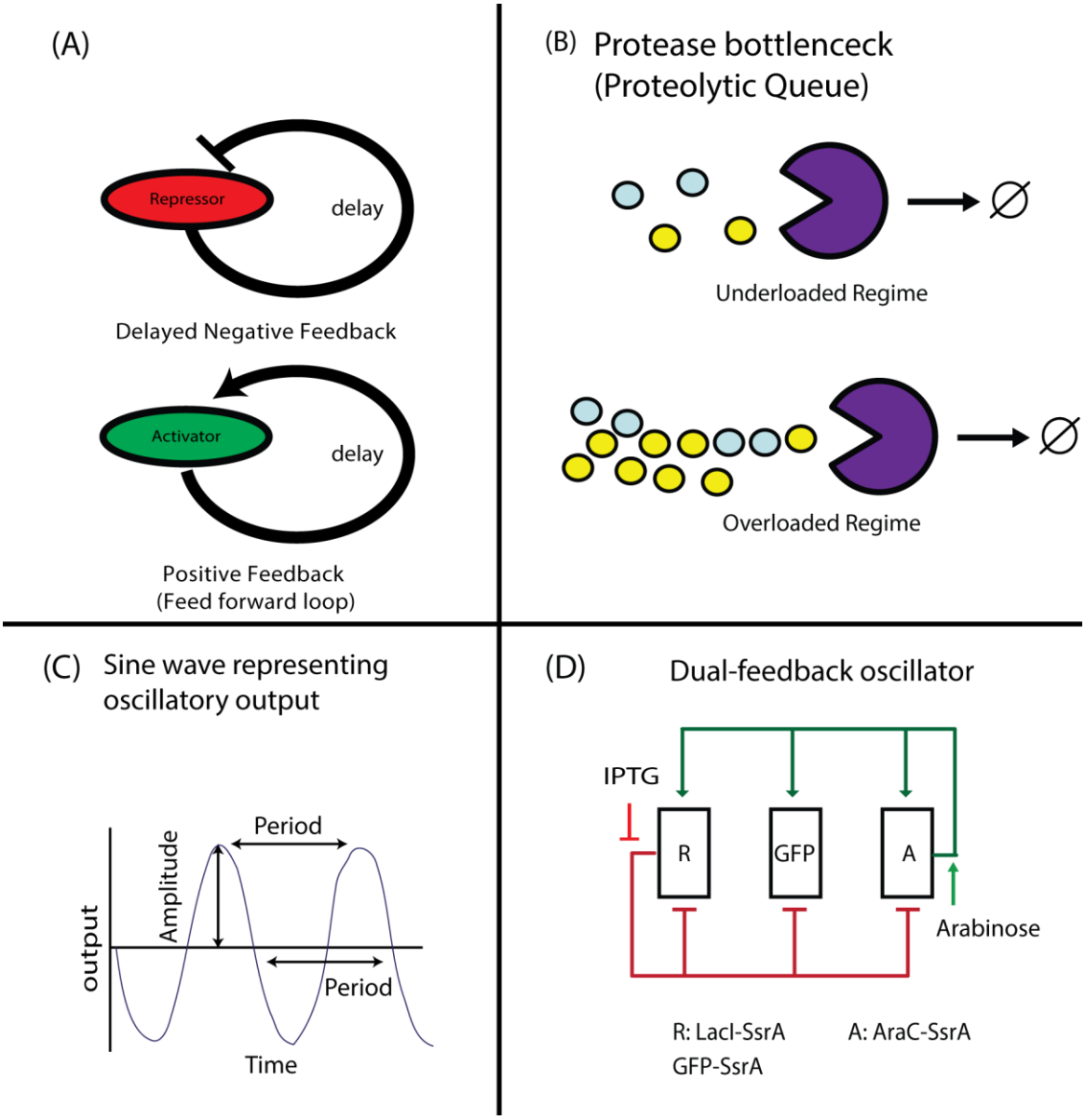


Fig. 4.1. A. Delay negative feedback loop and positive feed forward loop. **B.** Protease bottleneck causing queue. When few proteins are produced, they are degraded faster in underloaded regime. In overloaded regime, large number of proteins targeted to proteolysis causes proteins to form a queue**. C.** Oscillatory output that represents as a sine wave with period and amplitude. Period is the time between two peaks or valley. Amplitude is the height of the sine wave**. D.** The conceptual diagram of dual-feedback oscillator.

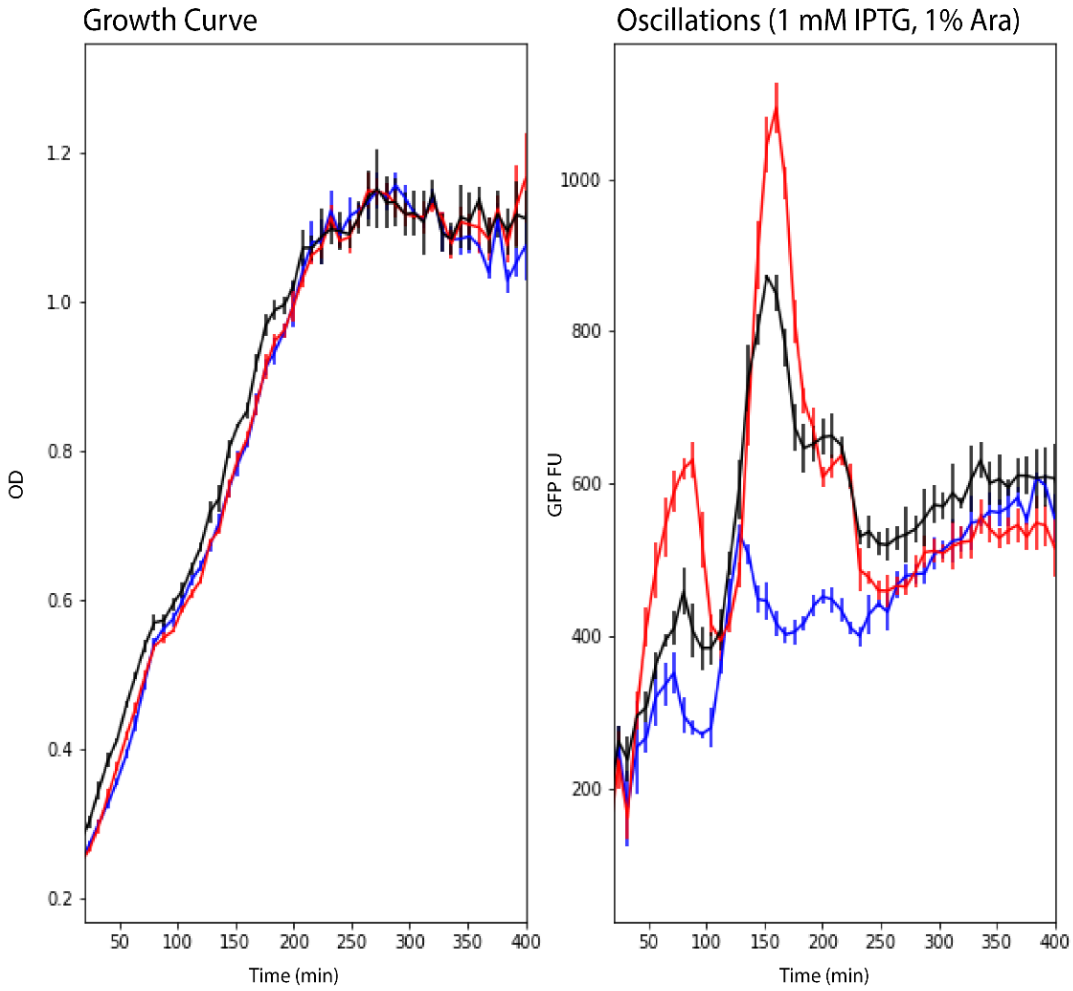
The proteolytic queue was first discussed in dual feedback oscillator[110]. The design of dual feedback oscillator[114] is comprised of a repressor (l*acI*) and an activator (*araC)* under same promoter, $P_{lac/ara1}$ (Fig. 4.1D). The regulator elements LacI and AraC, and marker, GFP, all are fused with SsrA tag that are targeted to ClpXP proteases. Hence, ClpXP proteolytic queue forms with tagged proteins (AraC, LacI and GFP). The time delay of repressors corresponds to the length of the queue. Considerable yet short queue forms with only a negative feedback loop (shorter time delay), where the stochastic nature of the cell interferes and further limits the unified response[251, 252]. But dual-feedback oscillators have added a positive feedback loop (e.g., feed-forward loops) (Fig. 4.1A, D) with the addition of an activator (AraC) that increases the time delay to achieve a more unified response and lessen the effect of stochasticity on the output[251-253].

In this study, we seek to understand the effect of queue on the oscillatory output. We replaced the SsrA tag from dual feedback oscillator to ultra-high affinity LAA-LAA tag to change the turnover rate of the regulatory elements. We further tested the effect on the oscillatory output when we change the key component, ClpX in the queue.

4.3. Results.

4.3.1. The ultra-high affinity tag (LAA-LAA tag) increases amplitude but not period of the oscillatory output at population level.

The dual feedback oscillator function with a negative regulator, LacI and a positive regulator, AraC using only one promoter $P_{lac/ara1}$. The $P_{lac/ara-1}$ promoter is a hybrid of promoters P_{BAD} and P_{Llac01}[232]; AraC acts as an activator for P_{BAD} promoter and LacI is a repressor for P_{Llac01} promoter. AraC and LacI activity is externally controlled by arabinose and IPTG respectively, where arabinose enhances AraC binding and IPTG inhibits LacI binding. Hence, arabinose and IPTG together control the activity of the $P_{lac/ara-1}$ promoter. Since the function of the repressor LacI is primarily operates the oscillator, we first replaced the SsrA tag of LacI to novel ultra-high affinity, LAA-LAA tag. Then we replaced the marker, GFP tag to compare between two tags. We also checked if there is any difference in growth rate to avoid the effect of cell division on output. Figure 4.2 (left) shows that there is no change in growth between three constructs. We induced cultures with 1 mM IPTG and 1 % Ara and compared the oscillatory output at batch culture in 96-well plate. We observed increased in amplitude (Fig. 4.2) between dual feedback oscillator (Graph color: Blue, LacI-SsrA, GFP-SsrA, AraC-SsrA tag) and two constructs harboring LAA-LAA tag. Construct pKmPJ260 (Graph color: Red, LacI-LAA-LAA, GFP-SsrA, AraC-SsrA) has LAA-LAA tag fused to only LacI while pKmPJ266 (Graph color: Black, LacI-LAA-LAA, GFP-LAA-LAA, AraC-SsrA) construct has LacI and GFP fused with LAA-LAA tag.



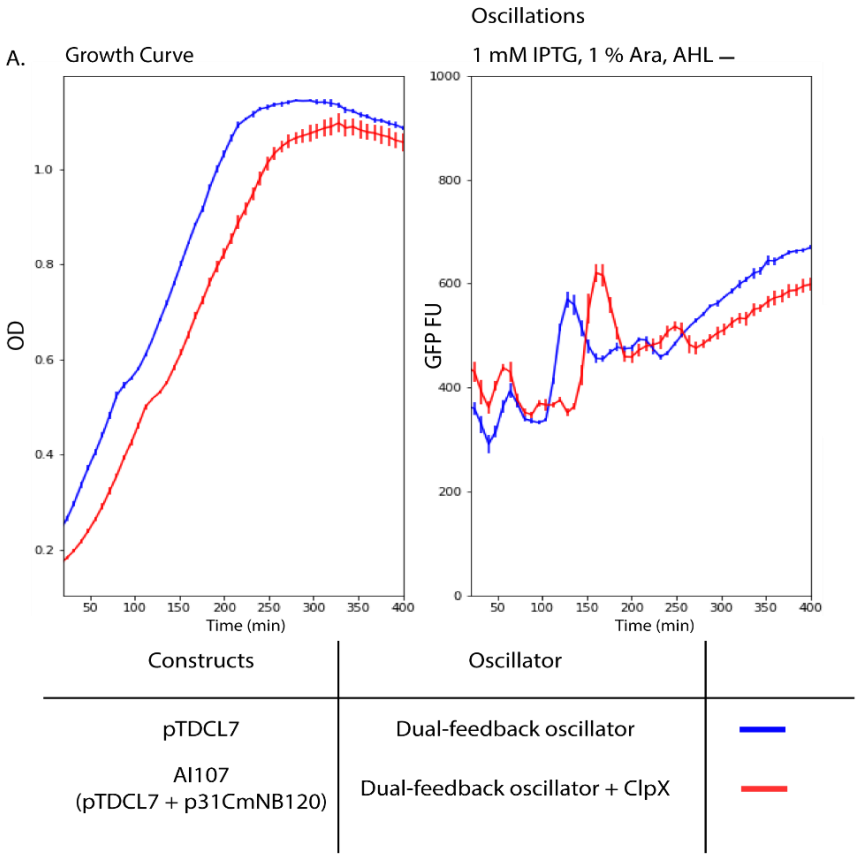
Regulatory elements	LacI	AraC	GFP	Constructs	
Degradation tags	SsrA	SsrA	SsrA	pTDCL7	
	LAA-LAA	SsrA	SsrA	pKmPJ260	
	LAA-LAA	SsrA	LAA-LAA	pKmPJ266	

Fig. 4.2. Comparison of oscillatory output at batch culture, 96-well plate. Cultures were induced with 1 mM IPTG and 1 % Ara and GFP FU recorded at 8 min interval. Dual feedback oscillator with regulatory elements tagged as shown in the table. The original dual-feedback oscillator represents as blue color. Red and black colors represent the variations in dual-feedback oscillator. Left: Average growth curve for three constructs. Right: GFP FU output. FU: arbitrary fluorescence unit.

Comparing the oscillatory output of three constructs in batch culture, we observed few significant differences. First peak for pKmPJ260 and pKmPJ266 were 1.8 and 1.3-fold higher than pTDCL7. Second peak for pKmPJ260 and pKmPJ266 were 2.05 and 1.63-fold higher than pTDCL7. However, the average period slightly increases from 56.01 min for pTDCL7 to 72.03 min for pKmPJ260 & pKmPJ266 between 1^{st} peak and 2^{nd} peak. However, period between 2^{nd} and 3^{rd} peak are 72.03 min for pTDCL7 and 56 min for pKmPJ260 & pKmPJ266 (Fig. 4.2). Normally, increasing amplitude causes increase in period. Here we used two different affinity degradation tags targeted to ClpXP proteolytic system that allows us to separate the peak and amplitude dependency. We are currently analyzing results from single cell data collected using microscope.

4.3.2. Change in ClpX chaperone increases the period of dual feedback oscillatory output.

In previous study (Chapter 3), we identify chaperone ClpX is responsible for ClpXP proteolytic queueing. We constructed ClpX under P_{lux} promoter where ClpX levels can be controlled with inducer AHL. We retained the native ClpX in the cell. We added dual-feedback oscillator plasmid with ClpX construct in the same strain. We first compared the dual feedback oscillator output when ClpX construct is present and absent to check if there is any leakage of AHL promoter to have an effect on the output. We also compared the growth profile between two strains. We induced culture with 1 mM IPTG and 1 % Ara, and measured GFP levels at population level in 96-well plate reader over time. The two strains containing dual feedback oscillator construct with or without ClpX construct showed no change in the growth curve (Fig. 4.4A, left). They also showed similar oscillatory output when ClpX is not induced (AHL-) (Fig. 4.4A, right).


A.
Growth Curve
Oscillations
1 mM IPTG, 1 % Ara, AHL –
OD
GFP FU
Time (min)
Time (min)
Constructs
Oscillator
pTDCL7
Dual-feedback oscillator
AI107
(pTDCL7 + p31CmNB120)
Dual-feedback oscillator + ClpX

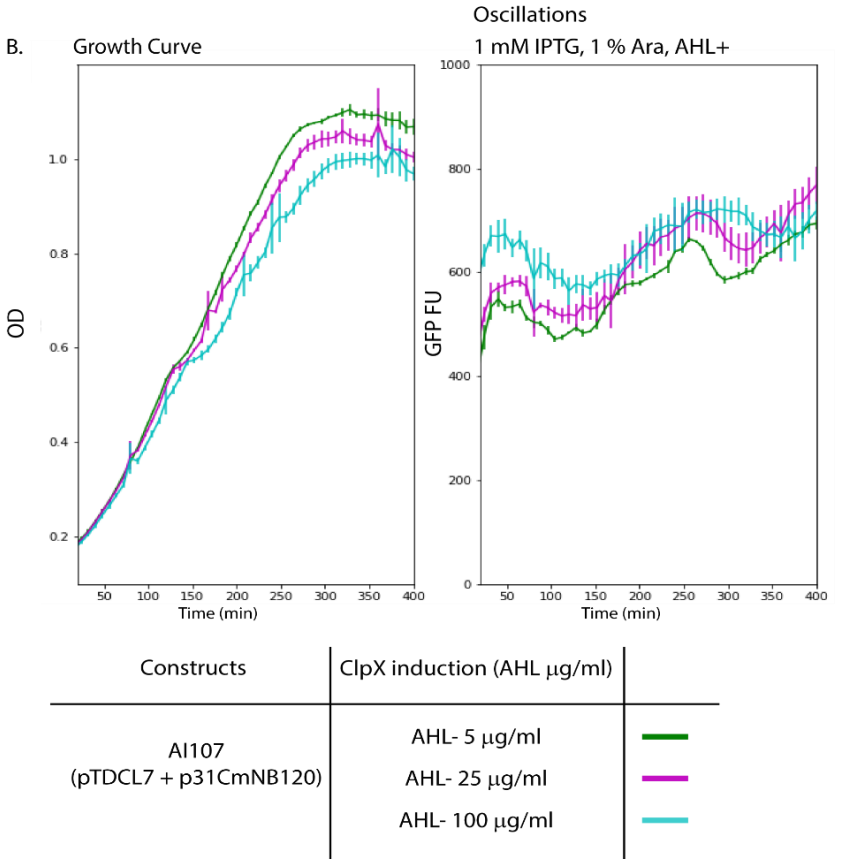

B.
Growth Curve
Oscillations
1 mM IPTG, 1 % Ara, AHL+
OD
GFP FU
Time (min)
Time (min)
Constructs
ClpX induction (AHL µg/ml)
AI107
(pTDCL7 + p31CmNB120)
AHL- 5 µg/ml
AHL- 25 µg/ml
AHL- 100 µg/ml

Fig. 4.3. A. Dual feedback oscillator output with addition of ClpX construct, does not change when ClpX is not induced. **B.** Change in oscillatory output when ClpX is induced with 5, 25, 100 μg/ml AHL. With ClpX production, oscillator period increases. Bottom- information of construct, Left- average growth curve, Right- GFP FU output. FU: arbitrary fluorescence unit.

Since Inducer AHL controls the ClpX expression. When we induced ClpX with addition of AHL, the oscillation period doubles for 5 μg/ml and 25 μg/ml AHL but increases 2.3-fold for 100 μg/ml AHL than no ClpX induction (Fig. 4.4A & B). We speculate that due to faster degradation of LacI in the cell due to increase in bottleneck, the time required for repressor LacI to reach the threshold increases. Therefore, the period of the oscillation increases. However, only slight increase in amplitude was observed with AHL induction.

4.4. Discussion.

Many researchers have leveraged queueing to intentionally couple circuits, e.g., via synchronization and entrainment of a synthetic oscillator[112, 135, 238]. The design exploits the knowledge of two unrelated pathways in the network can be coupled due to the same bottleneck, specifically proteases. It was previously shown to entrain dual-feedback oscillator using the unrelated protein with the same degradation tag, SsrA tagged protein[112]. The coupling occurs at the protease level because they are a part of the same proteolytic queue. A similar concept of coupling was applied with two different synthetic oscillators where the output of two oscillators were synchronized[135] at ClpXP proteases. Hence, queue modulation is of importance that can allow us to fine-tune the oscillators based on queueing.

In the previous chapter, we developed SsrA tag variant, an ultra-high affinity tag that forms similar queue as compared to SsrA tag but gets processed faster. The faster processing leads to the faster turnover rate of the proteins. We replaced the repressor LacI from dual feedback oscillator. The output is mainly dependent on the levels of the repressor LacI and hence, with changing the levels of LacI using ultra high affinity tag, we could change the output. We saw increase in amplitude when used ultra-high affinity tags over SsrA tagged proteins. Surprisingly, the period of the oscillations did not change. However, the oscillation variations can only be measured at single cell levels.

Similar to amplitude, we achieved to change the period without changing the amplitude. We accomplished that with increasing the levels of ClpX, a key component for queue. Since we increased the bottleneck, the time delay increases to reach to the saturation level for LacI to repress the oscillations. As we retained the native ClpX, we could only

increase the current period and could not shorten it. In future, using ClpX mutant strain we could be able to control the oscillator period on a vast range.

4.5. Materials and Methods.

4.5.1. Strains and Plasmids.

E. coli strain, NB001 is used that is LacI and AraC mutant[233]. The plasmid pTDCL7 (dual-feedback oscillator) was a gift from Dr. Jeff Hasty from university of California, San Diego. The SsrA tag in pTDCL7 is replaced with ultra-high affinity tag variant, LAA-LAA from LacI (pKmPJ260) and LacI-GFP (pKmPJ266) using PCR amplification. The ClpX protein is present under P_{lux} promoter (p31CmNB120).

All the experiments were carried out in MMB+ media which is a modified MMA media. The composition of MMB+ is as follows: K2HPO4 (10.5 mg/ml), KH2PO4 (4.5 mg/ml), (NH4)2SO4 (2.0 mg/ml), C6H5Na3O7 (0.5 mg/ml) and NaCl (1.0 mg/ml), 2 mM MgSO4 x 7H2O, 100 µM CaCl2, thiamine (10 µg/ml), 0.5% glucose and amino acids (40 µg/ml). Corresponding antibiotics are used depending on the strain and plasmids. All cultures were incubated at 37ºC, and broth cultures were shaken at 250 rpm.

4.5.2. Batch culture experiment.

Cells were diluted 1:100 from overnight cultures and was grown to OD 0.2-0.3 in MMB+ media. Then they load to individual wells in 96-Well Optical-Bottom Plate with Polymer Base (*ThermoFisher*) for fluorescence measurement using *FLUOstar Omega* microplate reader. Inducers IPTG (1 mM) and Ara (1%) and/or AHL (5 µg/ml-100 µg/ml) depending on experiment. The fluorescence for GFP (485/520) was measured every 8 minutes intervals with intermediate shaking to decrease variability between wells. Minimum of 3 replicates for each treatment were measured to determine Mean, SD and SEM for fluorescence.

5. CHAPTER 5: OVERALL CONCLUSION.

In this study, we used top-down and bottom-up approaches to teased apart biological pathways to study them independently and developed modular parts for a synthetic biology toolbox. Here, I implemented a design-build-test-learn cyclic approach. Each iteration provided us with more information about the native pathway, and we were able to improve the design using the learned information. Similar to natural systems where transcriptional, translational, and post-translational controls are used concurrently, my engineered synthetic circuits utilized all three controls. Chapter 2 assessed the importance of proteolytic queueing in synthetic circuits with a particular focus on dynamic systems, e.g., oscillators. The most commonly used degradation tag, SsrA, from *E. coli* has some drawbacks. I resolved some of these drawbacks by engineering new degradation tags with greater affinity (Chapter 3). When we employed high affinity variants in synthetic oscillator instead of the SsrA tag, it displayed a change in the output (Chapters 4). Another disadvantage of tagging proteins with the native SsrA amino acid sequence is that multiple proteases can recognize it. This can cause crosstalk between proteases (interference of other proteases during protein degradation). We identified that ClpX binding sequence and not SspB binding sequence is responsible for crosstalk. Hence, tags that only depends on the SspB binding are effective to reduce crosstalk phenomenon with Lon and ClpAP proteolytic systems (chapter 3).

To better understand the ClpXP proteolytic bottleneck, we studied each component of this system. From this study, I identified ClpX as the fundamental chaperone for the queue (Chapter 3). Future work may involve leveraging ClpX levels inside the cell to modulate protein output or queue formation in a more controllable manner. I further

analyzed increasing ClpX levels on synthetic oscillator output where queue is being processed faster (Chapter 4). My work focused on building a library of degradation tags in Chapter 3 and identifying ways to alter ClpXP proteolytic queue, because synthetic biologists and engineers are in great need of new methods to modulate protein levels in a controllable/predictable manner independently of the transcriptional regulation. Furthermore, identifying the orthogonal pair is advantageous in building orthogonal circuits that can be used in different species. Analyzing the circuit output at population and single cell levels informs us of the system's variations. Further application of mathematical models for developed circuits helps us predict the output for a wide range of input numbers. I am working with other researchers in the Butzin lab alongside collaborators to build a mathematical understanding of queueing using my data. Hence, the circuits developed in this study can be used as modular parts to build more complex networks for industrial and medical applications.

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