FRET-based Synthetic Biology Approach for SUMOylation Cascade in Bacterial Cell and Interaction with Influenza A Virus

My Linh Le, Department of Bioengineering
Chelsy Cervantes, Department of Bioengineering
Chuchu Liu, Graduate Student, Department of Bioengineering
Runrui Dang, Ph.D. Student, Department of Bioengineering
Chen Ling, Graduate Student, Department of Bioengineering
Kalvin Yuwen; Department of Biological Sciences; University of California, Irvine
Jiayu Liao, Ph.D., Department of Bioengineering

ABSTRACT

The post-translational modification SUMOylation plays a critical role in regulating protein function and stability in both physiological and pathological processes, such as cancers, immune responses, and virus infections. Namely, SUMOylation enhances cell proliferation of the influenza A virus (IAV) M1, making the study of SUMO1 protein interactions significant to viral research. However, research in this field requires mammalian cell cultures that are both labor intensive to maintain and costly to scale up for drug screening. The purpose of this project is to design a SUMOylation pathway in bacterial cells that can eliminate these setbacks, aiding future research in being more efficient. Using synthetic biology techniques, we reconstituted the SUMOylation cascade into *E. coli* and attached a FRET donor CyPet to screen its fluorescent protein interaction with our target substrate YPet-IAV M1. The most suitable among the 13 *E. coli* strains, BL21(DE3) PlysS, showed a successful fluorescent energy shift from the donor CyPet to acceptor YPet, suggesting SUMO1 binding to IAV M1 within 1-10 nm. We also performed SUMOylation inhibitor testing to validate our SUMOylation construct FRET efficiency. Our novel engineered SUMOylation cascade was able to simulate SUMO conjugation to substates in bacterial cell cultures similar to their native counterparts in mammalian cells, offering an alternative to eukaryotic models in future viral interaction and drug screening research.

KEYWORDS: SUMOylation, quantitative Förster Resonance Energy Transfer, synthetic biology, influenza A virus, viral proteins, protein interactions

FACULTY MENTOR - Dr. Jiayu Liao, Department of Bioengineering

Dr. Liao, a founding faculty member of UCR's Bioengineering Department, researches host–virus interactions and develops advanced qFRET technology for both basic and translational science. Before UCR, he was a senior research fellow at the Novartis Genomic Institute and Scripps Research. As the founding scientist of the GPCR platform at GNF, his work on FTY720 led to the discovery of SEW2871, contributing to the development of ozanimod for multiple sclerosis. He has authored over 70 publications and holds 32 patents.

My Linh Le

My Linh Le is a current 4th-year undergraduate student in bioengineering. For the past year, she has been following under the guidance of Dr. Liao in research on biotechnology and molecular engineering. Her work focuses on qFRET analysis of E3 ligase interaction with IAV M1 proteins along with other SUMOylation enzymes. She aspires to further her professional career in research and plans to pursue a master's in bioengineering, with a concentration in cellular and molecular engineering.

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INTRODUCTION

Influenza is a seasonal respiratory virus that infects almost 1 billion people worldwide and causes 290,000 – 600,000 deaths annually.[24] Its most common strain, Influenza A (IAV), has led to disease outbreaks such as the 2009 H1N1 and the 2013 H7N9 pandemics in recent medical history, which emphasizes the demand for innovations in influenza prevention.[23] Current research suggests that the IAV M1 protein exploits the host SUMOylation cascade to facilitate viral proliferation.[23] However, the study of SUMO-IAV M1 interaction faces challenges as it requires mammalian, specifically human, cell cultures that are both costly and labor intensive to maintain.[23] The goal of this project is to utilize synthetic biology technology to design a polycistronic SUMO pathway inside *E. coli* which can reduce traditional SUMO-IAV M1 research drawbacks. Quantitative Förster Resonance Energy Transfer (qFRET) is used to examine the protein expression of polycistronic SUMO in different *E. coli* strains and validate interaction of SUMO1 peptides with IAV M1.

One notable application of synthetic biology is the ability to engineer bacteria to perform complex functions, such as the SUMOylation pathway, which typically only exists in eukaryotic cells (Fig. 1). SUMO, or small ubiquitin-related modifier proteins, covalently binds to target proteins at lysine residues, a process made possible by its structure similar to ubiquitin.[3] Three enzymes facilitate the conjugation reactions: an E1 activating enzyme, a specific E2 conjugating enzyme (*UBC9*), and a few E3 protein ligases such as PIAS2. SUMOylation primarily modifies a molecule already attached to a SUMO protein, influencing aspects like the protein's subcellular location, protein-protein interactions, and the activity of the SUMOylated protein.[2,3] SUMOylation plays critical roles in several cellular processes directly relevant to cancers, such as immune regulation, signal transduction, DNA damage, cell cycle, cancer metastasis, and stem cells.[17,18,19]

Although the SUMOylation cascade is complex, synthetic biology allows its reconstruction in *E. coli* through artificial cloning.[5,2] The artificial cloning of genetic material enables the introduction of the SUMOylation pathway into *E. coli*. Specifically, the bacteria are synthetically engineered

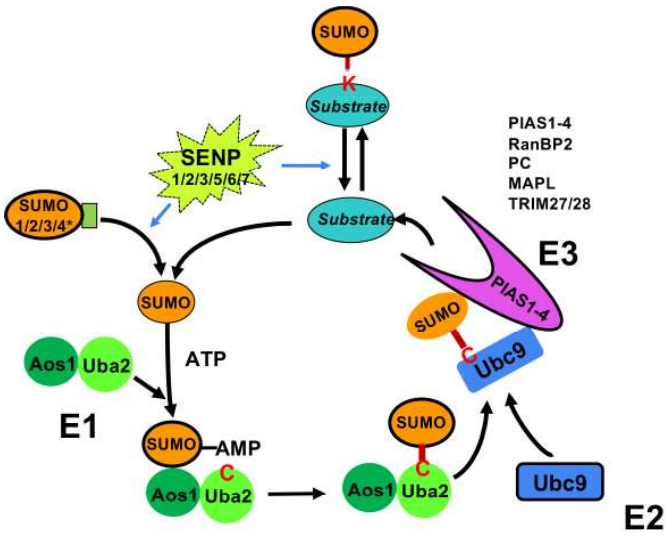

Figure 1: Illustrated SUMOylation cascade in eukaryotic cells. SUMO peptide is first expressed as a pro-SUMO precursor and then processed to mature SUMO peptide by SENP protease. The mature SUMO peptide is then activated by the E1 activating enzyme, Aos1/Uba2 heterodimer, before being transferred to the E2 conjugating enzyme, Ubc9. The E3 ligase improves substrate specificity to help SUMO1 bind to substrates through Lys residues. The conjugated SUMO1 peptide can then be removed by SENP, cycling through its reversible post-translational modification.

to incorporate the DNA necessary for expressing SUMO proteins, along with the E1, E2, and E3 enzymes.[2] A more efficient gene cloning method, facilitated by polycistronic DNA synthesis technology, can generate the necessary sequences to encode this pathway.[4] This combination allows for the effective reconstitution of the SUMOylation assay[5].

To evaluate SUMOylation activity, Förster Resonance Energy Transfer (FRET) is a technique that monitors protein-protein interactions within *E. coli*, enabling the quantification of SUMOylation efficiency and providing essential information about the genetically engineered bacteria[6]. The FRET technique relies on the non-radioactive transfer of energy via electrostatic attractions between two fluorophores, a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP), CyPet and YPet, respectively,

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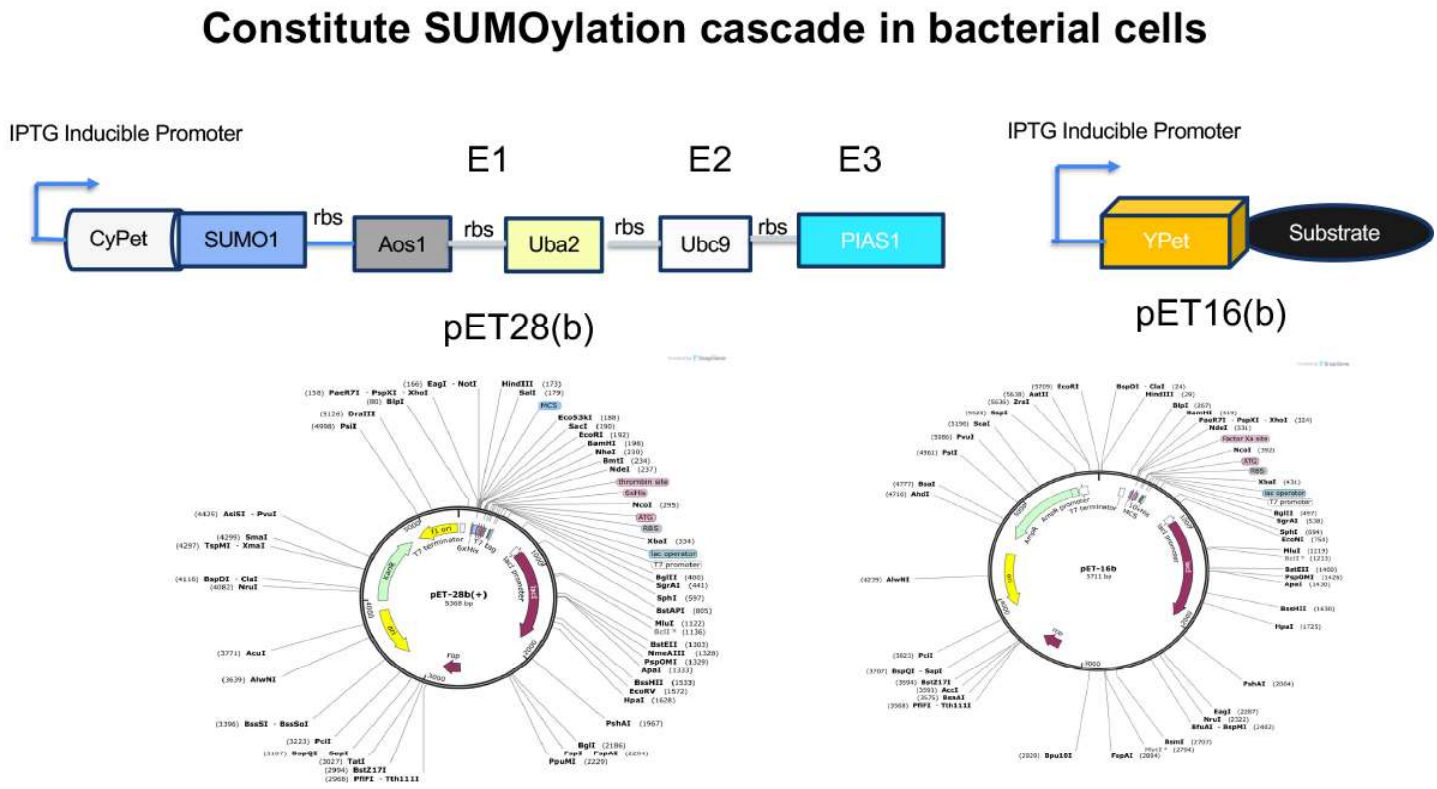

Figure 2: Reconstitute human SUMOylation cascade in bacterial cells using synthetic biology approach for polycistronic expression. To constitute the SUMOylation cascade as a FRET assay in bacterial cells, all the SUMOylation enzyme genes E1, E2 and E3, and CyPet-SUMO gene were constructed in one bacterial expression vector, pET28(b) (Kan+), with ribosome binding sites (RBS) and translation enhancement sequences between each genes (Left). The substrate gene, YPet-M1 was constructed in another bacterial expression vector, pET16 (Amp+)(Right).

in this project, when they are 1-10 nm apart.[6,7,8,9] Unlike traditional FRET settings in well plates, this process occurs directly within *E. coli*, containing the genetic material necessary for SUMOylation.[6,7,8,9] To successfully perform FRET, several conditions must be met. The donors and acceptor fluorophores need to be within the optimal distance to allow energy transfer. The fluorescence environment must also be supportive, maintaining favorable pH and ionic strength to ensure effective fluorescence.[7,9] The experiment begins by exposing the donor fluorophore to a light source, which excites the molecule. Energy transfer occurs through dipole-dipole coupling, exciting the acceptor fluorophore. As the acceptor's electrons return to their ground state, they release photons, and the donor's emission can be measured to confirm that FRET has occurred.[6,7,9]

This synthetic biology approach for SUMOylation pathways significantly reduces the need for iterative cloning, especially tedious protein expression and purification procedures.[1, 4, 5,10] With this synthetic biology approach, only the final SUMOylation conjugation needs to be examined. By applying the synthetic biology approach of SUMOylation, the cost of time and resources can be significantly reduced, allowing significant efficiency improvement in drug discovery and research.[1,10]

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MATERIALS AND METHODS

Designing the Polycistronic SUMO Construct as a Synthetic Biology Approach

The synthetic SUMOylation cascade construct was designed by attaching CyPet to SUMO1 and E1 activating, E2 conjugating, and E3 ligase enzymes via an engineered ribosome binding site (RBS), AAAGAGGGGAAA, and translation enhancer, TTAACTTA, between each gene. This construct provides all the necessary enzymes to promote the SUMO conjugation to a substrate, which is Influenza A Matrix protein M1 (IAV M1) in this study. The E3 enzyme, PIAS1, was chosen based on our lab's past research that observed a high affinity for IAV M1 protein.[11] The polycistronic SUMO sequence was cloned downstream of the IPTG inducible promoter to promote protein expression and, consequently, the SUMOylation cascade of CyPet-SUMO1 to YPet- IAV M1. The plasmid was synthesized by GenScript and cloned into E. coli TOP10 cells via electroporation. Colonies were cultured in 2xYT medium, and DNA was extracted using Biomiga Express Plasmid Midiprep Kit. Concentration was confirmed using a NanoDropTM spectrometer, with a target concentration above 200 ng/μL.

Determination of Polycistronic SUMO protein expression in E. coli

Having cloned polycistronic SUMO plasmid DNA, we transformed it into different strains of electrocompetent *E. coli* bacteria, all specialized for high-efficiency protein expression via electroporation. The following list of *E. coli* bacteria used to amplify the plasmid construct includes: SHuffle® T7, Rosetta 2(DE3), Rosetta 2(DE3) PlysS, BL21(DE3) PlysS, BL21-CodonPlus, BL21(DE3), BL21-CodonPlus (DE3)-RIL, Rosetta (DE3)PlysS, OverExpress™ C43(DE3), OverExpress™ C43(DE3) PlysS, E.Cloni® 10G (SOLOs), OverExpress™ C41(DE3), and ArcticExpress (DE3)RP.

After successfully transforming polycistronic SUMO to yield greater than 24 colonies for each competent cell strain, we picked the colonies into 96-well round bottom trays with LB media to screen for CyPet-SUMO1 protein expression. We prepared two sets of identical trays to incubate overnight: uninduced (UN) and induced (IN) of each strain. Once an optical density (OD) of greater than 0.6 at 600-nm absorbance was reached, we added 0.3-μM IPTG into the IN plates to promote protein expression. We screened for competent cell strains for CyPet fluorescence in 16, 24, and 48-hour time courses. Using the Tecan Infinite® 200 PRO plate reader, we performed a fluorescent spectrum scan on each UN and IN plate at an excitation 414 nm and an emission range from 450 nm to 600 nm, which contained the range of CyPet fluorescent emission at 475 nm.

SUMO Conjugation of SUMO to IAV M1 in *E. coli*

From the CyPet protein expression qFRET measurements of polycistronic SUMO in the 13 screened electrocompetent cells, we standardized all data by dividing by their respective O.D. at 600-nm absorbance at the time of screening. Next, we chose the strain with the highest relative fluorescence unit (RFU) value to directly transform with YPet-IAV M1 via electroporation. Three colonies were picked and inoculated in 3 mL of LB broth with a 1:1000 ratio of Kanamycin and Ampicillin, then incubated overnight. The next day, we separated the cultured tubes into new induced (IN) and uninduced (UN) tubes containing the same amounts of LB broth and antibiotics to shake until a desired OD of greater than 0.6 was reached at 600-nm absorbance. Only IN tubes had 0.3-μM IPTG added to promote CyPet and YPet protein expression. Following centrifugation, the resulting pellets were resuspended in 1x PBS buffer and transferred into black, clear-bottom 384-well plates for fluorescence screening. Each double-transformed UN/IN culture sample underwent a 24-hour time course scan to observe CyPet-YPet protein interaction. Fluorescence spectrum scans were performed on each UN/IN sample using the Tecan Spark® multimode microplate reader, with excitation at 414 nm and emission collected from 450 to 650 nm. This wavelength range contains the overlap between CyPet emission and YPet excitation at 475 nm, which provides optimal observation of CyPet donor transferring fluorescent energy to YPet acceptor, signaling that the polycistronic SUMO and IAV M1 proteins successfully interacted within a range of 1-10 nm of each other.

SUMO Inhibitor Testing

The pET28b constructs encoding polycistronic SUMO and the pET16(b) IAV M1 plasmid were used to transform BL21(DE3) PlysS *E. coli* cells. The ideal signal colony was cultivated at 37 °C and 250 rpm after being injected into 3

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mL of liquid LB with 50-mg/mL kanamycin and rapamycin culture. At 16°C and 180 RPM for 48 hours, 0.3-mM IPTG and 250-µM SUMOylation inhibitor simulated culture expression. After 48 hours at room temperature and 13,000 RPM for three minutes, the bacterial cells were extracted. 60 µL of 1x PBS (pH 7.4) was used to resuspend the bacterial cell pellet. We then conducted a fluorescent spectrum scan using the Tecan Spark® Multimode microplate reader with excitation at 414 nm and emissions between 450 and 600 nm.

RESULTS

After screening all 13 strains, we transfected the polycistronic SUMO pathway into E. coli and monitored CyPet protein expression at 16, 24, and 48 hours post-transfection. These timestamps correlated to all our samples' growth, peak, and degradation periods. **Table 1** summarizes the highest CyPet protein expression measurements among the 24 samples of every E. coli strain, with BL21(DE3) PlysS presenting the highest fluorescence at 26459 RFU. Specifically, this peak is shown in the 24-hour induced polycistronic SUMO in BL21(DE3) PlysS qFRET scans at 475 nm (**Fig. 3**); the expected emission wavelength of CyPet when excited at 414 nm.

The resulting scans of Polycistronic SUMO in BL21 (DE3) PlysS (**Fig. 3**) at an excitation of 414 nm revealed a single broad emission peak consistent with the expected fluorescence profile of CyPet. Emission was measured from 450 nm to 600 nm, and all spectra were normalized to allow comparison between the 24 clones. While variations in intensity are observed, the overall shape of the emission curves remains consistent. The absence of a secondary peak supports the conclusion that CyPet is the sole fluorescent species present in the system. IAV M1 was introduced to the system as a YPet acceptor fluorophore to monitor the potential of protein-protein interactions via FRET. This positive peak was then cloned three times and re-induced as described in the SUMO Conjugation of SUMO to IAV M1 in *E. coli* section.

The high CyPet protein expression of Polycistronic SUMO in BL21(DE3) PlysS was further analyzed by comparing the mean CyPet fluorescence among all IN 13 electrocompetent *E. coli*. A confidence interval (CI) of 95% was used to estimate the true means of all strains, which, as observed in **Fig. 4**, supports CyPet high expression in BL21(DE3) PlysS and highlights its suitability as an E. coli host for the polycistronic SUMO construct. With a calculated CyPet expression mean of 19166.683 RFU, the true population mean was determined to be within [17491.548, 20841.818], the highest among the 13 transformed strains.

Electrocompetent E.Coli	CyPet protein Expression (R.F.U)
SHuffle® T7	18828
Rosetta 2(DE3)	22731
Rosetta 2(DE3) PlysS	21370
BL21(DE3) PlysS	26459
BL21-CodonPlus	19513
BL21(DE3)	25140
BL21-CodonPlus (DE3)-RIL	18368
Rosetta (DE3)PlysS	22210
OverExpress™ C43(DE3)	17843
OverExpress™ C43(DE3) PlysS	17618
E.Cloni® 10G (SOLOs)	19936
OverExpress™ C41(DE3)	25488
ArcticExpress (DE3)RP	22039

Table 1: Comparison chart of the highest CyPet fluorescent expressions at 24 hours of IN polycistronic SUMO in each 13 Electrocompetent E. coli listed, with the highest expressing strain underlined in black. control conditions.

To improve the consistency across the experiments, SUMO and M1 cultures were diluted to maintain a uniform starting optical density (OD) of 0.6 at 600 nm. This helps ensure reliable comparisons between induced and uninduced samples while reducing variability in fluorescence, which could be observed in **Fig. 5**, leading to consistent qFRET signals with positive CyPet-YPet energy transfer. FRET was used to validate the conjugation of SUMO1 peptide to the IAV M1 substrate. This allows for the detection of molecular proximity between two tagged proteins, CyPet (donor) and YPet (acceptor), when they are within 10 nm apart. Our system's shift from a single peak (donor only at 475 nm) to a double peak emission (donor and acceptor at 475 nm and 535 nm, respectively) demonstrates physical approximation between the SUMO enzymes and M1 protein.

These three clones again show the double peak pattern with one peak around 475 nm (CyPet) and a second distinct peak

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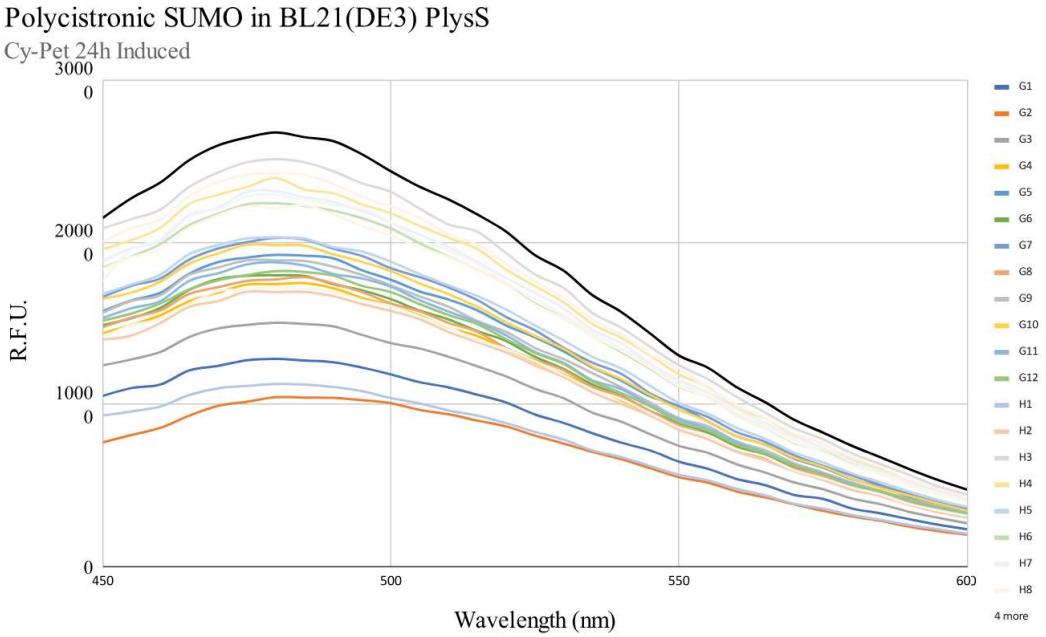

Figure 3: Fluorescence spectrum of polycistronic SUMOylation cascade containing CyPet-SUMO1 in bacterial cells. The pET28-polycistronic plasmid was transformed into the BL21(DE3) PlysS strain. 24 colonies were picked up and cultured for inductions of all proteins for 24hrs. Then bacterial cells were determined for the emission of CyPet-SUMO1 at the peak of 475 nm. The strain with the highest Relative Fluorescence Unit (RFU) was H11 in the BL21(DE3) PlysS strain, highlighted in black.

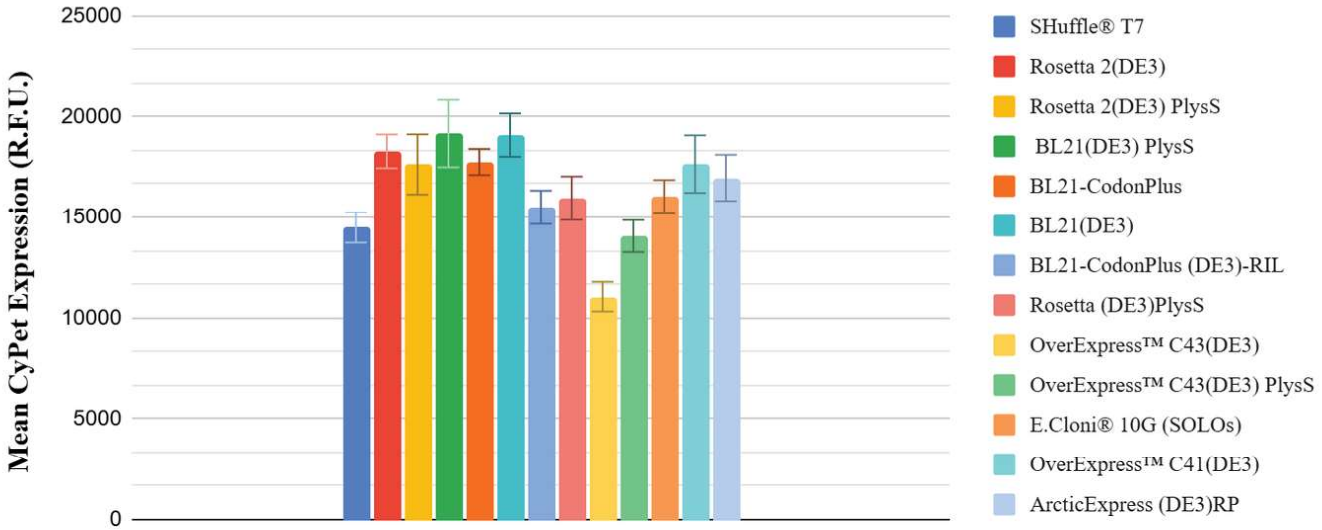

Figure 4: Mean CyPet Expression of Polycistronic SUMO in Electrocompetent cells: SHuffle® T7, Rosetta 2(DE3), Rosetta 2(DE3) PlysS, BL21(DE3) PlysS, BL21-CodonPlus, BL21(DE3), BL21-CodonPlus (DE3)-RIL, Rosetta (DE3) PlysS, OverExpress™ C43(DE3), OverExpress™ C43(DE3) PlysS, E.Cloni® 10G (SOLOs), OverExpress™ C41(DE3), and ArcticExpress (DE3)RP.

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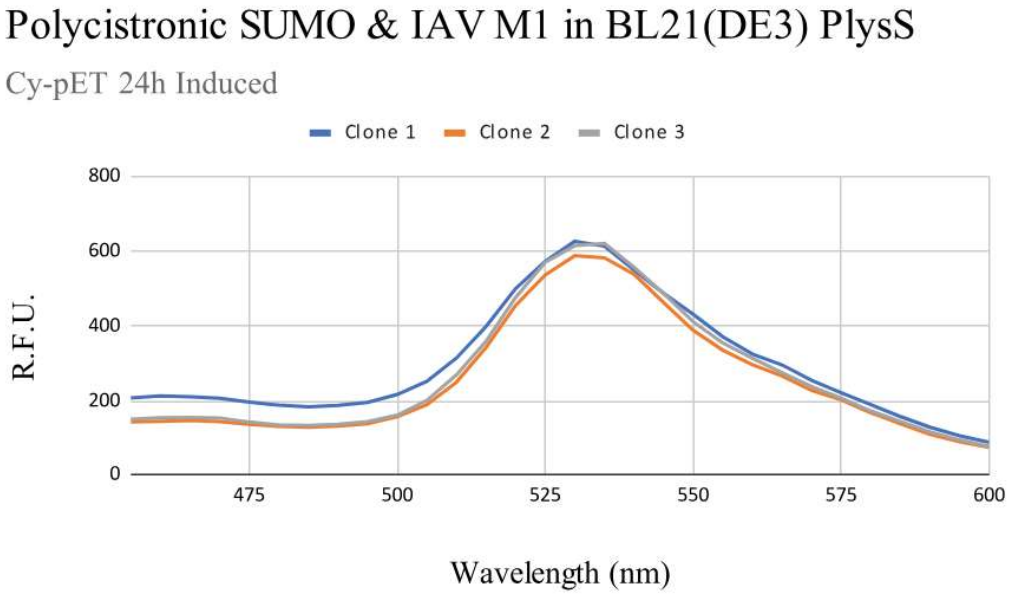

Figure 5: FRET emission of polycistronic SUMOylation cascade and YPet-IAV M1 in bacterial cells. The bacterial cells containing polycistronic SUMO were transformed into the plasmid containing SUMOylation substrate, pET16-YPet-IAV M1. After induction for 24hrs, the bacterial cells were examined for the FRET signal at 535 nm when excited at 414 nm for the FRET donor CyPet. The emission of bacterial cells at 530 nm indicates successful SUMOylation conjugation. (SOLOs), OverExpress™ C41(DE3), and ArcticExpress (DE3)RP.

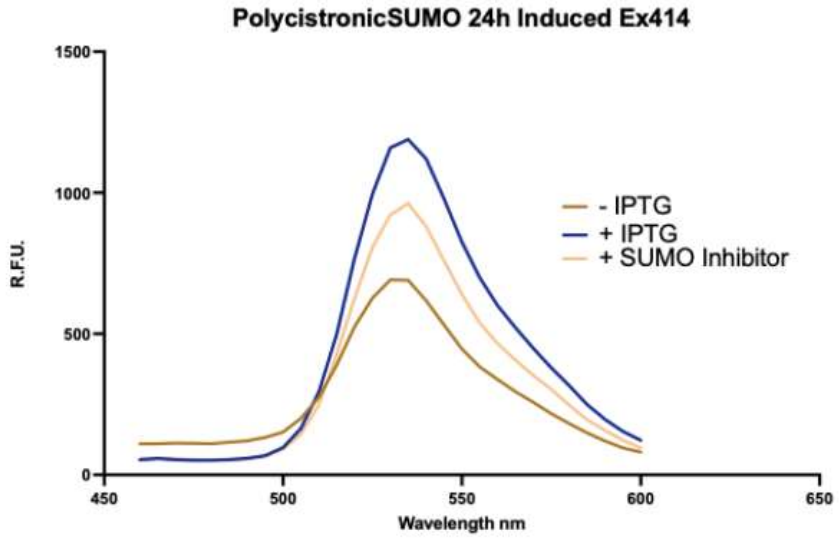

Figure 6: Polycistronic SUMOylation system in inhibitor evaluation. The reconstituted FRET-based SUMOylation system with synthetic biology in bacterial cells was used to evaluate the activity of SUMOylation inhibitor, STE, discovered in our lab. After the double-transformed bacterial cells were cultured to O.D. of 0.6 at 600 nm absorbance, both IPTG and SUMOylation inhibitor were added for another 24 hrs before the FRET signal was examined. The decreased FRET signal with SUMOylation inhibitor suggests that SUMOylation cascade is successfully inhibited in this assay with synthetic biology design of SUMOylation cascade.

near 520-530 nm (YPet). These emission scans revealed that the YPet peak signal was significantly more substantial than the CyPet signal, indicating the successful expression of both fluorophores. The observed increase in YPet emission, followed by the diminished CyPet fluorescence, is consistent with efficient FRET. This strongly suggests that the donor CyPet and YPet acceptor fluorophores are within close proximity, reflecting a successful interaction of the engineered protein system.

The M1 protein, which is SUMOylated in IAV, is essential for viral RNA trafficking and assembly. Following the SUMOylation enzymatic cascade to receive the FRET donor, CyPet, the M1 protein of IAV was first fused with the FRET pair acceptor YPet to function as a substrate in the SUMOylation experiment. Due to the proximity of the FRET donor and acceptor, the FRET signal was highly significant following the conjugation of the SUMO to the M1 (**Fig. 6**, +IPTG). The FRET signal showed that the inhibitor hindered this SUMOylation reaction (**Fig. 6**, +SUMO inhibitor). Bacteria pellets of Polycistronic SUMO are determined by Western Blot. The primary antibodies used in this study were anti-His (1:1000 dilution), and detection was performed using an anti-mouse secondary antibody (1:1000 dilution).

DISCUSSION

This study developed a successful polycistronic SUMOylation construct for expression in *E. coli*, representing a significant advancement in the application of synthetic biology and SUMO-IAV M1 research. By reconstituting the eukaryotic SUMOylation cascade in the prokaryotic system, we have begun to create an efficient platform for SUMO conjugation process to target substrates. Namely, the synthetic polycistronic SUMO construct was able to conduct the complete SUMOylation cascade to attach to IAV M1 protein in E. coli, as observed through the successful CyPet-YPet FRET in **Fig. 5**. This protein-protein binding was from the conjugation of CyPet-SUMO1 expressed in the polycistronic construct to substrate IAV-M1 through the catalytic reactions of E1, E2, and E3 (**Fig. 2**). The reconstituted SUMOylation pathway in *E. coli* overcomes limitations of in vitro mammalian systems, where maintaining cell culture and conducting enzymatic assays are costly and labor intensive.[23] The findings in our project can significantly aid in future SUMOylation research, providing an *E. coli* system alternative capable of quantitative analyses, similar to standard mammalian cultures.[23]

The polycistronic SUMO construct allows for the simultaneous expression of SUMO1 and the three enzymes (E1, E2, and E3), facilitating efficient protein expression and SUMO conjugation reaction to the substrate. From the 13 bacterial strains tested, BL21 (DE3) PlysS yielded the highest CyPet expression. This was because of the suppressed T7 polymerase expression in the PlysS plasmid that decomposes the T7 polymerase before induction to overcome the possible toxicity of downstream proteins. This enables both promoted polycistronic SUMO protein expression through IPTG induction and reduction of basal expression of the gene of interest under the control of T7 promoters, which is critical in protein expression of toxic proteins in *E. coli*.[15]

Limitations and Future Research

While polycistronic expression of the SUMOylation cascade in *E. coli* offers an accessible platform, the system presents limitations in assay reproducibility. A primary concern is the gene order within the polycistronic vector, as genes positioned downstream of the promoter tend to exhibit reduced expression levels. This effect could imbalance the relative amounts of E1, E2, E3 proteins being expressed. An imbalance could hinder modification since the SUMO pathway needs simultaneous activity of all three enzymes.[5,13] Additionally, intercistronic spacing between genes can influence translational efficiency. Both short and very long distances between ribosome binding sites (RBS) and start codons could potentially impair translation re-initiation.[14] This affects downstream protein levels, introducing the complex issue of tuning expression levels across the construct where stoichiometric ratios are essential for pathway functions.[14]

Another potential drawback involves translational coupling, a design used to enhance expression continuously in polycistronic operons.[13,14] This design is intended to improve downstream gene expression but can have unintended effects, such as ribosome drop-off or secondary structure formation interfering with internal RBS exposure. Such

unpredictability can destabilize expression and prevent efficient translation of SUMO1 or PIAS1.[14] A potential solution to determine if these issues are significant within our polycistronic SUMO system is to use a Western blot to verify protein expression of E1, E2, E3, and SUMO1. Certain modifications in the synthetic SUMOylation construct can also improve gene expression, such as adjusting the spacer region between the ribosome binding sites to the start codon. We can further utilize synthetic biology technology to identify optimal ribosome binding site distances to maintain translational efficiency while preventing ribosome drop-off and formation of secondary structures.[13] Additionally, we can include an upstream leader peptide in our construct to improve downstream gene regulation and overcome variability in re-initiation efficiency.

CONCLUSION

The synthetic biology polycistronic SUMO technology examined in this project shows great potential for future clinical applications. From our qFRET observations, the engineered construct yielded high protein expression rates. It reproduced the full SUMOylation cascade to bind to target substrate IAV M1 in *E. coli*, which opens possibilities for improved recombinant protein research and high-throughput screening in drug discovery. Eukaryotic cells are often complex and costly to maintain, especially when studying SUMOylation modifications with other target substrates. The Polycistronic SUMO construct allows for convenient and efficient induction of SUMO protein expression. It activates its cascade of supporting enzymes to express SUMO in bacteria without the tight regulation of other eukaryotic molecules in the system. Our engineered construct also avoids other eukaryotic post-translational modifications that can interfere with SUMOylation, isolating the pathway into bacterial cells to study specific SUMOylation effects.

Having shown high SUMO protein expression in bacteria, our polycistronic SUMO system can also be a more cost-effective method for drug screening, allowing ease to scale up in bacteria models and abundant proteins for interactions with different small molecule inhibitors. In recombinant protein research, our polycistronic SUMO construct creates a simultaneous expression of SUMO enzymes and proteins, allowing for better solubility, stability, and protein folding in large scale protein production. This is particularly valuable for difficult-to-express eukaryotic proteins, which often aggregate in *E. coli* due to improper folding. The ability to reproduce native SUMOylation pathways in bacteria allows us to study native and controlled SUMO-mediated modifications on various substrates outside of IAV M1, extending the synthetic SUMO construct application to structural biology, enzyme engineering, and functional protein assays.

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