"Expanding the Yeast Modular Cloning (MoClo) Toolkit for Optimizing Heterologous Protein Secretion in *Saccharomyces cerevisiae*"

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Introduction

Yeast, specifically *Saccharomyces cerevisiae (S. cerevisiae),* is widely recognised as a favoured host for synthesizing recombinant proteins. Yeast expression systems have several advantages over other potential host cells. This is due to yeast being a single-celled organism, that experiences rapid growth, with yeast cells doubling approximately every 90 minutes (Bergman et al, 2001). The ability of yeast to be easily genetically manipulated, coupled with eukaryotic features such as a secretory pathway, makes it an ideal host for recombinant protein production. In addition, *S. cerevisiae's* "generally recognised as safe"(GRAS) status is essential for proteins used in food and biopharmaceutical industries. (O'Riordan et al) .

A recently developed toolkit that utilises modular cloning (MoClo) principals, which allows for rapid screening of a library of certain well-characterized signal peptides, allowing for efficient secretion of a target protein (O'Riordan et al., 2024). MoClo utilizes Golden Gate Cloning for the assembly of multiple DNA fragments in a predetermined order. This method has gained popularity due to its efficiency and versatility in synthetic biology using type IIs restriction enzymes. The key concepts of MoClo include the type IIS restriction enzymes that cut DNA at a specific distance from their recognition site, creating overhangs as well as allowing efficient assembly of expression vectors. The DNA overhangs facilitate the assembly of the DNA fragments in a predefined sequence. The use of Golden Gate Assembly combines the restriction enzymes and ligase in a single reaction allowing for simultaneous cutting and ligation.

Aims of the Project

During my placement the main aims of my project was to identify 10 candidate "secretion boosting" genes from the literature. Once these candidates were identified, primers incorporating BsmBI and BsaI restriction sites were designed and ordered. Once primers were received and prepared they were used to clone these genes into the pYTK001 vector. Yeast expression constructs were then created with a strong promoter and HIS3 marker. In subsequent steps, these constructs were co-transformed with a plasmid for low-efficiency secretion of mRuby2, and the efficiency of mRuby2 secretion was evaluated. The 10 'secretion boosting' genes were:

1.COG5 **2** ERV2 **3** MNS1 **4.** SEC65 **5.** SRP14 **6.** SRP54 **7.** SSA1 **8.** SWA2 **9.** SEC16 **10** CWH41

Methods Used

A wide range of techniques were used throughout the project. The initial step involved the preparation of primers and conducting PCR reactions using Phusion Hot Start II High-Fidelity DNA Polymerase. Once the PCR products were run on a gel the samples were purified using a PCR purification kit.

Purified PCR products were organised and labelled 1-10. Golden Gate Assembly was conducted on the purified product to place them into the entry vector. During the Golden Gate Assembly reaction, LB agar plates containing the antibiotic carbenicillin were prepared. Subsequently, containing the target genes, were transformed into competent *E. coli* cells, which were then plated on the prepared LB agar plates and incubated overnight to allow for colony growth. Restriction digests were conducted to confirm correct insert before samples were sent for sequencing. Four of seven sequences were moved forward to the next step of BsaI based assembly. They were then transformed into *E.coli* cells. Mini preps were carried out on successful colonies as well as digestion to check for correct inserts, these were checked against virtual digests. Those plasmids that had the correct digest were then

cotransformed into yeast cells (BY4741 strain) together with a vector that directs low efficiency secretion of mRuby and plated Colonies that grew on SD -LEU – HIS agar were picked and inoculated into MOPS & K_2PO_4 buffered SD -LEU -HIS media on a 96 well plate with. mRuby fluorescence and absorbance at 600nm was then measured using a Tecan plate reader.

Results and outcomes:

Based on the readings from the Tecan plate reader, it was observed that ERV2 is a potential "secretion-boosting" gene, as its reading exceeded that of the positive control. With high levels of secreted mRuby2 in the supernatant compared to levels in the cell. The remaining genes are just below the positive control indicates that the gene does not boost secretion. However, it does show that the gene is functional and capable of secreting the protein, albeit not at an enhanced level.

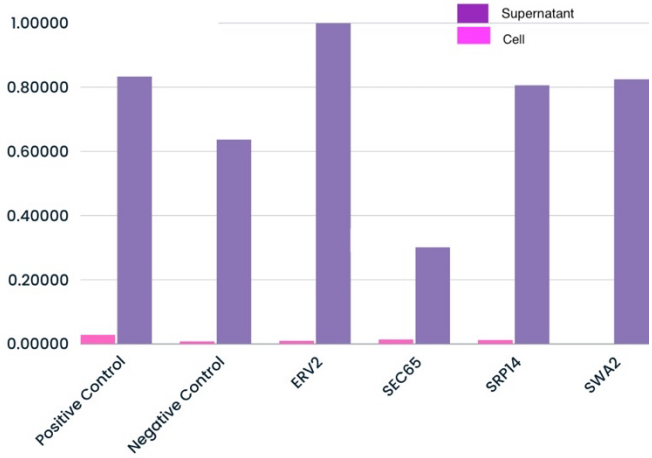

Fig 1. ***Normalised results for mRuby fluorescence of yeast cells expressing the indicated genes.*** *Overexpression of ERV2 enhanced mRuby secretion into the media (supernatant) while secretion for SRP14 and SWA2 was slightly below positive control threshold.*

Departures from original project plan

A number of the genes initially identified through the literature as “secretion boosting” had to be excluded due to time limitations in the project. The main reasons for excluding these genes were difficulties with inserting them into the entry vector or incorrect sequences that prevented them from being moved to the next step of being placed into the expression vector. Instead of spending a significant portion of the project trying to resolve these issues, it was decided to proceed with as many genes as possible where the sequencing was successful and to revisit the excluded genes at a later time.

Future Directions in which the project could be taken

Fluorescence readings using a Tecan plate reader provides quantitate data on protein secretion, but western blots offers even more advantages. It confirms the specificity and molecular weight of the protein, detects post-translational modification and asses the proteins integrity. The next step could involve revisiting genes that were previously dropped and carry out western blots on these genes as well as ones successful during the project, to evaluate mRUBY2. Optimizing conditions for genes with correct sequences but readings below the positive control threshold may also be investigated.

Value of studentship to the student and to the research group

To the student - During this unique and exciting opportunity, I have gained invaluable transferable skills. I have deepened my understanding of a range of molecular biology techniques these skills included cloning, PCR, DNA gel electrophoresis, and the propagation and transformation of bacteria and yeast. These skills along with improving my analytical and problem-solving abilities will be extremely beneficial to me for my final year at university, also being invaluable for further studies I hope to pursue in the future.

To the lab- The studentship has proven immensely valuable to the research group by directly allowing the extension of their existing work on protein secretion in yeast. The data gathered throughout the project will be a help to those continuing the work on expanding the MoClo toolkit.

References:

Bergman, L.W. (2001) ‘Growth And Maintenance Of Yeast’, Two-Hybrid Systems, Volume 117, Pages. 009–014. https://doi.org/10.1385/1-59259-210-4:009

O’Riordan, N. M., Jurić, V., O’Neill, S. K., Roche A. P. & Young P. W. (2024) 'A Yeast Modular Cloning (MoClo) Toolkit Expansion for Optimization of Heterologous Protein Secretion and Surface Display in Saccharomyces cerevisiae', ASC Synthetic Biology, Vol 13/Issue 4 https://pubs.acs.org/doi/10.1021/acssynbio.3c00743

Acknowledgements

I would like to thank Dr Paul Young and all those in Paul Young’s lab for this invaluable opportunity and their support over the six weeks. We sincerely appreciate the Biochemical Society's funding of the studentship. This program has helped achieve their strategic objectives, by promoting and sharing knowledge and support career development for everyone.

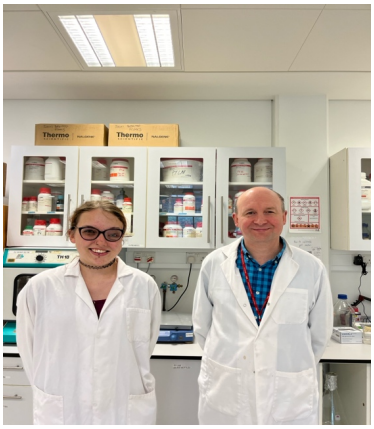

Figure 2. Lia Teahan O'Connor (left) and Dr.Paul Young (right)