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Introduction

Post-translational modification of proteins is vital in the generation of functional enzymes. Cells depend on assembly pathways to incorporate cofactors and prosthetic groups into apoproteins in order to manufacture functional proteins Specifically, the c-type cytochromes represent a class of proteins involves the covalent attachment of heme cofactors to cysteines found within CXXCH motifs in their amino acid sequences, resulting in the formation of holoproteins. In certain bacterial species, genes encoding cytochromes containing 20-80 CXXCH motifs have been identified (Kranz et al., 2009). However, the functional characteristics of these cytochromes remain elusive due to the challenging nature of recombinant gene expression. The high number of heme groups within these cytochromes has been proposed to hinder successful recombinant expression (He et al., 2017). This research aims to investigate the expression of a recombinant cytochrome FioB, containing a recurring 3-heme motif within a single gene containing 24 hemes. The length of the cytochrome can be increased until expression is no longer possible by gradually adding stop codons. This approach serves as a valuable tool for exploring the limitations of recombinant cytochrome expression.

A current obstacle faced in the research of proteins and the characterisation of their function lies in the expression of recombinant proteins Cytochromes with numerous heme groups likely play a crucial role in electron transfer pathways within bacterial cells, yet their properties and functions remain poorly understood due to the difficulties associated with their expression and isolation. Little is known about the primary function of these genes with regard to the electron transport mechanism and cellular metabolism.

Project Aim:

The purpose of the project was to investigate the level of expression of truncated variations of the Slit_0867 gene. By systematically adding of primer engineered stop codons after each 3-heme motif an upper limit of possible cytochrome length can be determined. To achieve this we needed to obtain truncated plasmid based copies of Slit_0867 by PCR site directed mutagenesis then transform plasmids into E. *coli* BL21 pEC86 and finally run the expression trial of successful Slit_0867 variants

Results

Primer design

The primers were designed to place a stop codon after the 3rd, 6th, 9th, 12th, 15th, 18th and 21st hemes respectively (Figure 1).

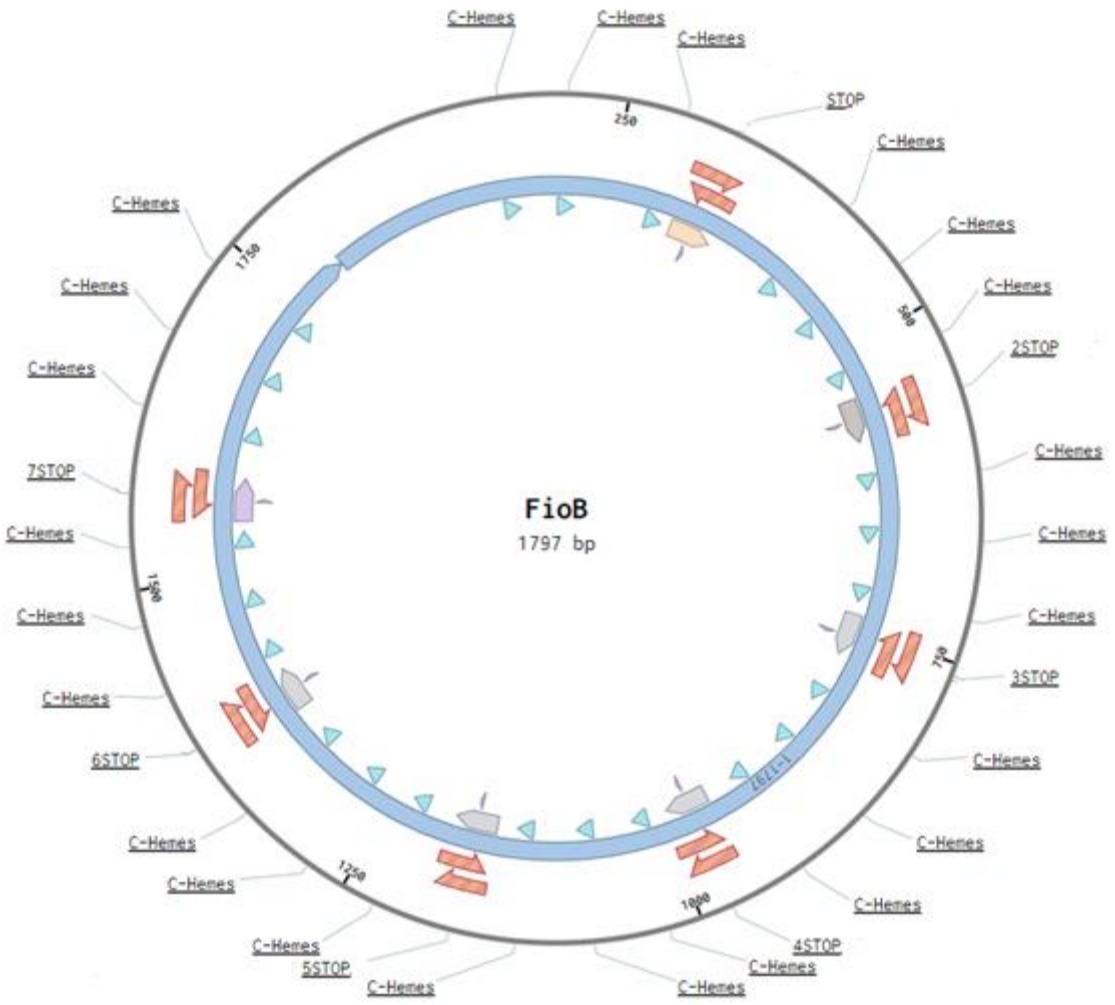

Figure 1: Illustrating gene DNA for p*FioB* and locations of stop codons after each 3 heme motif

Transformation of pMEGGAX_*FioB* into T10 E. *coli*

An aliquot of 2 µL of pMEGGAX_*FioB* was added to a culture of 50 ul Top 10 Escherichia *coli* and incubated on ice for 1 hour. The T10 E. *coli* was then heat-shocked for 40 seconds at 42 °C, then immediately put on ice. To the cells 200 µL of LB broth was added, which was then incubated in a heat-block at 37 °C, 350 rpm for 1 hour 30 minutes. After this the solution was plated onto LB agar with added Kanamycin (Kan) 30 ng/ul and incubated at 37 °C overnight. Colonies were

harvested from this plate, and used to inoculate a solution of 10 µL Kan + 10 mL LB broth, which was then incubated in a shaker at 37 °C overnight.

Addition of stop codon primers

Kan + LB broth overnight was collected and pMEGGAX_*FioB* was isolated by use of GenElute™ Plasmid Miniprep Kit protocol. A Nanodrop Spectrophotometer was then used to identify the best sample of the plasmid whose identity was confirmed by use of agarose gel electrophoresis (Figure 2).

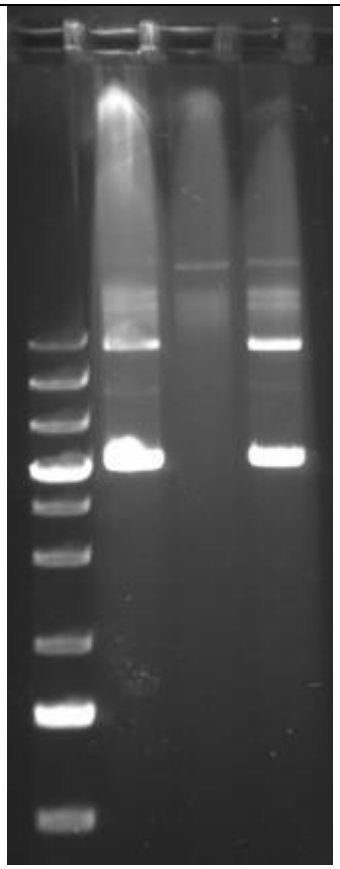

Figure 2: Agarose gel electrophoresis of plasmid prep of T10 pMEGGAX_*FioB* with banding confirming this identity. Lane 1: 1Kb+ ladder, Lane 2-4: T10 pMEGGAX_*FioB*

Standard PCR protocol was then carried out on this sample with each respective stop codon primer. Agarose gel electrophoresis was then ran on the samples to identify successful annealing (Figure 3). Each successful sample was then transformed into T10 E. *coli* and plated on Kan + LB agar plates, incubated overnight at 37 °C.

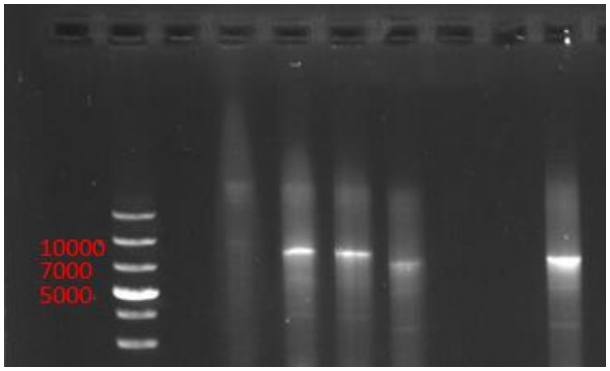

Figure 3: Agarose gel electrophoresis of pMEGGAX_*FioB.1*, Lane 2: 1Kb+ ladder, Lane 4-7 and Lane 10: pMEGGAX_*FioB* PCR Mutagenesis with stop codon after 3rd heme. Banding at around 7,000 bp aligns with plasmid size of 6,623 bp.

Expression trial

Successful plasmids from PCR were transformed in to E. *coli* BL21 with pEC86 plasmid, the purpose of the added pEC86 being to allow for cytochrome c maturation. The expression trial consisted of 5 x 50 ml LB + Kan + Chloramphenicol with 1% inoculum which were then incubated overnight at 37 °C. Cultures were then grown to an OD_{600} of 0.4 then induced with arabinose, at concentrations: 0 mM, 0.1 mM, 0.5 mM, 1 mM and 5 mM respectively. These cultures were then left overnight at 37 °C. Following this, the cultures were harvested by centrifugation, then the pellets were resuspended in 1 ml 50 mM Tris 100 mM NaCl pH8, cultures were lysed via sonication. Whole cell SDS was run by taking 50 µL of sample and mixing with 50 µL of SDS loading dye, then boiled for 30 mins at 90 °C. The SDS gel was ran and stained with peroxidase linked heme stain which can be seen in Figure 4.

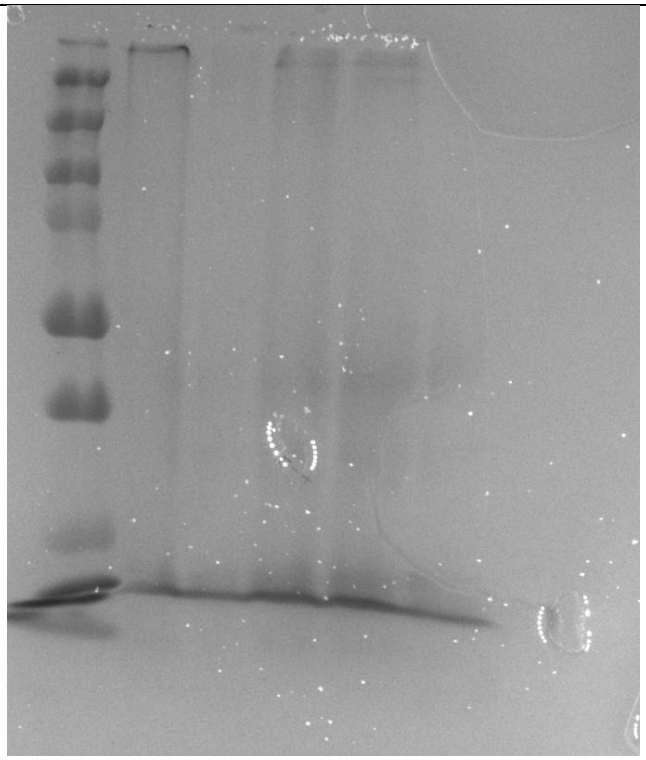

Figure 4: SDS-PAGE with gel visualised by heme staining, banding seen confirms transformation of pMEGGAX_*FioB.3* (STOP codon after 9th heme) into E. *coli* BL21 + pEC86.

With the success of the expression trial we were able to show that it is possible for the expression of a recombinant multiheme cytochrome that contains a 9 hemes.

Future trajectory of the project

Further efforts to express the 12, 15, 18 and 21 heme motifs are required to find where the limit of expression is as well as protein purification of the expressed proteins and cyclic voltammetry to confirm activity. In addition to this, transformation into *Shewanella oneidensis* should also be attempted to trial expression to identify if the heme limit is different.

My takeaways from this studentship

This studentship has been a valuable opportunity for my academic and professional growth. It not only provided brilliant mentorship and refinement of prior lab skills but also introduced me to the critical aspect of repeated trial and error and problem-solving, which I had not dealt with to that extent in my scientific journey leading up to that point. I gained essential skills, including primer design, plasmid prep, PCR site-directed mutagenesis, and transformation just to name a few. Furthermore, the studentship connected me with experienced scholars, expanding my professional

network. It significantly advances my career prospects and lays the foundation for a successful scientific career. Ultimately, I believe the studentship provided a brief yet accurate representation of what it is like to work in research and how to trouble shoot and adapt accordingly to challenges encountered along the way.

References

He, S., Barco, R.A., Emerson, D. and Roden, E.E. (2017). Comparative Genomic Analysis of Neutrophilic Iron(II) Oxidizer Genomes for Candidate Genes in Extracellular Electron Transfer. Frontiers in Microbiology, 8. doi:https://doi.org/10.3389/fmicb.2017.01584.

Kranz, R.G., Richard-Fogal, C., Taylor, J.-S. and Frawley, E.R. (2009). Cytochrome c Biogenesis: Mechanisms for Covalent Modifications and Trafficking of Heme and for Heme-Iron Redox Control. Microbiology and Molecular Biology Reviews : MMBR, [online] 73(3), pp.510–528. doi:https://doi.org/10.1128/MMBR.00001-09.