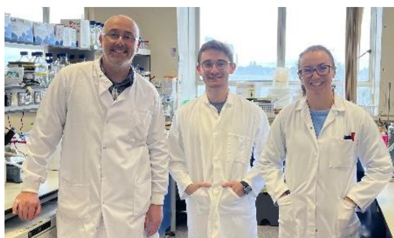

Left to right: Dr Timothy Knowles, Henry Box, Hannah Johnston

Investigating the potential inter-membrane phospholipid transport function of paraquat-inducible ABC (PqiABC) with fluorescent proteoliposomes

Student: Henry Box
Supervisor: Dr Timothy Knowles

Introduction

For more successful antibacterial drug development, a more thorough understanding of the cell envelope is required as it maintains the integrity and shape of the cell. In gram negative bacteria (which constitute 9 of 12 priority pathogens identified by the World Health Organisation [1]), the envelope is an essential structure composed of two membranes sandwiching a peptidoglycan layer. Although this structure has been known for some time, its assembly mechanisms have only recently begun to emerge. Components of the outer membrane (which makes contact with the host) are delivered by complex protein machinery: proteins via the Bam complex, lipoproteins via the Lol pathway and lipopolysaccharides via the Lpt pathway. Mechanisms for phospholipid transport remain elusive, though.

The first pathway correlated with phospholipid transport was the Mla pathway but has since been postulated not to be responsible for the bulk of phospholipid transport and rather more responsible for maintaining outer membrane asymmetry [2, 3]. However, a protein complex with structural similarity to Mla is believed to span the entire intermembrane space with a hydrophobic tunnel and could be responsible for the bulk movement of phospholipids. This machinery is called paraquat-inducible ABC (PqiABC).

Understanding the mechanisms of phospholipid transport, and so potentially the mechanism PqiABC, could provide a new avenue for antimicrobials development. If such mechanisms are conserved across gram-negative bacterial species, then such knowledge could contribute to the fight against a significant number of diseases caused by bacterial infection. This aligns with the strategy set out by the Biochemical Society as they aim to highlight the importance of gathering biochemical knowledge in relation to global health and fighting disease, as was demonstrated recently during the COVID-19 pandemic.

Aims

This project aimed to confirm preliminary evidence gathered by the Knowles laboratory at The University of Birmingham which suggests that PqiABC is a phospholipid pump. Doing so will require an assay which can routinely assay the system in real time. Therefore, the first aim was to design and collect data from a FRET-based transport assay making use of proteoliposomes containing PqiABC and fluorescently-tagged phospholipids (Figure 1). We predicted that when the PqiABC system was complete, there would be a change in fluorescence signal as the fluorescently-tagged phospholipids were transported from one proteoliposome to the other. We also aimed to find the functional impact of the presence of metal chelator versus supplemented zinc ions as analysis of the PqiA structure with an AlphaFold model implies zinc coordination (Figure 2).

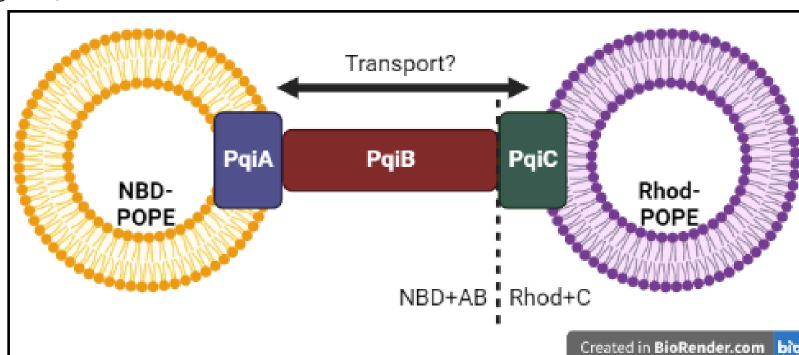

Figure 1 FRET-based phospholipid transport assay with PqiABC. In this example, POPC proteoliposomes containing NBD-tagged POPE (NBD-POPE) and PqiAB or Rhodamine-tagged POPE (Rhod-POPE) and PqiC are mixed, hence the "NBD+AB, Rhod-C" format in Figure 4.

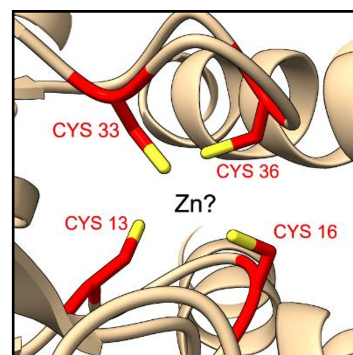

Figure 2 AlphaFold [4, 5] *model of PqiA showing four cysteine residues (red) potentially coordinating a zinc atom.*

Departures from original project plan

I began work on an alternative assay for the routine measurement of phospholipid transport between proteoliposomes. This would rely on the activity of the cardiolipin synthase A (ClsA) to convert POPE into cardiolipin after they were transported from a proteoliposome without ClsA to one with ClsA. A thin-layer chromatograph would reveal cardiolipin presence, and therefore PqiABC transport. The main aim for me was to purify this protein successfully as the most recent protocol was published in the 1990's [6].

Methods

PqiAB: Production of strep-tagged PqiAB involved transformation of plasmid pET26b and growth in C43 *E. Coli*, then affinity-based and size exclusion chromatography. **Proteoliposome preparation:** Liposomes containing set proportions of un-tagged POPC and NBD-tagged or rhodamine tagged POPC were prepared in a fume cupboard then sonicated before adding either PqiAB or PqiC to each. This was done in duplicate – with EDTA or zinc chloride supplemented within the buffers. **FRET assay:** Baseline fluorescence was measured with the NBD-tagged PqiAB or PqiC proteoliposomes. Fluorescence was measured over time upon addition of the rhodamine-tagged proteoliposomes with the corresponding PqiAB or PqiC component. **C-terminal His-tagged ClsA purification and construct modification:**

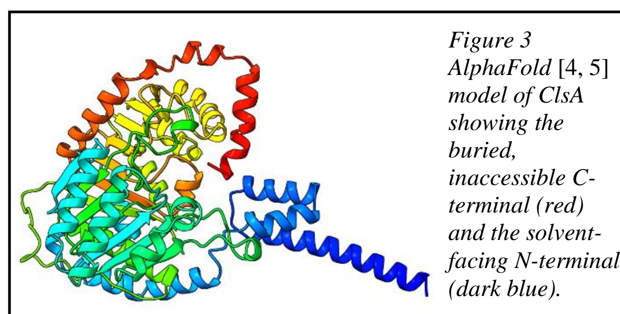

Figure 3 AlphaFold [4, 5] *model of ClsA showing the buried, inaccessible C-terminal (red) and the solvent-facing N-terminal (dark blue).*

Protocol inspired by Hiraoka *et.* al [6]. Having found that C43 *E. coli* expressed the protein better than BL21 in post-induction Western blots, the protein was unfortunately absent after Ni-NTA column chromatography. An AlphaFold model suggests that the His-tag would be better situated on the N-terminal (Figure 3), though, so primers were designed to remove the C-terminal tag and add it to N-terminal on the previous DNA construct.

Results

The overarching result of the FRET-based transport assay is largely inconclusive with regards to the phospholipid-transporting function of PqiABC and its functional dependence on zinc. When chelator (EDTA) was present, there was no significant sign of transport because the levels of FRET due to NBD quenching are insignificant as indicated by the difference between the assays and the sonicated controls (representing maximal NBD-rhodamine mixing and so maximal NBD quenching). The same was true for the assays containing zinc (Figure 4). Therefore, this assay design has not shown PqiABC phospholipid transport between proteoliposomes.

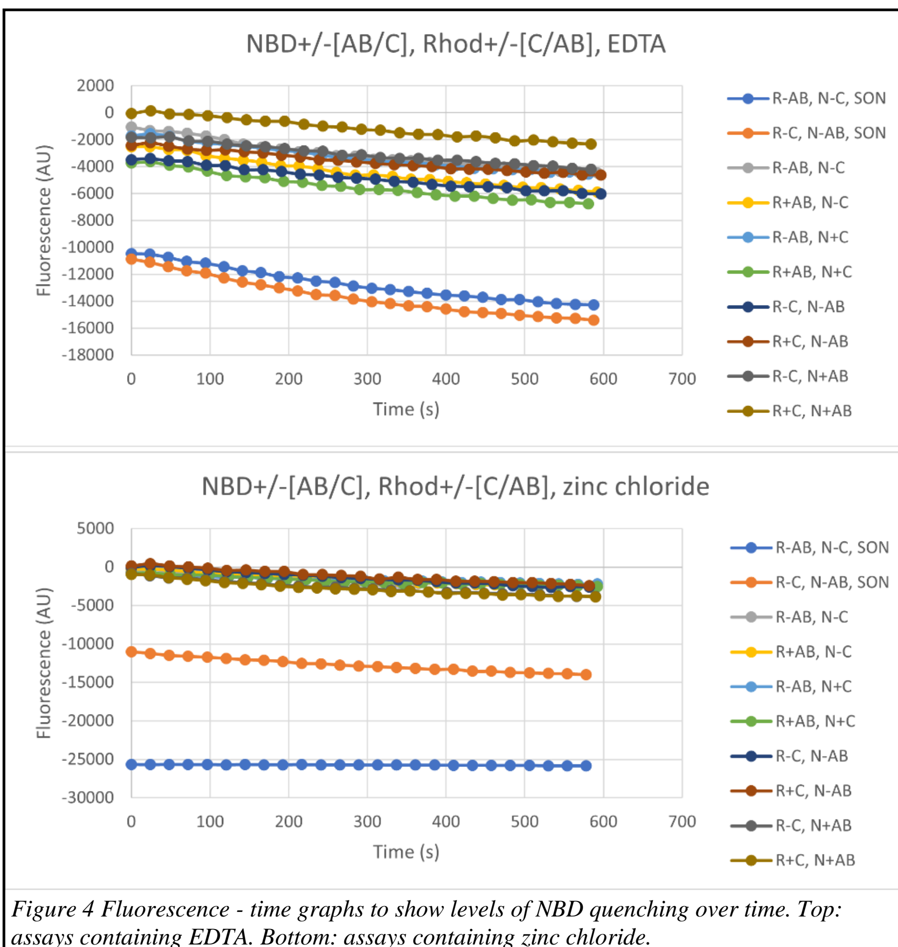

Figure 4 Fluorescence - time graphs to show levels of NBD quenching over time. Top: assays containing EDTA. Bottom: assays containing zinc chloride.

Future directions

There are many reasons why this assay may not have worked. Most fundamentally, PqiABC might not be a phospholipid transporter. Alternatively, the fluorescent tags could be sterically interfering with transport through the protein. There might even be an energetic requirement not factored into this design by proton motive force (PMF). The ClsA-based proteoliposome assay could help clarify these possibilities by removing the requirement for fluorescent tags. However, a method to ensure that the proteoliposomes do not burst and mix due to increasing pH while testing PMF will need to be considered carefully. It is uncertain, though, whether the Knowles lab will return to the FRET-based assay due to these complexities. Therefore, the group will continue developing a ClsA purification protocol and perhaps find success monitoring transport this way.

Value of studentship

To the student

As I move into the lab-based fourth year of an MSci course, these six weeks have been an invaluable introduction to extended research projects. From participating in lab meetings to contributing to high level conversations about the design of the assays, there have been many transferrable skills developed in a new context such as project management and problem solving. Additionally, I can now convincingly evidence my enjoyment for scientific research, strengthening my desire to pursue a PhD in structural biology.

To the research group

"Henry's research has been hugely beneficial to the research within the Knowles lab. His excellent approach to experimental design, identifying the correct controls to use and careful consideration of normalising the data, has irrefutably confirmed that our approach to study the PqiABC complex, using a FRET based assay, was not the right direction to take. Furthermore, through discussions with him and his valuable input we have devised an alternative approach utilising a ClsA based assay, which we are currently investigating. Overall, his research has led to a far deeper understanding of working with PqiABC, that has fundamentally changed the direction of research within the Knowles group." – *Dr Knowles.*

Acknowledgements

All PqiC used in this project was previously prepared by Knowles lab PhD student Hannah Johnston, who also supervised me daily.

References

[1] WHO, "WHO publishes list of bacteria for which new antibiotics are urgently needed," *World Health Organisation*, Feb. 27, 2017. [2] J. C. Malinverni and T. J. Silhavy, "An ABC transport system that maintains lipid asymmetry in the Gram-negative outer membrane," *PNAS*, vol. 106, no. 19, pp. 8009–8014, May 2009, [Online]. Available: www.pnas.org/cgi/content/full/ [3] G. W. Hughes *et al.*, "Evidence for phospholipid export from the bacterial inner membrane by the Mla ABC transport system," *Nat Microbiol*, vol. 4, no. 10, pp. 1692–1705, Oct. 2019, doi: 10.1038/s41564-019-0481-y. [4] M. Varadi *et al.*, "AlphaFold Protein Structure Database: Massively expanding the structural coverage of protein-sequence space with high-accuracy models," *Nucleic Acids Res*, vol. 50, no. D1, pp. D439–D444, Jan. 2022, doi: 10.1093/nar/gkab1061. [5] J. Jumper *et al.*, "Highly accurate protein structure prediction with AlphaFold," *Nature*, vol. 596, no. 7873, pp. 583–589, Aug. 2021, doi: 10.1038/s41586-021-03819-2. [6] S. Hiraoka, K. Nukui, N. Uetake, A. Ohta, and I. Shibuya, "Amplification and Substantial Purification of Cardiolipin Synthase of Escherichia coli," 1991. [Online]. Available: https://academic.oup.com/jb/article/110/3/443/819328