Daphne Jackson Trust Fellowship application, Oct 2018

**Applicant:** Dr. Elizabeth Hughes

**Supervisor:** Dr. Edward Wallace

**Host Organization:** Institute for Cell Biology, School of Biological Sciences, Edinburgh University

**Project Title:**  Dynamic mRNA processing in response to environmental stimuli in the fungal pathogen *Cryptococcus neoformans*.

During my undergraduate I was drawn to molecular biology and how it relates to the pathogenesis of disease. My PhD looked at the infecting HIV viral population and its relationship to disease progression, primarily the evolutionary analysis of isolates infecting lymphoid and non-lymphoid tissues (E S Hughes, Bell, & Simmonds, 1997; Elizabeth S. Hughes, Bell, & Simmonds, 1997). This led to the discovery of a previously unknown dormant HIV population in the brain and a first author paper with over 100 citation (E S Hughes et al., 1997).

Subsequently, I examined the replicative processes of HCV by determining whether the NS5B protein (predicted to possess an RdRp activity) was capable of directing HCV replication. I optimized bacterial expression systems and purified the NS5B fusion protein.

Following this I investigated the structure and function of the major outer membrane proteins (MOMP’s) of Chlamydia where I cloned and expressed wild type and VS4 domain mutated proteins and functionally reconstituted them at the single-channel level. Reconstitution in planar lipid bilayers showed that the VS4 domain was not required for pore formation but may help to form the channel vestibule where it may interact with other protein loops (E. S. Hughes, Shaw, & Ashley, 2001).

I then joined a leading bio safety testing facility as the research and development scientist and developed a wide range of molecular based assays for clients, and provided technical training and support for colleagues.

I chose to take a career break to raise my family. My husband works in the marine industry as a consultant in risk assessment/safety management and this job takes him away from home on a regular basis. Taking this into account and the prohibitive costs of childcare we decided it would be best if I put my career on hold to raise our family. During this time I have taken over the administrative side of our business.

Both of my children now attend high school and I am confident that they are mature and resilient enough for me to return to my chosen career. I am excited to re-engage with the scientific community. I find the challenges associated with research both rewarding and enlightening and I look forward to returning to the lab environment.

The Daphne Jackson Fellowship with MRS presents an ideal platform for me to resume my biomedical research career. The provision of mentoring, support and retraining are invaluable. This fellowship will give me the opportunity to update my existing skills while learning new invaluable ones to help me develop as a person and move my career forward.

Word count 398

**Research Summary: Dynamic mRNA processing in response to environmental stimuli in the fungal pathogen *Crytococcus neoformans*.**

1. To date most *in vitro* studies have been carried out in rich media which does not accurately reflect the conditions *C. neoformans* will encounter upon infection of the lung. I would like to design an *in vitro* system which more closely mimics *in vivo* conditions of the lung/alveoli. To do this I will test different cell culture conditions and establish a reproducible minimum growth medium and conditions for yeast cells and spores. Having established a reproducible *in vitro* system I will compare yeast cells and spores under different environmental conditions and examine the pattern of gene expression to generate a comprehensive picture of what genes are active in each species. This will help me to gain a deeper understanding of what transcriptional activity may contribute to infection.
2. Interaction with other organisms have been shown to effect infectivity and virulence. For example, bacteria can stimulate spore germination and, previously, bacterial cell wall components have been shown to modify the morphology of *C. neoforman*s yeast cells from normal cells to a titan cell. I would like to examine the changes in gene expression during this event to gain a deeper understanding of what transcriptional activity contributes to this morphological change.

Modular cloning for sysnthetic biology

CRISPR transformation

High throughput sequencing library preparation

Lab automation

Scientific abstract.

Section 4: Host Organisation

Dr. Edward Wallace.

Sir Henry Dale Fellow (Wellcome Trust/Royal Society Early Career Research Fellow).

Institute for Cell Biology, School of Biological Sciences, Edinburgh University.

Project funding remit: The lab is funded by Wellcome Trust/opal Society. Innovative research on fungal pathogens falls under the MRC’s health strategic aim.

Dr. Edward Wallace, of the institute for Cell Biology in Edinburgh University, has agreed to be my supervisor for the duration of the fellowship. Dr. Wallace in a renowned RNA scientist and working with him will allow me to build on my existing skills in the field of molecular biology but also learn new techniques including modular cloning for synthetic biology, CRISPR transformation, high throughput sequencing and library preparation, lab automation and computational data analysis of large data sets. This will provide me with crucial work experience in one of Scotland’s world-class universities and equip me with specific skills in high demand in biomedical research and biotech industries.

Relevant collaborations?

How well equipped is the organisation to support the proposed research plan?

Names of people involved in retraining.

Section 5: Retraining program

Describe retraining program. Include planned research methods/techniques and personal development.

Distinguish between new and refreshed skills and include as many specific details as possible.

**Section 6: Proposed Research plan.**

**Background:**

*C. neoformans* is an opportunistic and facultative pathogen that is the causative agent of cryptococcosis, which has grown in prevalence in tandem with the HIV/AIDS epidemic (Harrison, 2009; Park et al., 2009). *C. neoformans* primarily infects immunocompromised individuals and is one of only a few fungal species that have been shown to cross the blood-brain barrier leading to cryptococcal meningitis that is fatal if left untreated (Harrison, 2009; Park et al., 2009). A global number of 223 000 cases of cryptococcal meningitis are estimated to occur annually, with over 70% of cases in sub-Saharan Africa, and an estimated 181 100 resulting deaths (Rajasingham et al., 2017). Park et al., 2009; estimated the global burdens much higher at almost 1 million global cases and 600 000 deaths.

*C. neoformans* is found in the environment typically associated with pigeon guano, soil and decaying wood. It is a free living fungus with no requirement for mammalian virulence, however, it is able to adapt, survive and proliferate within a mammalian host to cause disease. Both the basidiospore and desiccated encapsulated yeast cells are postulated to act as infectious propagules, and inhalation of these is the primary route of infection.

The natural ecology and disease progression of *C. neoformans* is well characterized, however, few studies have examined in detail the differential gene expression and regulation of *C. neoformans* during early stages of infection. *C. neoformans* must undergo rapid changes in gene expression upon presentation within the alien environment of the lung. Indeed not many fungal pathogens can grow at 37 ⁰C, a characteristic virulence factor of *C. neoformans* and consistent with its role as a human pathogen(Perfect, 2006). The ability to investigate the genetic response to environmental stimuli is a powerful tool to elucidate the adaptive response/responses required for this accidental pathogen to survive in a hostile environment. I would like to examine in detail what happens when this organism reactivates within this alien environment.

**Aim 1: Determine environmental influences on gene expression in *C. Neoformans* in an *in vivo-like* system.**

Current knowledge of the early events in *C. neoformans* infection are based on research using animal models or *in vitro* culture methods primarily in rich media such as YPD or YPDA. While these culture methods have produced a lot of useful data, and much has been extrapolated about the infectivity and virulence of *C. neoformans,* they do not accurately reflect the lung environment where nutrients are likely to be scarce. I would like to design an *in vitro* system which more closely mimics *in vivo* conditions of the lung. I will use minimal media and re-seed into a controlled environment to assess the response of *C. neoformans* to different environmental stimuli. Analysis of differential gene expression over a time-course will produce a snapshot of actively expressed genes and may elucidate the important steps for infection at early time-points. For example, modification of cell wall composition and structure will likely play a crucial step in the initial infection and establishment of *C. neoformans* in a mammalian host.

This work will carry on from investigations previously carried out in Dr E Wallace’s lab in collaboration with ……looking at the effect of temperature and nutrient availability. The difference in my studies will be utilizing a culture medium more consistent with the host lung for *C. neoformans* growth prior to re-seeding and examining further environmental stimuli, such as pH, ionic strength, divalent cations and mucus, which constitute the natural habitat of the lung and can have synergistic or antagonistic effects on anti-fungal activity.

Or temperature, nutrient limitation or influx, metals, pH and carbon dioxide concentration.

**Aim 2**: **Investigate how bacteria, that naturally colonize the mammalian lung, influence gene expression in *C. neoformans*.**

In many environments bacteria and fungi coexist and interact in nature competing for space and nutrients. Similar cross-species interactions are expected to occur between the wide ranges of micro-organisms that constitute the human microbiome. Many combinations of micro-organisms have been isolated from sputum specimens from patients with cystic fibrosis including bacteria and fungi (Bauernfeind et al., 1987). Direct contact between fungi and bacteria can result in important changes to their physiology and future interactions. These interactions can also be modified by the environment. For example *Candida albicans* has been shown to co-adhere with oral *Streptococcus* species under varying nutritional conditions (Nikawa et al., 2001). It has been previously shown that *Bacillus safensis* can interact with two human fungal pathogens found in the environment, *C. neoformans* and *Candida albicans*, blocking virulence factor production without affecting overall fungal growth (Mayer & Kronstad, 2017). Bacteria can stimulate spore germination in several fungi (Chandelier, Abras, Laurent, Debruxelles, & Cavelier, 2006; Hansen & Jakobsen, 1997; Hildebrandt, Ouziad, Marner, & Bothe, 2006) and it has been shown that germination of *C. neoformans* is essential for survival in phagolysosomes (Botts & Hull, 2010). Bacterial small molecules such as metabolites and extracellular molecules have been shown to affect the morphology and pathogenicity of fungi (Joyner et al., 2010; Mowat et al., 2010; Noverr & Huffnagle, 2004).

I hypothesize that bacteria, or perhaps bacteria bits, may influence the infectivity and virulence of *C. neformans* during the early stages of infection. I would like to examine the effect bacteria, residing in the lung, have on infecting yeast or spores by incubating with sera containing bacterial components (human, murine, FBS). Previously bacterial cell wall components have been shown to modify the morphology of *C. neformans* from a normal yeast cell to a titan cell (Dambuza et al., 2018). I would like to examine the gene expression in *C. neformans* during interaction with bacterial proteins to decipher possible cell surface modifications that may enhance infection of this pathogenic fungi.

Mention previous work on titan cells here?

Following these pilot studies I would like to further characterize potential components that modulate gene expression in *C. neoformans* and the changes in phenotype/morphology observed. Understanding how bacteria and fungi interact within specific niches in the body and how these interactions affect pathogenesis is critical for identifying potential novel targets for drug development.

Lungs of immunocompromised individuals are frequently colonized by bacteria, especially *Pseudomonas aeruginosa*, and fungi such as *Candida albicans* and *Aspergillus fumigatus* (Müller & Seidler, 2010; Peleg, Hogan, & Mylonakis, 2010).

**Aim 3**: **Investigate how component of the innate immune system influence gene expression in *C. neoformans*.**

Following inhalation fungi will first encounter the mucosal surface of the lung which is covered in a film of surfactant that maintains surface tension during respiration and contains soluble effector molecules with antimicrobial properties such as the collectins and host-defense peptides.

Collectins (Surfactant Protein-A (SP-A), Surfactant Protein-B (SP-B) and Mannose Binding Lectins (MBL)) have all previously been shown to bind to *C. neoformans* (Chaka et al., 1997; Schelenz, Malhotra, Sim, Holmskov, & Bancroft, 1995; van Asbeck, Hoepelman, Scharringa, Herpers, & Verhoef, 2008; van de Wetering, Coenjaerts, Vaandrager, van Golde, & Batenburg, 2004; Walenkamp, Verheul, Scharringa, & Hoepelman, 1999). *In vitro* studies suggest SP-A can bind acapsular and capsular yeast cells but with a reduced affinity compared to SP-D and *C. neoformans* was shown to be resistant to SP-A binding once capsule synthesis had been induced (Giles, Zaas, Reidy, Perfect, & Wright, 2007). In contrast SP-D binds acapsular yeast with a high affinity (and capsular yeast with a lower affinity) causing profound aggregation, increased phagocytosis and enhanced fungal survival (Geunes-Boyer, Beers, Perfect, Heitman, & Wright, 2012b; Geunes-Boyer et al., 2009; Schelenz et al., 1995; van de Wetering et al., 2004).

Previous studies, using animal models, have suggested a protective role for SP-D in *C. neoformans* infection (Geunes-Boyer, Beers, Perfect, Heitman, & Wright, 2012a). In contrast, other studies suggest that SP-D mediates host protection by opsonizing fungi prior to presentation to phagocytes (ref). I would like to investigate whether SP-D binding affects *C. neoformans* directly by analyzing fungal gene expression before and after binding of purified recombinant SP-D. Direct binding may alter gene expression and enhance virulence factors, such as capsule production/cell wall rearrangements, thereby enhancing fungal survival.

**Methods:**

I will use a variety of high throughput methods and functional profiling to identify enriched pathways during the early phase of *C. neoformans* adaptation to the host lung environment.

**Preparation of cDNA libraries:**

I will prepare cDNA libraries from mRNA extracted from yeast cells prepared under the variable conditions described above. I will use a recently described multi-plex method to prepare cDNA libraries (Shishkin et al., 2015) for RNA-seq analysis.

Workflow:

* Experimental set up
* Extraction of total RNA from sample using the QIAgen plant and fungal RNA extraction kit and QIAgen RNA mini plant kit according to the manufacturer’s instructions..
* Assessment of RNA concentration and quality.
* cDNA Synthesis through reverse transcription using Superscript VI for qPCR analysis.

**Quantitative real-time RT-PCR**: Workflow of gene expression analysis using RT-qPCR.

I will use SYBR-green fluorescence based RT-qPCR to analyze gene expression in response to environmental stimuli.

* Experimental design.
* cDNA template preparation for qPCR.
* qPCR to measure expression levels of target genes.
* Data analysis using appropriate normalization methods.

**RNA sequencing and quantitative analysis:**

Workflow of gene expression using RNA-Seq.

* Experimental design.
* cDNA template preparation for sequencing from cDNA libraries prepared.
* Sequencing of cDNA library on the XXX instrument.
* Data analysis of resulting short read sequences.

**Challenges:**

Total RNA of each sample was treated with DNase (Turbo DNA-free kit; Ambion) to avoid genomic DNA contamination. Reverse transcription was performed using the XXX. XXX genes were selected for validation of RNA-seq results using RT-PCR. Primer pairs were designed to span exon-exon junctions using Primer3, and ACT1 was used as an internal control. All the primer sequences are as follows (5′ to 3′): ACT1, CCACACTGTCCCCATTTACGA (forward) and CAGCAAGATCGATACGGAGGAT (reverse); CNAG\_05431, AAGCCCCCTGAGAGACCTAG (forward) and GGAGCTCCAACAGACTCGA (reverse); CNAG\_06493, AACAGACGGCATCGAAGGTT (forward) and GTTACAGAATGCTGCGCTCG (reverse); CNAG\_00654, TGTCAGAACCAGCATGAGGC (forward) and CCGCCCTACCTTCCGTAATC (reverse); CNAG\_00815, ACCTGGCATCGATGGGTTTT (forward) and TGGAGGTTGCGGTCACAATA (reverse).

The amplifications were conducted in a total volume of 20 µl, containing 1× SYBR green (iTaq universal SYBR green supermix; Bio-Rad), 300 nmol/liter of both primers, and 1 µl of diluted cDNA. The amplification was conducted as follows: 5 min at 95°C, followed by 35 cycles consisting of 30 s at 95°C, 30 s at 54°C, and 30 s at 72°C. Finally, melting curve analysis was performed from 60°C to 95°C, with increments of 0.5°C per 10 s. Amplification, melting curve analysis, and detection were conducted with the MyiQ single-color, real-time PCR detection system (Bio-Rad).

**Microscopy**:

**Bioinformatics and Data Analysis**:

**Lab Automation**:

Modular cloning and CRISPR

I would like to examine the host-pathogen interaction comparing yeast and spores under previously optimized conditions and look at the cell surface modifications that occur. I would like to compare host epithelial and alveoli macrophages during the infection process and compare the resulting gene expression profiles to delineate what is up or down regulated and from this to infer what may be important at the point of infection. Further understanding of the interaction of opportunistic or primary fungal pathogens will be important in understanding fungal pathogenesis.

I will construct cDNA libraries to give a snapshot of actively expressed genes under different environmental stimuli and use RT-qPCR and RNA-seq for quantitative gene expression analysis. Gene expression profiling enables you to investigate the effects of different conditions on gene expression by altering the environment to which the organism is exposed and determining which genes are expressed.

RT-qPCR is one of the most powerful and sensitive gene analysis techniques available. The sheer volume of expression data produced will necessitate sophisticated computational methods for analysis.

RT-qPCR is a very sensitive method for measuring gene expression. qPCR is one of the most powerful and sensitive gene analysis techniques available and can be used for quantitative gene expression analysis.

Knowing an organism is expressing certain genes provides a lot of information about how an organism is functioning and potentially new insights into which genes (and therefore proteins) are involved in certain behaviors

However, more information is needed than just the mRNA profile to establish function. It may be helpful to work out which proteins are synthesized through proteomics experiments

Technical considerations when using qPCR> data analysis.

Asumption: gene expression levels correspond to protein levels.

Few antifungal drugs are available for treatment. And devlopement of new therapies in complicated by the need for pathogen-specific targets. Euk targets not work in euks. Due to their eukaryotic nature and relatively close physiological similarity to human cells pathogenic fungi are notoriously difficult to target for clinical therapy.

Describe how your retraining will be useful for the project.

Describe the risks involved and how you will overcome them.

Section 7: Ethical approval and licences.

Not Applicable.

Section 8: Timetable

Simple diagrammatic workplan in a Gants chart or table illustrating research and retraining elements. Columns as months (1-36) rows as tasks/actions.

Indicate major outputs and landmarks.

Section 9: References

Use smaller font to keep to one page

Section 10: Future planning