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

**Background:**

*C. neoformans* is an opportunistic and facultative pathogen and the causative agent of cryptococcosis. 1,2*C. neoformans* is 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.

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. 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 the lung.

**Aim 1: What environmental stimuli in the host trigger an acute stress response in *C. neoformans*?**

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 fungal support media such as YPD or YPDA. While these culture methods have produced useful data about the infectivity and virulence of *C. neoformans,* they do not accurately reflect the lung environment, where nutrients are likely to be scarce.

This work will carry on from investigations previously carried out by Dr. E Wallace, in collaboration with Dr. Elizabeth Ballou, designed to dissect the contributions of host factors and temperature in shaping initial growth. In this pilot study a distinct physiological response (capsule induction) and differential RNA abundance was documented between the different conditions (See Appendix 1).

**Objective:**

I would like to identify what causes this phenotypic shift. I will analyze differential gene expression using qPCR over a time-course to produce a snapshot of actively expressed genes under different environmental stimuli. This may shed light on the important steps for infection at early time-points.

**Method:**

Initially I will inoculate growth-arrested *C. neoformans* yeast cells grown in YPD (GA-Cn-YPD) into RPMI-1640 media and YPD + serum and incubate at 25⁰C and 37⁰C. I will examine the cells to determine any phenotypic changes. I will extract RNA for analysis using qPCR (primers against differentially expressed genes previously detected by RNA-seq in the Wallace labs). I will compare results from these and the previous experiments to determine how the gene profile alters in relation to phenotype.

Analysis of this data will determine future studies. If the addition of serum to media is sufficient for capsule induction I will incubate GA-Cn-YPD in RPMI-1640 + purified albumin and RPMI-1640 + charcoal stripped FBS (CS-FBS). This will allow me to compare the effect of albumin and reduced levels of endotoxins and hormones (these are greatly reduced in CS-FBS) on capsule induction. (See Appendix 2 for experimental plan).

If serum is not sufficient I will dissect RPMI-1640 and look at the impact removing each component individually has on capsule induction. (See Appendix 3).

**Aim 2**: **Investigate how bacterial cell wall components influence gene expression in *C. neoformans*.**

Peptidoglycan and cell wall fragments are increasingly being recognized as important signaling molecules that can inhibit growth and virulence factors and influence morphology of other microbes. The addition of fetal calf serum (FCS) to media induces capsule formation in *C. neoformans*3. Bacterial cell wall components (in serum) have been shown to modify the morphology of *C. neoformans* from a normal yeast cell to a large, highly ploidy titan cell 4. The peptidoglycan subunit muramyl dipeptide was identified as a component of serum associated with titan cell induction.

**Objective:**

I would like to dissect the role bacterial cell wall components play in the pathogenesis of *C. neoformans* in the lung. I will identify which components influence gene expression and examine changes in phenotype to determine the molecular mechanisms underlying these changes.

**Method:**

I will incubate growth arrested GA-Cn-YPD and GA-Cn-YNB in serum-free media with purified components of bacterial cell walls. Namely, peptidoglycan subunits N-acetyl glucosamine (NAG), N-acetyl muramic acid (NAM) and muramyl dipeptide found in gram positive and negative bacteria; lipopolysaccharide found in gram negative bacteria and teichoic acid (a major surface antigen) found in gram positive bacteria. These are all commercially available. I will incubate cells at 25⁰C and 37⁰C if capsule was induced at both temperatures in aim 1. Otherwise, I will only carry out these experiments at 37⁰C. Depending on results from pilot studies I will further characterize the effect of lipopolysaccharide by incubating yeast cells with the subunits lipid A (antigenic), core polysaccharide and/or o-polysaccharide.

I will examine the cells to determine any phenotypic changes under each condition. I will extract RNA for further qualitative and quantitative analysis using qPCR and RNA-seq.

**Aim 3**: **Investigate how SP-D influences 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 proteins A and D and mannose binding lectins (SP-A, SP-D and MBL’s), have previously been shown to bind to *C. neoformans* 5–9. *In vitro* studies suggest SP-A has little effect on *C. neoformans* 10. 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 6,7,11,12.Previous studies, using animal models, have suggested a protective role for SP-D in infection 13.

**Objective:**

I would like to investigate if binding of SP-D affects *C. neoformans* directly by analyzing fungal gene expression before and after binding of purified recombinant human SP-D (rh-SP-D). Direct binding of rh-SP-D may modulate gene expression of surface receptors in a synergistic or antagonistic way and could therefore reciprocally modulate virulence factors, such as capsule production/cell wall rearrangements, thereby enhancing fungal survival.

**Method:**

I will incubate rh-SP-D (commercially available) with yeast cells in a microtiter plate at various concentrations. I will analyze the plates for agglutination of *Cryptococci* and extract RNA for further qualitative and quantitative analysis using qPCR and RNA-seq.

Functional profiling under differing stimuli may identify enriched pathways during the early phase of *C. neoformans* adaptation to the host lung. Analyzing the transcriptome of *C. neoformans* over a time-course under these selective environmental pressures may lead to micro-evolutionary adaptation of *C. neoformans* in the host lung and pave the way for possible new drug targets aimed at de-regulating virulence instead of directly killing the fungus.

**Challenges:**

Going from yeast cultures to identification of gene expression involves a multi-step approach including harvesting cells, RNA isolation, removal of genomic DNA, cDNA synthesis and qPCR/RNA-seq. This multi-step approach can introduce inter- and intra-sample variation which must be normalized in order to make sense of the data produced.

Normalization is very important when comparing data from different experimental conditions in a quantitative and qualitative manner. The amount of mRNA produced may vary considerably. I will normalize RNA levels by spiking in a 1:100 ratio of methanol-fixed *Schizosaccaromyces pombe*. I will normalize using three reference *S.pombe* genes. The reverse transcription step of cDNA synthesis has been proposed as the source of the most variability in qPCR experiments. I will use an external RNA control to normalize for external global mRNA changes by spiking extracted RNA samples with equal amounts of control RNA prior to cDNA synthesis. This will allow me to control for the presence of inhibitors of RT and DNA polymerase. Analysis of qPCR data can result in misrepresentation of the expression profiles obtained 14 I will overcome this by selecting 3-4 stable expressed reference controls and the geometric mean of these will be taken. These will allow me to normalize differences in the amount and quality of starting material as well as in the efficiency of the reaction. It is common to use ubiquitously expressed genes. qPCR will be carried out under MIQE guidelines15.

Manipulation during the construction of the cDNA libraries can complicate the analysis of RNA-seq reads where PCR artefacts of short identical reads can be confused for genuine reflection of the RNA present. In order to overcome this limitation I will run 2 or 3 biological replicates and determine whether the same sequences are observed in each sample.

Normalization is an important prerequisite for any quantitative data analysis of gene expression. Different normalization approaches can have significant effects on the distribution and calculation of significant values (*P*-values)16,17. I will analyze RNA-seq data with DESeq2 in R. The package DESeq2 provides methods to test for differential expression by use of negative binomial generalized linear models.

During this project I will be dealing with many samples and carrying out qPCR on many genes. In order to make this practical I will use the sate-of-the-art lab automation facilities at the Edinburgh Genome Foundry to automate qPCR plate loading thereby reducing human error through repetition.

Word count: 1473 (1500)

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Appendix 1:

YPD medium-no capsule induction at 25⁰C or 37⁰C and RPMI-1640 + serum-capsule induction at 37⁰C but not 25⁰C).

Appendix 3: Composition of RPMI-1640

One liter of RPMI 1640 contains (Moore G):

Glucose (2 g)

pH indicator (phenol red, 5 mg)

Salts (6 g sodium chloride, 2 g sodium bicarbonate, 1.512 g disodium phosphate, 400 mg potassium chloride, 100 mg magnesium sulfate, and 100 mg calcium nitrate)

Amino acids (300 mg glutamine; 200 mg arginine; 50 mg each asparagine, cystine, leucine, and isoleucine; 40 mg lysine hydrochloride; 30 mg serine; 20 mg each aspartic acid, glutamic acid, hydroxyproline, proline, threonine, tyrosine, and valine; 15 mg each histidine, methionine, and phenylalanine; 10 mg glycine; 5 mg tryptophan; and 1 mg reduced glutathione)

Vitamins (35 mg i-inositol; 3 mg choline chloride; 1 mg each para-aminobenzoic acid, folic acid, nicotinamide, pyridoxine hydrochloride, and thiamine hydrochloride; 0.25 mg calcium pantothenate; 0.2 mg each biotin and riboflavin; and 0.005 mg cyanocobalamin)

Appendix 4: Commercially available bacterial cell wall components.

N-acetyl glucosamine: uk.vwr.com and www.sigmaaldrich.com

N-acetyl muramic acid: [www.sigmaaldrich.com](http://www.sigmaaldrich.com)

Lipopolysaccharide: www.sigmaaldrich.com

Lipoteichoic acid: www.sigmaaldrich.com