2013 CFID Undergraduate Summer Research Award Summary Report

Exploring the overexpression of a central regulator of *Aspergillus fumigatus* asexual sporulation, *brlA*, as a way to reduce virulence through reduced vegetative growth

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Background

Aspergillus fumigatus is a ubiquitous environmental mold that causes a frequently fatal invasive pneumonia in immunocompromised patients. The success of the fungus is linked to its ability to produce and efficiently disperse high numbers of airborne asexual spores (conidia). Humans inhale several hundred spores each day, but these are efficiently eliminated by innate immune responses. However, in immunocompromised patients, conidia escape immune defenses and germinate into filamentous hyphae which invade host cells and penetrate blood vessels reaching other organs, resulting in a form of invasive pneumonia that is often fatal to patients (Yu et al., 2006). In 1992, IA was responsible for approximately 30% of fungal infections in patients dying of cancer, and it is estimated that IA occurs in 10 to 25% of all leukemia patients, in whom the mortality rate is 80 to 90%, even when treated. Interestingly, although *A. fumigatus* is only one of the *Aspergillus* species, it causes 90% of Invasive Aspergillosis (Latge, 1999). Evidence suggests that the virulence of *A. fumigatus* is encoded in a collection of genes all of which contribute to its resistance against pressures that act on suppressing its vegetation (Askew, 2008). As a result, the overall fitness of the fungus is improved and its ability to grow and invade host cells is promoted. The growth rate of this organism plays a key role in its ability to cause disease, and strains with reduced hyphal growth show attenuated or no virulence.

Sporulation in *A. fumigatus* relies on a genetically programmed sequence of events and is associated with a cessation of vegetative growth, usually in response to nutrient limitation (Mah and Yu, 2006). Interestingly, in the human host, the fungus does not sporulate. This absence of sporolation allows the fungus to concentrate its energy towards vegetative growth. **We therefore hypothesize that forcing the induction of conidiation of *A. fumigatus* during IA will**

result in impairment of vegetative growth, leading to a reduction of the fitness of the fungus and an impaired virulence. If this hypothesis is confirmed, developing molecules that activate sporolation will be explored as a new therapeutic tool.

The balance that exists in *A. fumigatus* between asexual sporulation and vegetative growth has been described in the non-pathogenic model *A. nidulans*. In *A. nidulans*, the asexual development pathway relies on the activation of a key downstream master switch required for controlling sporulation, *brlA* (Aguirre, 1993; Seo et al., 2006). In our lab, we have previously identified the *brlA* orthologue in *A. fumigatus*. Through gene disruption studies and whole organism genome profiling, *brlA* was confirmed to control both sporulation and some metabolic pathways in *A. fumigatus* (Twumasi-Boateng et al., 2009). In this report, we present the work that we have initiated to prove that suppression of sporulation is required for virulence in IA, and inversely that undue activation of sporulation impairs the virulence ability of the fungus.

Experimental Approach – Principle.

To test our hypothesis, we examined the effect of forced expression of blrA in *A. fumigatus* as a mechanism to inhibit vegetative growth. This approach uses a regulatable promoter system based upon the prokaryotic regulatory element in the *E. coli* tetracycline-resistance operon to control *brlA* expression. This system also requires that the expression of the gene of interest be controlled by a trans-activator protein, rtTA2S-M2, that binds to the operon sequence (*tetO*) in the promoter/enhancer region of the gene of interest only in the presence of tetracycline. In this system, doxycycline, a tetracycline paralog, can be substituted for tetracycline. Moreover, Vogt *et al.* has demonstrated that doxycycline has no effect on *A. fumigatus* growth (Vogt et al., 2005).

Results

1. Modification of p500 (vector construction)

To place *brlA* under control of the Tet-Operator, the *brlA* ORF was cloned downstream the operator $TetO_7$, acting as the transcriptional controller, into p500, a plasmid constructed by another group to regulate gene expression in eukaryotes (Vogt et al., 2005). Two restriction sites were introduced into the plasmid. First, the *SbfI* restriction site was introduced downstream the mini-*gpdA* promoter, Pmin, by PCR amplifying the vector with primers that were flanked with *SbfI* restriction sites. The PCR product was then digested with *SbfI* and self-ligated. The resulting ligation products were transformed into mach1® *E. coli,* and bacterial

clones were selected on Ampicillin enriched growth medium. Transformants were selected, plasmids were extracted and analyzed by enzymic digestion to test for *SbfI* site insertion. Plasmid with the correct diagnostic fragment sizes was sent for sequencing and the *SbfI* site insertion was confirmed. Then, a *PacI* restriction site was introduced in the p500::*SbfI* plasmid, downstream the *hph* ORF, using the same method. The subsequent plasmid was also validated by restriction enzyme digestion and sequencing. The *PacI* was inserted for cloning of another development regulator, *gliP*, but was not used for *brlA* cloning. The new plasmid was named p500SP.

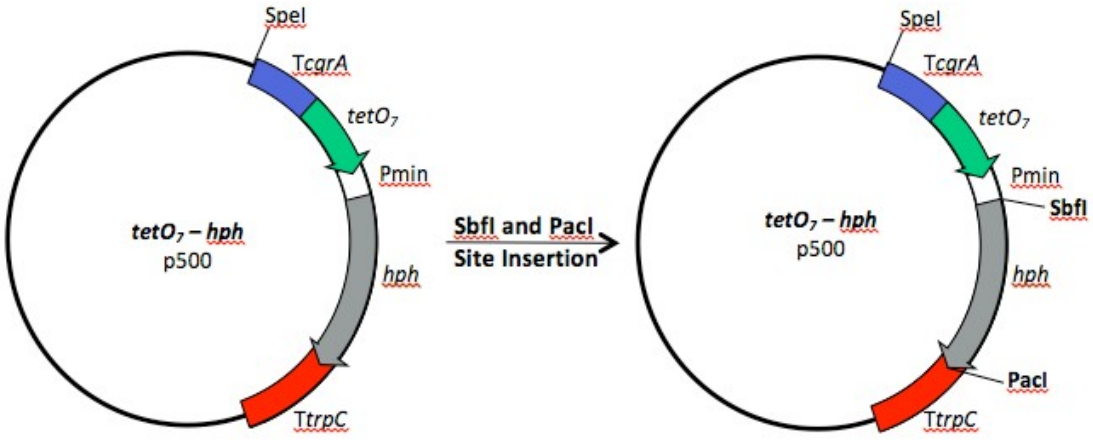

Figure 1. Schematic representation of the first modification of the Tet Operator bearing plasmid (Vogt et al., 2005).

2. Insertion of brla *in p500SP.*

The 1.7 kb *brlA* ORF was PCR amplified from wild type *A. fumigatus* DNA. During PCR, a *SbfI* site was added upstream, and a *SacII* site was added downstream. PCR product was digested with *SacII* and *SbfI*, as well as the p500SP plasmid. As a result, the *hph* ORF (encoding for hygromycin resistance) was excised. The 1.7 kb segment was ligated with the 5.0 kb backbone of the p500 plasmid following a vector ligation protocol and later transformed into mach1® *E. coli*. Plasmids were extracted from transformants obtained on Ampicillin rich medium, and diagnostic restriction digestions were performed to select for the correct transformation. The final product was sequenced to confirm correct construction.

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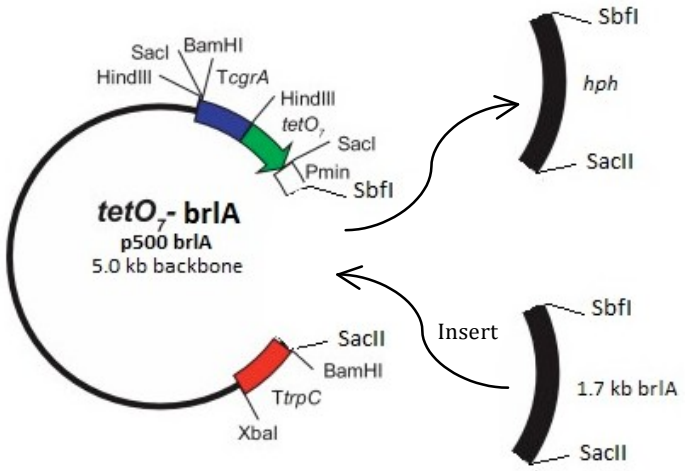

Figure 2. Schematic representation of the second modification of the Tet Operator bearing plasmid (Vogt et al., 2005)

3. Transformation into A. fumigatus mutant strain ΔbrlA:

A transformation protocol prepared by Sheppard lab was followed (Gravelat et al., 2012). The main steps are described below:

a. Protoplasting

A previous student in our lab created a *ΔbrlA* mutant strain that was used in this project for transformation (Twumasi-Boateng et al., 2009). *ΔbrlA* strain was allowed to grow into a fungal biomass. A cocktail of enzymes was utilized to digest the carbohydrate rich cell wall of *A. umigatus*, thereby releasing membrane bound protoplasts, some of which contain intact nuclei. The number of protoplasts was monitored at regular intervals, and when the quantity of protoplasts was sufficient, the protoplasts were isolated and resuspended in iso-osmotic buffer until transformation.

b. Protoplast Transformation

Protoplasts were co-transformed with p500::*brlA* and p480. This p480 plasmid is required since it bears a transactivator. In the presence of tetracycline, or any of its analogs, the direct interaction with rtTA2S-M2 and binding to the operator turns on expression. As a result, *A. fumigatus* protoplasts were co-transformed by heat-shock with TetO::*brlA* and the plasmid bearing the operator activator rtTA2S-M2 (p480). Protoplasts were then plated on iso-osmotic plates that were incubated at room temperature overnight without drug in order to allow the protoplasts to reconstitute their cell wall.

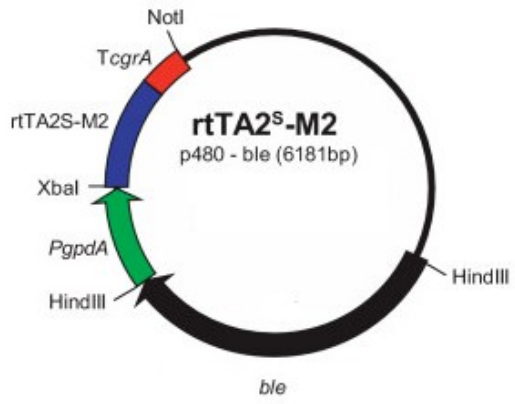

Figure 3. Schematic representation of the second modification of the Tet Operator bearing plasmid (Vogt et al., 2005)

c. Selection of Transformants

The p480 plasmid bears the *ble* cassette that encodes for phleomycin resistance. By overlaying all OSM plates with 10 ml of top agar containing phleomycin, we selected for transformants that incorporated the cassette and most probably the transactivator coding gene (rtTA2S-M2) along with it. Successful transformants penetrated through the overlay agar and were allowed to grow for 4 days after which another overlay of doxycycline enriched growth medium was placed on top. Transformants that successfully also incorporated TetO::*brlA* reinitiated sporolation: these clones were expected to appear as green mycelium colonies on a white lawn, made of non sporolating mycelium. At this point, we have not isolated a co-transformant, but I plan on continuing this work during the school year, using a modified protocol as described below. This continuation of the project will be part of an undergraduate research course at McGill University.

Significance and Future Directions

This study outlines a novel approach to manipulating the fungal life cycle in order to attenuate virulence. We anticipated that doxycycline induced expression of *brlA* will result in mistimed sporulation and reduced vegetative growth *in vitro*. Since growth rate plays a key role in virulence, reduction or cessation of growth should improve survival in animal models. If these experiments provide promising results, new therapeutic tools that induce sporulation may be useful for invasive aspergillosis prophylaxis. Since co-transformation showed a low rate of success in our hands, a "*ΔbrlA* + rtTA2S-M2" strain that expresses the transactivator following exposure of the fungal strain to tetracycline or doxycycline will be constructed. Once this

strain has been created and validated, it may be used for transformation with p500::*brlA*, as described in paragraph 3 of the "Results" section. Following expression of these constructs in *A. fumigatus* the *in vitro* mutant phenotype will be characterized, especially with respect to growth and morphological changes. Strains showing conidiation in response to doxycycline will be tested for virulence in worms and later in an inhalation mouse model of invasive aspergillosis as developed in the Sheppard lab (Sheppard et al., 2006).

Summary of Achievements:

During this summer period I successfully constructed the necessary plasmids and molecular tools to test the hypothesis that manipulation of sporulation can be a potential virulence strategy. In working as part of this research team, I have developed many skills invaluable to an undergraduate student: teamwork, problem solving, stamina, and dedication. I have become familiar with many basic techniques used in a molecular biology laboratory including but not limited to enriched growth media preparation, plasmid and RNA extraction, DNA transformation into *A. fumigatus,* running PCR, following protocols and troubleshooting for mistakes. Most of the molecular biology work I was involved in required knowledge about the DNA sequence. The online software "A Plasmid Editor" provided the tools I needed to carry on the plasmid construction and the enzymic tests performed. Through this project, I gained a greater understanding of the research field and what it is like to conduct basic science research in microbiology. Reflecting on my experience, I learned that laboratory work is demanding. It requires careful planning, acquiring new skills, attention to details, active documentation, and an ability to look at results from many prospectives to interpret them. But I also learned that I truly enjoy this demanding environment. While the repetition of experiments or the generation of negative results can be frustrating at times, I love the intellectual challenge and the thrill of taking part in a new discovery. I am also interested in becoming a physician and my interest in medicine is actively stimulated by shadowing doctors and volunteering in a medical setting. This enjoyment makes me confident that I want to peruse a scientist clinician career, and equally confident that I will succeed.

References

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