

STUDIES ON THE ANAEROBIC FUNGUS *PECORAMYCES RUMINANTIUM* SP.
C1A: SPORE COLLECTION AND GENETIC MANIPULATION APPROACHES

By

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Abstract: The overall aim of this dissertation is to develop means for spore collection and growth synchronization, long-term storage, and gene knockdown protocols in the anaerobic gut fungal isolate *Pecoramyces ruminantium* strain C1A (C1A). A novel technique for the growth of strain C1A on agar medium in serum bottles and subsequently flooding the observed aerial growth to promote spore release from sporangia into the flooding suspension was developed. This surface growth-aerial flooding approach was to achieve three different goals hitherto unfeasible in strain C1A liquid cultures. First, surface growth was shown to be an excellent cryopreservative- and freezing temperatures-free approach for AGF long-term storage, and the utility of the approach was verified in multiple strains in addition to strain C1A. Second, the developmentally synchronized C1A spores collected allowed for real time PCR (RT-PCR) transcriptional analysis of focal adhesion (FA) genes at various stages of development. This study showed for the first time that FA scaffolding proteins are indeed transcribed during growth in the absence of an extracellular matrix anchor, suggesting an alternative function for such proteins in the anaerobic gut fungi and hence highlighting the diverse functionalities of FA scaffolding proteins in basal fungi. Finally, the collected spores were shown to be naturally competent, and such ability was exploited to develop and optimize an RNA interference (RNAi)-based protocol for targeted gene silencing in C1A. Germinating C1A spores readily uptook chemically-synthesized small interfering RNA (siRNA) oligonucleotides coding for the D-lactate dehydrogenase (*ldhD*) gene resulting in marked target gene silencing; as evident by significantly lower *ldhD* transcriptional levels, a marked reduction in the D-LDH specific enzymatic activity in intracellular protein extracts, and a reduction in D-lactate levels accumulating in the culture supernatant. Collectively, results from these studies have opened the door not only for developmental biology studies, but also for targeted gene manipulations in this understudied fungal clade.

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Preface

The anaerobic gut fungi (AGF) belong to the phylum *Neocallimastigomycota*, which constitute an asexual basal fungal lineage that resides in the rumen and alimentary tract of herbivores. AGF have been of particular interest recently, as they could have a potential application in biofuel production due to their lignocellulolytic and fermentative capabilities.

Before I began my graduate studies, other laboratory members successfully isolated a pure culture of the AGF, *Pecoramyces ruminantium* strain C1A (C1A), sequenced its genome and transcriptome, and successfully identified and quantified the fermentation pathways and acid products of C1A when grown on various substrates. Oxygen tolerance studies were also conducted in order to assess the extent of the strict anaerobic nature of C1A.

The anaerobic nature of AGF isolates, the lack of reliable long-term storage procedures, and the tendency for senescence upon long-term continuous culturing make these organisms difficult to maintain in a laboratory setting. Consequently, research on AGF has lagged dramatically behind their aerobic fungal counterparts. There is currently no genetic system available for AGF. This dissertation focuses on (1) developing a means for spore collection and long term storage of AGF, and (2) utilization of collected spores for transcriptional studies and genetic manipulations of the anaerobic rumen fungus

Pecoramyces ruminantium strain C1A (C1A), with the ultimate goal of its potential use as a genetic system for AGF.

Chapter I serves as a general introduction for AGF, with a focus on what's currently known about C1A. I will discuss the reasons behind the challenging aspects of working with AGF in a laboratory setting that has resulted in the lag in AGF research. Chapter I will also introduce the importance of the following chapter projects in order to provide a means to remedy some of the challenging aspects that will be addressed.

Chapter II addresses a reliable long-term storage and zoospore collection method for AGF (similar to that of aerobic fungal counterparts) and how it was critical for developmental and molecular biological studies to move forward for AGF. Thus, Chapter II presents the novel anaerobic flooding technique that I developed and optimized, which functions not only as a means of long-term culture storage, but also allows for collection of viable, competent, and developmentally synchronized C1A spores. This work is published in the *Journal of Microbiological Methods*.

Chapter III reports on how the developed anaerobic flooding technique, described in Chapter II, was utilized to conduct a transcriptional study on focal adhesion (FA) genes from samples of C1A collected at various lifecycle stages when grown with or without a solid substrate as a carbon source. This in-depth developmentally timed transcriptional study was possible because of the newfound ability to collect developmentally synchronized C1A spores. For the first time, results from this study revealed that FA components are in fact transcribed during growth in the absence of an extracellular matrix anchor, and proposed alternative functions for FA scaffolding

proteins in AGF, highlighting their diverse functionalities in basal fungi. This work is published in *PLoS ONE*.

Chapter IV addresses the lack of an established genetic system in AGF, subsequently hindering in-depth functional gene investigations. Chapter IV reports the development of an RNAi-based protocol for targeted gene knockdown in the AGF isolate C1A. This study highlights even further the breadth and significance of the anaerobic flooding technique for spore collection, as the collected germinating spores were demonstrated to uptake chemically synthesized short double stranded siRNA, resulting in successful targeted gene silencing of the D-lactate dehydrogenase (*ldhD*) gene. This work has been submitted for review in *PeerJ*.

CHAPTER I

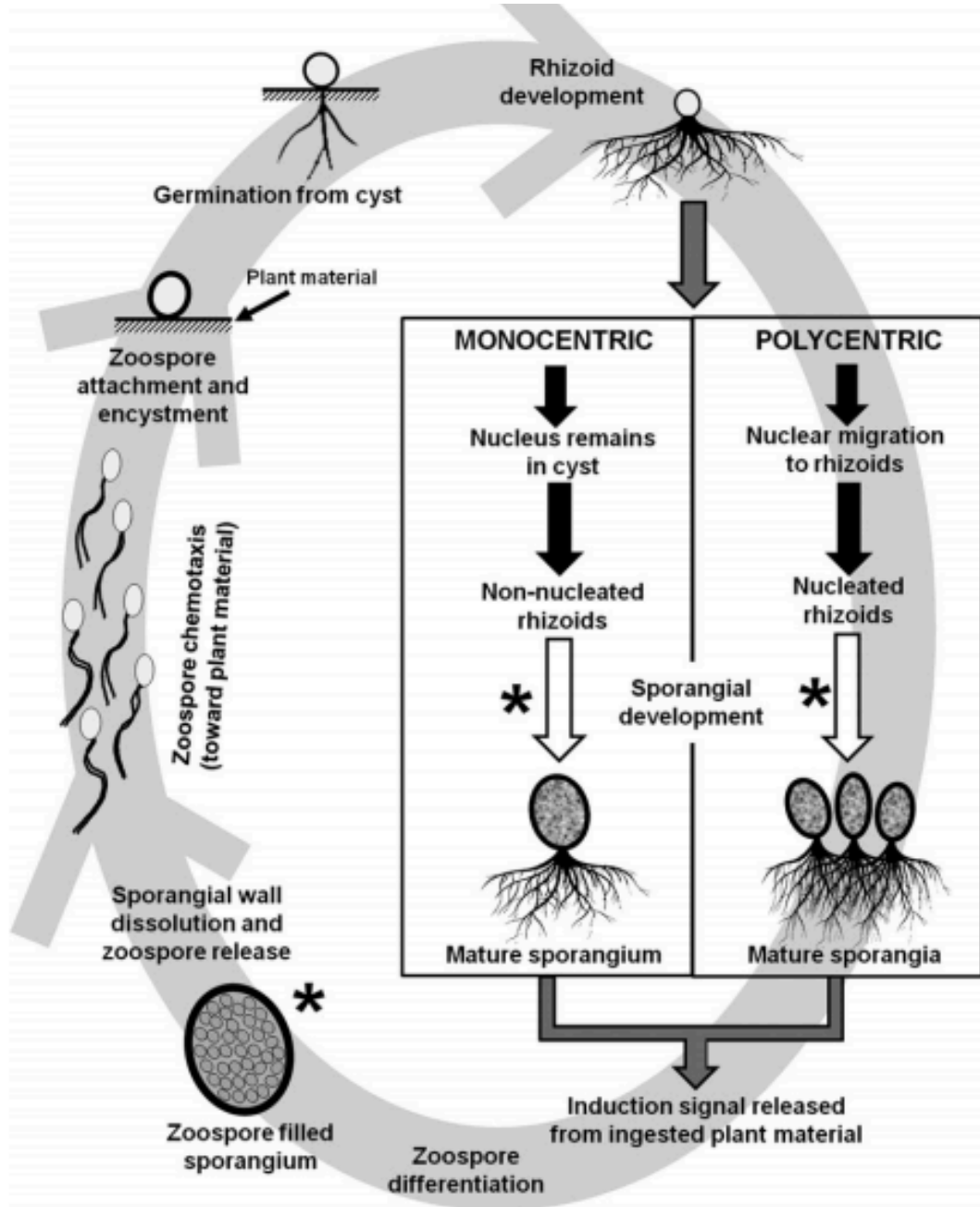
INTRODUCTION

Abstract

Anaerobic gut fungi (AGF) comprise a distinct phylum (*Neocallimastigomycota*) that possesses the unique ability to survive in the rumen and alimentary tract of mammalian herbivores. AGF have been shown to play an important role in the degradation of plant materials ingested into the herbivorous gut. AGF have sparked particular interest recently due to the potential of exploiting such capabilities for the production of biofuels and bio-based chemicals. However, the inherent fastidious and strict anaerobic nature of AGF has complicated the study of AGF, resulting in a dramatic research lag compared to their aerobic counterparts. This dissertation aims to provide a means to remedy some of the challenging aspects in working with AGF.

Anaerobic gut fungi (AGF). The Anaerobic gut fungi (AGF) are the sole fungal representatives that exist in the rumen and alimentary tract of mammalian, and some reptilian, herbivores and represent the unique fungal phylum *Neocallimastigomycota* [1, 2]. AGF constitute a basal fungal lineage that reproduces asexually by the production and release of flagellated zoospores from sporangia [1]. These flagellated zoospores are motile until they come in contact with a carbon source or solid substrate where they will attach, encyst and germinate, leading to the development of extensive rhizomycelia that function as an anchor for the production of zoospore-laden sporangia to repeat the lifecycle [1]. AGF are strictly anaerobic, and possess a complete arsenal of lignocellulosic enzymes, which, once attached to plant material, enable saccharification and fermentation resulting in the degradation of a wide range of plant polysaccharides [1, 3]. Thus, not only do AGF play an important role in enhancing plant biomass metabolism by the host animals [4], but they also have multiple potential biotechnological applications; as a source of lignocellulolytic enzymes [5-11], direct utilization of AGF strains for sugar extraction from plant biomass in enzyme-free biofuel production schemes [12], as additives to biogas production reactors [13, 14], and feed additives for livestock [15-21].

Figure 1-1. Asexual life cycle of anaerobic fungi (from Gruninger et al., 2014) [1].



Challenges associated with AGF maintenance, storage, and genetic manipulation.

Even though the ecological importance and biotechnological potential of AGF is undisputed [4-21]; overall progress in various aspects of AGF biology has been relatively slow. Indeed, progress towards understanding the various aspects of AGF molecular biological and developmental biological research is in stark contrast to the rich body of knowledge available for their aerobic counterparts [22-36]. This is due to the strict anaerobic nature, the lack of reliable storage procedure, and the lack of genetic tools for the manipulation of AGF. The eukaryotic and strict anaerobic nature of AGF makes it difficult to maintain these organisms in a laboratory setting. As a result there is a lack of reliable long-term storage and maintenance procedures [37], and therefore cultures must be continuously subcultured. Unfortunately, there is a propensity of many strains for senescence upon continuous subculture [38]. Thus, development of a reliable storage procedure for AGF is necessary for moving research forward in this understudied field. Moreover, there are currently no established protocols for transformation, gene insertion, gene deletion, or sequence-specific homologous recombination-based genetic manipulations in AGF, as procedures involving plating and colony selection are unfeasible under strict anaerobic conditions. Additionally, with the exception of microscopic-based observations [37-47], studies seeking to understand the physiological, structural, regulatory, and gene expression patterns associated with various developmental stages of the AGF life cycle have been exceptionally sparse.

Spore collection as a starting point for maintenance, developmental studies, and genetic manipulation in AGF. Methods for the collection of viable, developmentally

synchronized spores has been established in aerobic fungi, where spore release is induced upon exposing aerial sporangia that has been grown on a solid substrate, to flooding solution [48-51]. These methods have proven useful towards the advancement of molecular biological protocols in aerobic fungi using an *Agrobacterium*-mediated transformation (AMT) [23, 25, 26], and RNA interference (RNAi) approaches [22, 24, 27], as spores are amenable to nucleic acids uptake. For example, germinating spores from *Aspergillus* species could uptake synthetic small interfering RNAs (siRNA) for targeted gene silencing using an RNAi approach, thereby eliminating the need for transformation and subsequent transformation [24].

The ability to collect intact, viable, developmentally synchronized AGF spores would allow for synchronized growth experiments enabling detailed physiological investigation of spores at various lifecycle stages, transcriptional studies evaluating gene expression levels during spore encystment and germination [30, 48], as well as open the door for potential genetic manipulations [22-27]. Unfortunately, the strict anaerobic nature of AGF hinders the use of the established aerobic method for spore collection. Therefore, development of a similar procedure with extensive adaptations and safeguards to allow for aerial sporangial growth and the collection of structurally intact viable spores, under anaerobic conditions, is a critical step towards achieving progress on the molecular biological and developmental biological fronts in AGF.

***Pecoramyces ruminantium* strain C1A (C1A): History and biotechnological potential.** *Pecoramyces ruminantium* strain C1A (C1A) was isolated from fecal samples of an Angus steer [52], and has been maintained in anaerobic rumen fluid media by

continuous subculturing since 2009 [37, 52-55]. C1A genomic analysis depicted a unique evolutionary history for AGF based on the discovery of the presence of multiple genes involved in pathways that are only present in early branching fungal lineages and non-fungal Opisthokonta that are completely absent in Dikarya genomes [52]. Furthermore, genomic, transcriptomic, and experimental analyses were conducted on C1A in order to assess the biotechnological potential of AGF members. Results of these studies revealed that C1A has an extensive repertoire of lignocellulolytic machinery capable of simultaneous saccharification and fermentation, resulting in a remarkable ability to degrade the cellulosic and hemicellulosic fraction of plant biomass [12, 52, 53]. Subsequent studies towards uncovering the potential of C1A for use in biofuel production demonstrated that C1A could efficiently metabolize both untreated and hydrothermolysis-treated switchgrass and corn stover, resulting in the production of lactate, formate, acetate, and ethanol. This study also demonstrated that degradation of pretreated plant material by C1A resulted in an increase in the amount of ethanol and lactate products, and a decrease in acetate and formate production [54]. Further examination of the transcriptional response of C1A grown on a variety of lignocellulolytic substrates led to the identification of transcripts belonging to glycoside hydrolase (GH) families that are known to play a critical role in mediating cellulose and xylan degradation [53]. Identification of these highly expressed GH family members led to the development of a defined lignocellulolytic enzyme cocktail of selected C1A genes, that were cloned and overexpressed in *E. coli*, that were capable of producing hydrolysis yields comparable, and in some cases superior, to those of commercially available cocktails [8].

Storage, synchronized growth, and genetic manipulations of strain C1A. Each of the studies that constitute the remaining chapters of this dissertation were conducted with the overall aim to develop a means for spore collection (Chapter II), and utilize that method for transcriptional studies (Chapter III) and genetic manipulations (Chapter IV) of the anaerobic rumen fungus *Pecoramyces ruminantium* strain C1A (C1A). My efforts towards the quest for AGF spore collection resulted in the development and optimization of a novel anaerobic flooding technique [56]. This multifaceted anaerobic flooding technique has proved to be invaluable for each study project moving forward in the following ways: (1) it provided a means for culture storage and regeneration to avoid senescence that occurs from continuous culturing; (2) allowed for the separation of spores from other lifecycle stages (i.e. hyphae, sporangia, etc.), for collection of samples that only consist of spores; (3) allowed for collection of viable, competent, and developmentally synchronized C1A spores (i.e. flagellated zoospores, encysted spores, germinating spores) [56].

The second project, comprising Chapter III, was a transcriptional study aimed to examine the expression patterns of focal adhesion (FA) associated genes in C1A at different developmental stages when grown under conditions that provided a solid substrate versus soluble substrate conditions. The presence of genes in C1A genome coding for a nearly complete focal adhesion (FA) machinery is interesting, as the integrin adhesome and its function in focal adhesion was originally believed to be metazoan specific [38]. This study evaluated the evolutionary significance related to the presence of FA genes in the C1A genome [57]. I utilized the developed anaerobic flooding technique to synchronize growth of C1A cultures [56, 57], in order to collect separate samples of

various spore stages. Results from qRT-PCR analysis of FA-associated genes revealed for the first time that FA components are indeed transcribed during growth in the absence of an extracellular matrix anchor. Alternative non-adhesion functions for FA scaffolding proteins in C1A include involvement in hyphal tip growth during germination and flagellar assembly during zoosporogenesis, highlighting the possible functional diversity for FA scaffolding proteins in basal fungi.

Chapter IV aimed to evaluate the potential of targeted gene silencing in AGF using an RNAi approach. This study resulted in the development of an anaerobic RNAi-based protocol for targeted gene silencing in C1A, demonstrating the feasibility of RNAi in anaerobic fungi for gene silencing-based studies [58]. This work has been submitted for review in *PeerJ* [58]. Collectively, the projects within this dissertation have yielded promising techniques, which have opened the door for future developmental biology studies, as well as targeted gene manipulation studies in this understudied fungal clade.

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CHAPTER II

A FAST AND RELIABLE PROCEDURE FOR SPORE COLLECTION FROM ANAEROBIC FUNGI: APPLICATION FOR RNA UPTAKE AND LONG-TERM STORAGE OF ISOLATES

Abstract

Anaerobic gut fungi (AGF) represent a basal fungal lineage (Phylum Neocallimastigomycota) that resides in the rumen and alimentary tracts of herbivores. The AGF reproduce asexually, with a life cycle that involves flagellated zoospores released from zoosporangia followed by encystment, germination, and the subsequent development of rhizomycelia. A fast and reliable approach for AGF spores collection is critical not only for developmental biology studies, but also for molecular biological (e.g. AMT-transformation, RNAi) approaches. Here, I developed and optimized a simple and reliable procedure for the collection of viable, competent, and developmentally synchronized AGF spores under strict anaerobic conditions. The approach involves growing AGF on agar medium in serum bottles under anaerobic conditions, and flooding the observed aerial growth to promote spore release from sporangia into the flooding suspension. The released spores are gently collected using a wide bore sterile needle.

Process optimization resulted in the recovery of up to 7×10^9 spores per serum bottle. Further, the released spores exhibited synchronized development from flagellated spores to encysted spores and finally to germinating spores within 90 minutes from the onset of flooding. At the germinating spore stage, the obtained spores were competent, and readily uptook small interfering RNA (siRNA) oligonucleotides. Finally, using multiple monocentric and polycentric AGF isolates, I demonstrate that AGF grown on agar surface could retain viability for up to 16 weeks at 39°C, and hence this solid surface growth procedure represents a simple, cryopreservative- and freezing temperatures-free approach for AGF storage.

Introduction

The anaerobic gut fungi (AGF) constitute a basal fungal lineage (phylum Neocallimastigomycota) that is encountered in the rumen and alimentary tract of herbivores. The anaerobic gut fungi play an important role in breaking down ingested plant materials in the herbivorous gut by possessing a complete arsenal of lignocellulosic enzymes that enable the degradation of a wide range of plant polysaccharides. The AGF has a life cycle that involves the asexual production and release of flagellated zoospores from sporangia. These zoospores attach and encyst on solid surfaces, leading to the development of extensive rhizomycelia that function as an anchor for the production of zoospore-laden sporangia [1].

Due to their strict anaerobic nature [2], the propensity of many strains for senescence upon continuous subculture [3], and the lack of reliable long-term storage and maintenance procedures [4], progress on various aspects of AGF research has lagged behind their aerobic counterparts. For example, development of molecular biological approaches for genetic manipulations in the AGF (e.g. gene silencing, insertion, deletion, and mutation) is missing. Currently, there are no established procedures for genetic manipulations of AGF using any of the routinely utilized approaches in their aerobic counterparts (e.g. AMT transformation, RNAi). Similarly, apart from microscopic-based observations [5-12], developmental biology studies seeking to understand the physiological, structural, regulatory, and gene expression patterns associated with various stages of development within the AGF complex life cycle have been extremely sparse. The paucity in molecular biological and developmental biological studies is in stark contrast to the rich body of knowledge available on genetic manipulations [13-18] and

developmental biological [19-27] aspects of various aerobic fungal lineages.

Central to achieving progress on the molecular biological and developmental biological fronts in AGF is the ability to collect intact, viable developmentally synchronized spores (i.e. all spores at the same stage of development). Such collection is crucial, since molecular biological protocols e.g. RNAi approaches ([13, 15, 18], and AMT transformation [14, 16, 17] are conducted on spores due to their amenability to nucleic acids uptake. Similarly, the ability to collect viable developmentally synchronized spores will allow for synchronized growth experiments enabling detailed structural, behavioral, and physiological investigation of spores at various stages of development, as well as studies on gene expression during spore encystment and germination e.g. RT-PCR and transcriptomics studies [21, 28].

In aerobic fungi, harvesting viable, developmentally synchronized spores is straightforward, and involves flooding aerial sporangia grown on solid surface to induce spore release [28-31]. However, given their strict anaerobic nature, the process is challenging in AGF and requires extensive adaptations and safeguards to allow for aerial sporangial development under anaerobic conditions, as well as spore retrieval from anaerobic containers without transient oxygen exposure, or compromising spores' structural integrity via syringe-based manipulations.

Here, I present a simple and reliable approach for collection of viable developmentally synchronized spores from the AGF isolate *Orpinomyces* sp. strain C1A (henceforth referred to as C1A). The approach is based on multiple modifications of earlier procedures utilized for spore collection from the aerobic basal fungus *Phytophthora megasperma* [31]. I show that this protocol recovers almost exclusively (>90%) spores

compared to other life forms (e.g. sporangia, hyphal mats), that the obtained spores are viable, synchronized with respect to their development stage (swimming, encysted, or germinating), and are capable of uptaking small interfering RNA (si-RNA) oligonucleotides. Moreover, using multiple AGF monocentric and polycentric strains, I demonstrate the value of the developed solid surface growth procedure as a method for storage of AGF without the need for cryopreservative addition or subjecting cultures to sub-freezing temperatures.

Materials and Methods

Microorganisms and culture maintenance. Strain C1A was isolated from the feces of an Angus steer [32] and maintained by routine subculturing in an anaerobic, rumen-fluid-cellobiose medium (RFC) that was reduced by L-cysteine hydrochloride and dispensed under a stream of 100% CO₂. Media composition was as follows (per liter): 150 ml of minerals solution I (K₂HPO₄ 3 g.l⁻¹), 150 ml of mineral solution II (g.l⁻¹: KH₂PO₄, 3; (NH₄)₂SO₄, 6; NaCl, 6; MgSO₄.7H₂O, 0.6, and CaCl₂.2H₂O, 0.6), 1 ml Balch vitamins solution, 0.1 ml of Wolin's metal solution, cellobiose 3.75 g, sodium bicarbonate 6 g, and 150 ml of clarified sterile rumen fluid. Medium pH was adjusted to 6.6. Following autoclaving, the medium was amended with kanamycin, penicillin, streptomycin, and chloramphenicol from an anaerobic stock solution in order to provide final concentrations of 50 µg/ml, 50 µg/ml, 20 µg/ml, and 50 µg/ml, respectively. Strain C1A was used for the development and optimization of the approach described below for spore collection. In addition to C1A, five additional strains were used to examine the utility of the developed approach in long term storage of AGF isolates. These isolates are: strains G3, a monocentric species putatively identified as *Neocallimastix* and isolated from goat feces; G3G, C3G and C3J, polycentric species isolated from goat (G3G) and cow (C3G, C3J) feces and putatively identified as *Anaeromyces*; and S4B, a monocentric strain isolated from sheep feces and putatively identified as belonging to a novel genus that is phylogenetically most closely related to *Orpinomyces*.

A procedure for the recovery of viable, development-synchronized AGF spores.

i. Growth on agar media. C1A was grown in 160-ml serum bottles containing 45 mL of RFC medium supplemented with 2% agar (RFC-A) by transferring a 10% inoculum of an actively growing liquid culture. Three inoculation strategies were assessed, with the goal of identifying the strategy that yields the highest number of aerial colonies: 1. Seed inoculum i.e. inoculating C1A while the RFC-A medium was still in a liquid state allowing C1A to grow throughout the agar, 2. Inoculating C1A while the agar was partially solidified in a 'jelly-like' state allowing for growth throughout the top to middle layers of the agar, and 3. Surface inoculation of C1A after the agar completely solidified where the inoculum created a culture overlay on the agar surface. In all cases, following inoculation, the agar cultures in the serum bottles were placed on ice laying flat on their side until the agar was completely solidified (Figure 2-1).

ii. Flooding procedure for spore release and collection. Following growth of C1A on RFC-A with visible numerable colonies on the agar surface, 10 ml of a solution of sterile anoxic water with L-cysteine hydrochloride (0.05 g/l final concentration) as a reductant, and resazurin (0.0001% final concentration) as a redox indicator (SAW solution) was introduced to the surface of the agar culture by slow injection through the butyl rubber stopper while keeping the serum bottle on its side. The flooded serum bottle was then incubated statically on its side, and special care was taken in order not to disturb the culture so that no other life cycle stages will be dislodged from the agar surface into the flooding solution. I reasoned that such exposure to SAW will induce spore release from aerial sporangia, and hence only viable, newly released spores that are at the same development stage will be obtained, as previously suggested for *Phytophthora*,

Blastocladiella, and *Allomyces* strains [28-31]. Following incubation, the flooding solution (SAW plus the released spores) was gently collected anaerobically using a sterile syringe and a wide-bore needle into a pre-sterilized serum bottle with 100% CO₂ headspace. The procedure is outlined in Figure 2-1.

iii. Process optimization. Various operational parameters were evaluated to determine the optimal conditions that produce (i) the highest number of spores/ml, (ii) the highest percentage of spores as opposed to other life cycle stages, and (iii) viable spores capable of starting new cultures when inoculated in fresh media tubes. The numbers of obtained spores were counted using a hemocytometer counting chamber and a phase contrast Olympus BX51 microscope (Olympus, Center Valley, PA). Microscopic examinations were also utilized to visually determine whether other life cycle stages, e.g. sporangia, hyphal mats, were co-released with spores into the flooding solution. The spore: other life cycle stages ratio was determined empirically after examining multiple (at least 10) fields of vision. The viability of obtained spores was assessed by inoculating 9 mL of anaerobic RFC medium with 1 mL of the flooding suspension. Tubes were incubated at 39°C, and growth was scored every day (see the footnote to Table 2-1, Figure 2-2) for a week and compared to the growth of an active subculture of strain C1A.

The following parameters were evaluated in a single factorial design from a starting empirical condition of 7-day old culture of C1A on RFC-A media with 2% agar flooded with 10 ml SAW and incubated at 39°C for 60 minutes in the dark: (1) Concentration of agar in the serum bottles (1%, 1.5%, 2%, 2.5%), (2) culture age at the onset of flooding (5-9 days of growth on solid agar media), (3) Flooding solution composition (SAW versus a previously suggested sporulation solution of 1mM CaCl₂,

1mM Tris-Maleate with pH adjusted to 6.7 [33]), (4) incubation temperatures (i.e. temperature of incubation between flooding and recovery of spores into a sterile bottle (i.e. between steps 2 and 3 in Figure 2-1). The following temperatures were tested: 4°C, 22°C, and 39°C. (5) light vs. dark incubation between flooding and recovery, and (6) duration of incubation between flooding and recovery. Incubation times of 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 minutes were tested.

RNA oligomers uptake by collected spores. Dikarya spores in the germinating stage were shown to be amenable for RNAi and AMT-based transformation approaches involving nucleic acids uptake. Prior studies have clearly shown that viable spores of aerobic fungi are amenable for nucleic acids scavenging in a development-dependent manner, where germinating spores were found to accumulate the highest amount of exogenously added nucleic acids [34]. I hypothesized that the AGF spores released by flooding would be amenable to nucleic acids uptake at the germinating spore stage. To this end, I monitored the progress of C1A spore development during incubation with SAW solution on the agar surface. I observed that as the incubation time with SAW increases, the spores developed from swimming (<30 minutes), to encysted (50-60 minutes), to swollen elongated germinating spores (90-100 minutes).

Guided by C1A transcriptomic data [35], I designed a Cy3-labeled 21-nucleotide si-RNA (Sense: 5' UCGUUGGCGUGAGCUUCCAUU 3', and antisense 5' UGGAAGCUCACGCCAACGAUU 3'. The overhangs on each sequence are underlined) that theoretically does not anneal to any of the mRNA transcripts in C1A. The sense si-RNA was labeled with Cy3 at the 5' end to facilitate tracking of the si-RNA uptake by the germinating spores. The annealed (double stranded) si-RNA was purchased from

Dharmacon (Lafayette, CO). I added the annealed siRNA to the flooding solution after 75 minutes of the onset of flooding (where spores were observed to be at the onset of germinating), and allowed it to incubate at 39°C for an additional 15 minutes. The spores were recovered as described above, and transferred to tubes containing fresh RFC medium such that the final concentration of the annealed siRNA in the culture media was 20 nM. Tubes were incubated at 39°C, and at intervals samples were obtained, stained with DAPI, and examined for the uptake of the fluorescent si-RNA using an Olympus BX51 microscope (Olympus, Center Valley, PA), equipped with Brightline fluorescein isothiocyanate (FITC) filter set for Cy3, as well as a Brightline DAPI high-contrast filter set for DAPI fluorescence. Photomicrographs were taken with a DP71 digital camera (Olympus). The ratio of Cy3-labeled spores to the total number of spores (DAPI-labeled) was evaluated. The goal here was not only to study the ability of C1A germinating spores to uptake exogenously added si-RNA, but also to examine the effect of the uptake on the normal development of C1A when inoculated in fresh media.

Viability of anaerobic fungal cultures grown on agar surfaces. The spore recovery protocol described above starts with growth of C1A in serum bottles on agar media (step 1 in Figure 2-1). Fungal growth on solid media has been known to maintain a state of low metabolic activity for prolonged periods of time [36, 37]. As such, I reasoned that this growth procedure could lead to longer viability of AGF cultures compared to liquid media and could be used as a simple freezing- and cryopreservative-free approach for storing AGF. To this end, I inoculated C1A in agar-serum bottles with RFC-A media as described above, and incubated these cultures at 39°C in the dark for extended periods of time (ranging from 2-16 weeks). Serum bottles were flooded at various time intervals

(weeks 2, 4, 6, 8, 10, 12, and 16) and the released spores were collected as described above and used to inoculate fresh liquid RFC medium. Growth was monitored and scored in comparison to actively growing, routinely subcultured C1A cultures. The procedure was also tested on other anaerobic fungal isolates (strains G3, G3G, C3G, C3J, and S4B) described above to assess its suitability for long-term storage of a wide range of AGF isolates.

Results

Growth of AGF on agar surface in serum bottle. Strain C1A readily grew on agar media using the setting described above (Figure 2-1). Preliminary experiments demonstrated that the inoculation strategy employed greatly influences the number of colonies formed on the agar surface. Surface inoculation of agar resulted in patchy, uneven, growth that was loosely attached to the surface (Figure 2-3A). Seed inoculation of agar prior to solidification resulted in the majority of colonies residing deep within the agar block and hence inaccessible through the surface (Figure 2-3B). However, inoculation of agar during solidification, i.e. while it is still in a “jelly-like” state, yielded the largest number of colonies on the agar surface (similar to the one shown in Figure 2-1B-C), and hence was utilized in all subsequent experiments.

Flooding Process Optimization.

Effect of flooding conditions on the number of spores released. All of the six parameters examined, were shown to have a significant effect on spore recovery numbers, 1. Agar concentration: cultures grown on 2% agar produced 8 to 16-fold more spores compared to cultures grown on lower (1-1.5%) and higher (2.5%) agar concentrations (Figure 2-4A, p-value=0.00015). 2. Culture age: 7 day-old culture produced 10 to 16-fold more spores compared to younger cultures (5-day (p-value=0.00012), 6-day old (p-value=0.00014)), and 43-fold more spores compared to older cultures (9-day old (p-value=0.0001)) (Figure 2-4B). 3. Dark versus light incubation: Dark incubations during flooding produced 57-fold more spores compared to flooding in the ambient light (Figure 2-4C, p-value=0.0001). 4. Incubation temperature: Incubation during flooding at temperatures lower than 39°C resulted in 94- to 117-fold decrease in the number of spores released

(Figure 2-4D, p-value=0.0001) compared to cultures incubated at 39°C during flooding, 5. Flooding solution composition: SAW released 12.4-fold more spores than did the sporulation solution (Figure 2-4E, p-value=0.0002) previously used for maintaining *Blastocladiella* spores in the swimming stage [33], and 6. Duration of incubation: 60-minute incubation resulted in the largest number of spores obtained (Figure 2-4F, p-value=0.0001-0.0016 for number of spores obtained with 60 minutes flooding versus shorter and longer incubation times).

- *Effect of flooding conditions on the ratio of spores released to other life cycle stages:*

Table 2-1 shows the effect of changing flooding conditions on the percentage of spores obtained in the flooding suspension in relation to other life cycle stages including sporangia and hyphal mats. In the majority of conditions tested, the flooding procedure yielded >90% spores compared to other life forms. However, under few conditions, the flooding process yielded a relatively lower proportion (<90%) of spores. These include flooding 5-day old culture, and flooding for very short (2.5 minutes) or very long times (>90 minutes).

- *Effect of flooding conditions on viability of spores.* To test the viability of spores released, I used the obtained flooding suspension as an inoculum in fresh RFC media and evaluated the growth compared to a regular subculture of actively growing C1A. All conditions tested resulted in the release of viable spores capable of germinating and development into active C1A cultures. When spores obtained from flooding under the optimal conditions detailed above were used to inoculate fresh CBM media tubes, fungal biomass was similar to routine sub-cultures. On the other hand, specific sub-optimal

flooding condition, while they produced viable spores, resulted in lower fungal biomass (Table 2-1).

Collectively, these results suggest that the optimal conditions for obtaining the highest number and percentage of viable spores involve flooding 7-day old C1A cultures grown on 2% RFC-agar with 10 ml of SAW followed by incubation for 1h at 39°C in the dark.

C1A spores are capable of RNA oligonucleotide uptake. Interestingly, the duration of the flooding procedure (duration of incubation with SAW on the agar surface) not only impacted the number of recovered spores as shown above, but also their developmental stage. Microscopic examination revealed the exclusive release of very active swimming spores in incubations shorter than 30 minutes. As the incubation time increases, the proportion of resting (encysted) to motile spores steadily increase. In incubations longer than 80 minutes, the absolute majority of collected spores were in the resting stage, with few spores becoming elongated and some spores showing germ tube emergence. The 90 and 100 minutes incubation flooding produced exclusively germinating spores (Figure 2-5).

Prior research has demonstrated that germinating spores of *Aspergillus* were capable of uptaking oligonucleotides, and more specifically si-RNA molecules, and hence could be utilized for RNAi-based studies [13-15, 18]. In an attempt to test the feasibility of using the described flooding procedure with longer incubation time (90 minutes to obtain exclusively germinating spores) for future RNAi-based studies in anaerobic fungi, I added a Cy3-labeled double stranded siRNA (20 nM) that bears no sequence similarity to any of mRNA transcripts identified in C1A [35], while utilizing

75-minutes through a 90-minute incubation flooding of C1A cultures. At the end of the flooding duration, the solution containing the released spores and the Cy3-labeled siRNA was used to inoculate fresh RFC tubes. Cy3-labeled spores were observed starting 5 minutes following transfer to fresh media. The ratio of Cy3-labeled spores to the total number of spores reached its maximum (>90%) at 2 hours following transfer (Figure 2-6). These results clearly demonstrate the ability of germinating C1A spores to uptake siRNA, and that the exogenous addition of si-RNA to the spores had no effect on their development. RFC tubes inoculated with released spores in presence of the Cy3-labeled siRNA showed normal growth (data not shown).

Suitability of AGF solid surface growth as a long-term storage mechanism. Prior research has suggested that AGF colonies maintained on roll tubes could remain viable for extended periods of time [6, 38], although the process was often dependent on addition of plant biomass (sisal), and recovery required the addition of a carbon source and further incubation. I sought to determine whether the solid surface growth procedure utilized here could provide a long-term storage that is independent of sub-freezing temperatures, and the addition of cryopreservatives. To this end, I incubated strain C1A cultures on agar surface for various periods of time (2-16 weeks), as described above. At the end of these periods, I used the optimized flooding procedure to recover spores and used the obtained flooding suspension (water + spores) to inoculate fresh RFC tubes (Table 2-2). C1A cultures 2-16 weeks old were revived from the agar surface by flooding with water. Cultures older than 4 weeks did not produce enough zoospores in the flooding suspension to start a new culture. However, when the flooding solution was vigorously shaken, pieces of the mycelial mats dislodged from the agar surface and

released in the suspension were sufficient to start a new culture upon inoculating fresh RFC tubes.

To demonstrate the putative broad applicability of this storage process, I applied the same technique to five anaerobic fungal isolates (monocentric strains G3 and S4B putatively identified as *Neocallimastix* and a novel genus most closely related to *Orpinomyces*, and polycentric strains G3G, C3G, and C3J putatively identified as *Anaeromyces*). All cultures were successfully recovered after 4-16 weeks incubations on solid surface by flooding and inoculation into RFC media. The ease of the described technique plus the fact that it does not involve exposure to air or a change in temperature, both of which have proven detrimental for some isolates before (Table 2-3), makes it ideal for long-term storage of anaerobic fungal isolates.

Figure 2-1. (A) Cartoon depicting the flooding technique utilized for AGF spore collection. (B) A serum bottle with RFC-agar media inoculated with *Orpinomyces* C1A while the agar was partially solidified and incubated at 39°C for 1 week. Note that the colonies developed on the agar surface (arrows) as well as throughout the top to middle agar layers. A bottom view of a serum bottle with RFC-agar showing the plentiful colonies on the surface (arrows) is shown in C.

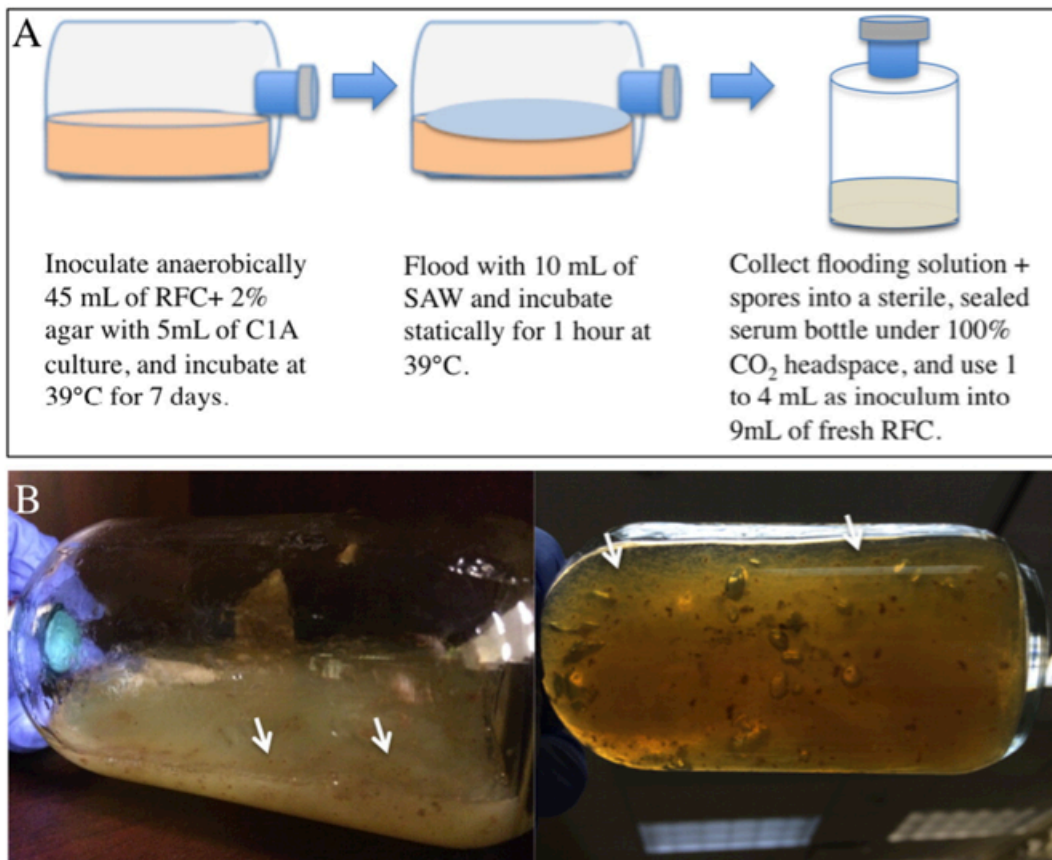


Table 2-1. Flooding optimization for strain C1A.

Condition		% Spores in the flooding suspension ^a	Viability ^b
Temperature	4°C	> 95%	++
	22°C	> 95%	+
	39°C	> 95%	+++
Dark vs. Light	Dark	> 95%	+++
	Light	> 95%	+++
Age of culture (days)	5	> 80%	++
	6	> 90%	++
	7	> 95%	+++
	9	> 95%	+++
Agar conc (% w/v)	1%	> 95%	+
	1.5%	> 95%	++
	2%	> 95%	+++
	2.5%	> 90%	+++
Length of incubation with SAW at 39°C (min)	2.5	> 85%	++
	5	> 95%	++
	10	> 90%	++
	20	> 90%	++
	30	> 95%	++
	40	> 90%	++
	50	> 90%	++
	60	> 95%	+++
	70	> 90%	++
	80	> 95% ^c	++
	90	> 90% ^c	++
	100	> 80% ^c	++
Flooding solution	Water	> 95%	+++
	SS ^d	> 90%	++

a: % of spores was determined during counting by recording the number of other life cycle stages (e.g. early sporangia, late sporangia, hyphal mats) per examined field of vision.

b: Viability scores: (-) no visible growth; (+) little growth; (++) moderate growth; (+++) excellent growth (examples shown in Figure S2).

c: spores were all resting with no flagella. Some have started producing a germ tube.

d: SS, sporulation solution (1mM CaCl₂, 1mM Tris-Maleate, pH 6.7).

Figure 2-2. Examples of the viability scores in the footnotes of Tables 2-1 and 2-2. (-) No growth was detected, growth score (+) where little growth is observed and small colonies are starting to form, growth score (++) where moderate growth is observed and small mats are starting to form, and growth score (+++) where excellent growth is observed with apparent biofilm and bigger mats are formed.

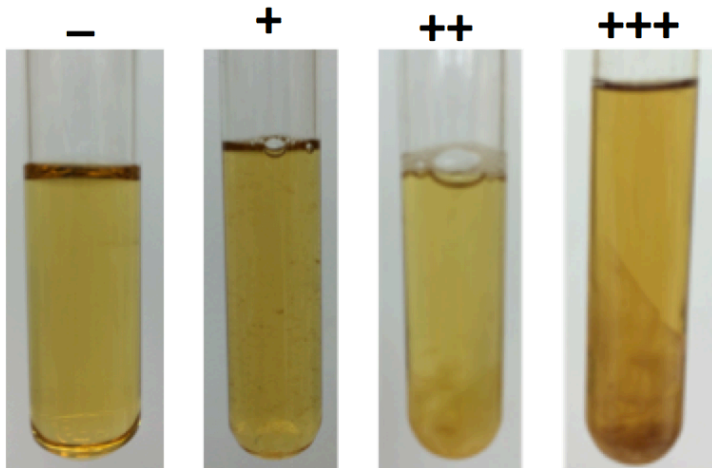


Figure 2-3. Inoculation procedures of C1A in solid agar media. (A) A picture depicting the surface inoculation of agar. Arrows pointing to the surface colonies. On the right is a light microscopy picture of the flooding solution following a 60 minute incubation on the surface of agar surface-inoculated with C1A. Note the presence and abundance of other life cycle stages (sporangia and hyphae in the sample). (B): A picture depicting the seed inoculation of the agar. Very few colonies are present on the surface (arrows).

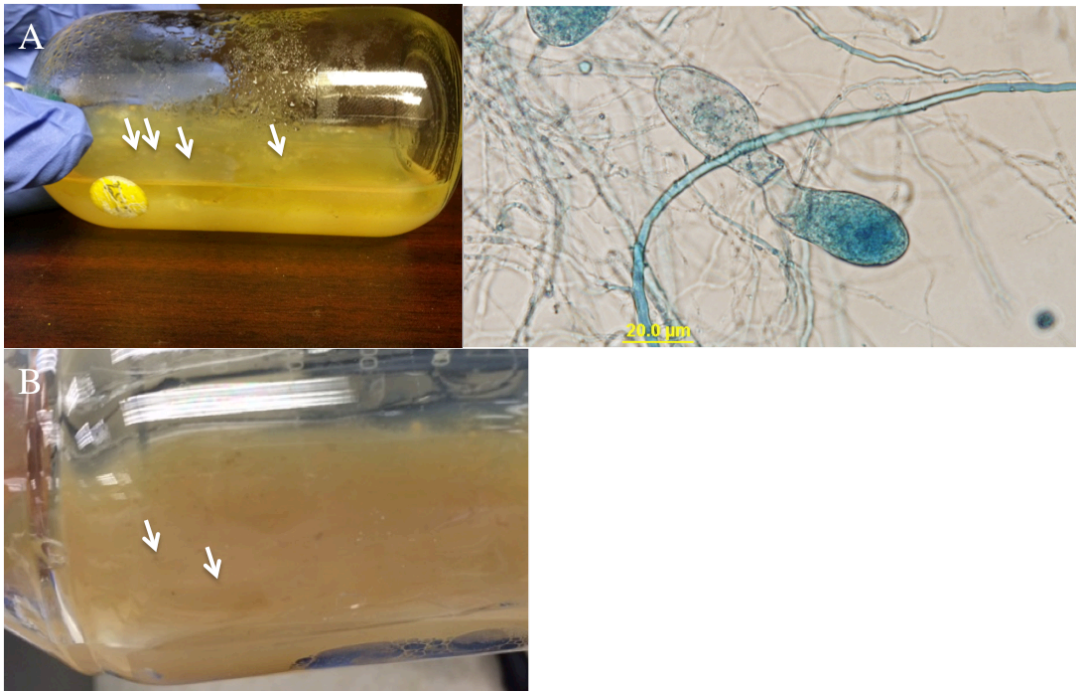


Figure 2-4. Results of flooding optimization showing the number of spores released per ml of the flooding suspension as agar concentration (A), culture age (B), light intensity (C), temperature (D), the nature of the flooding solution used (E), and the duration of flooding (F) were changed. The Y-axis is shown in a logarithmic scale and depicts 10^7 times the number of spores obtained per ml of the flooding solution. Error bars represent standard deviation from two counts.

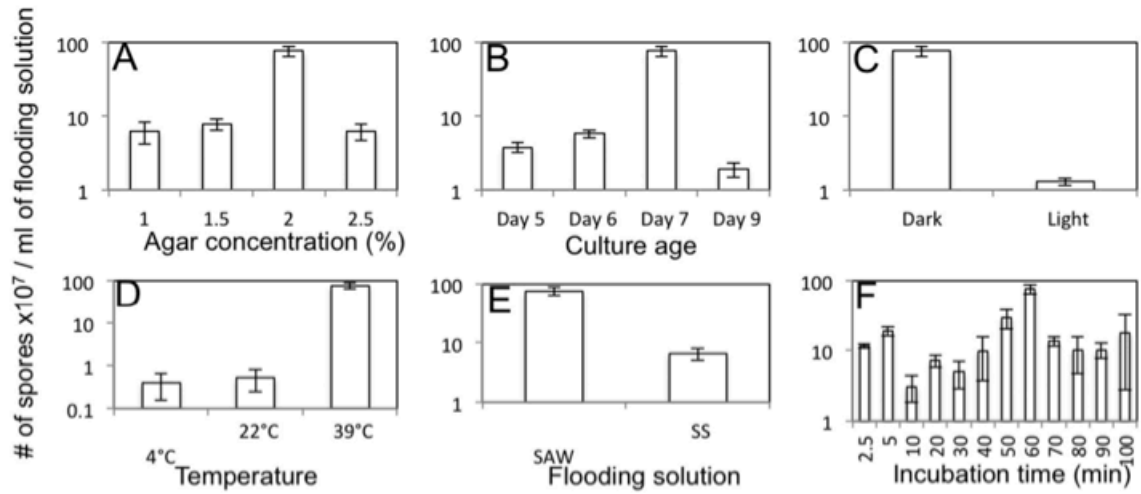


Figure 2-5. Phase contrast (A-C) and negative stain TEM (D-F) pictures of C1A spores obtained during flooding. (A, D) spores obtained by flooding agar surface with SAW followed by a 5-minute incubation at 39°C, arrows in A depict swimming spores, the flagella of which are shown in D (bar=100 nm). Longer incubation times (e.g. > 60 minutes) produced resting spores (B, E) that have started swelling (arrows pointing to swollen spores). Note the different sizes of spores and the absence of flagella in E (bar=500 nm). Incubation times longer than 90 minutes resulted in the production of swollen germinating spores (C, F) that have considerably increased in size compared to A. Arrows in F point to the germ tube starting to form on some of the spores (bar=500 nm).

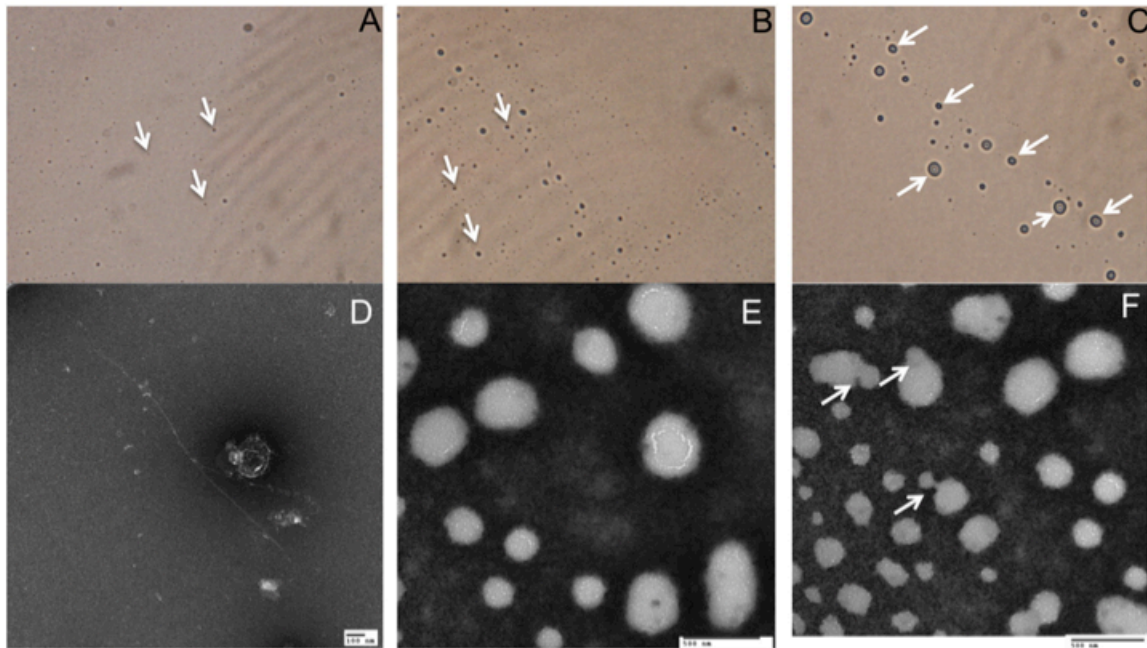


Figure 2-6. Germinating spores uptake of si-RNA. A 21-nucleotide double stranded si-RNA was added to the flooding solution 75 minutes after the onset of flooding followed by incubation for 15 more minutes at 39°C. The flooding solution was then used to inoculate 45 mls of fresh RFC medium and a sample was taken at regular intervals for visualization. The same field is shown for DAPI (A), and Cy3 (B)-labeled germinating spores 2 hours post inoculation (arrows point to the spores concurrently stained with DAPI and fluorescing green indicating the uptake of the Cy3-labeled siRNA) (bar=20 μ m).

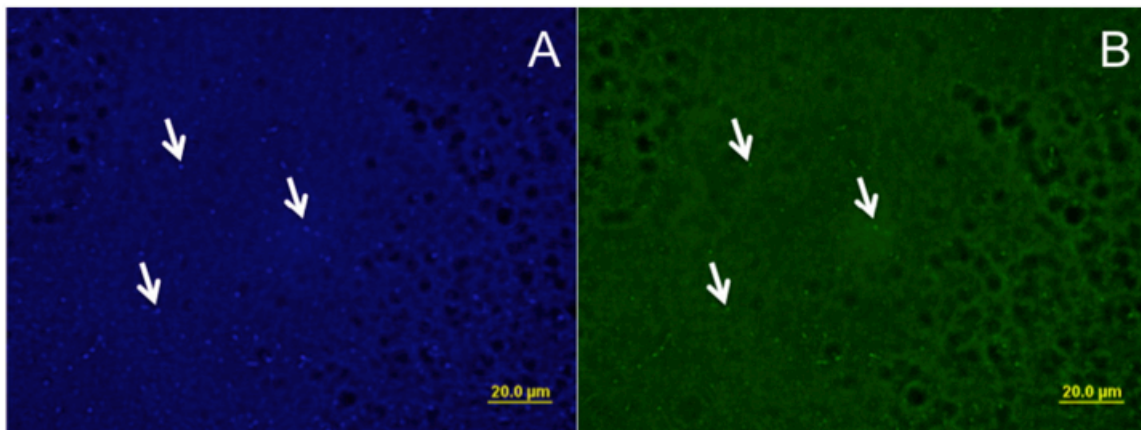


Table 2-2. Application of Growth on solid media for AGF long-term storage.

Isolate	Culture age (weeks) ^a	Viability post flooding and inoculation ^b
C1A	2	+++ (2)
	4	+++ (2)
	6	+++ (1)
	8	+++ (1)
	10	++ (1)
	12	++ (1)
	16	+++ (1)
<i>Anaeromyces</i> -like (3)	4	+++ (1)
	8	+++ (1)
	16	+++ (1)
<i>Neocallimastix</i> (1)	4	+++ (1)
	8	+++ (1)
	16	+++ (1)
Novel genus (1)	4	+++ (1)
	8	+++ (1)
	16	+++ (1)

a: cultures were grown on solid RFC media for the indicated periods of time (under culture age). For culture revival of 2-4 week-old cultures, SAW was anoxically added to the surface followed by incubation for 60 min at 39°C. The flooding suspension was then used to inoculate fresh RFC media (10% v/v). Post 6 weeks, the serum bottles were flooded with SAW and incubated undisturbed at 39°C for 60 minutes then were shaken vigorously to dislodge pieces of the mycelial mats before transfer of the flooding suspension to fresh RFC media (30% v/v) and recording growth.

b: growth was scored after inoculating the flooding suspension into tubes with fresh media. Viability scores: (-) no visible growth; (+) little growth; (++) moderate growth; (+++) excellent growth. The number in parenthesis refers to the number of days following inoculation until visible growth was seen in the tubes. Upon subculturing, growth was observed in all tubes and was comparable to the routine subculture.

Table 2-3. Prior methods for long-term storage of the anaerobic gut fungi.

Method of preservation	Fungal species	Survival
Adding DMSO to the growth media followed by gradual decrease in temperature and storage in liquid N ₂ [50].	<i>Neocallimastix patricarium</i>	Several months to 1 year
Cryopreservation in presence of ethylene glycol, cell-free rumen fluid, and a zoospore density of at least 5 x 10 ⁴ zoospores/ml, followed by gradual decrease in temperature [45].	<i>Piromyces communis</i> strain OTS1	More than 80% recovery was observed after 10 weeks of storage.
Cryopreservation with glycerol at -70°C [51].	Several <i>Ceacomyces</i> species	More than 90 days
Cryopreservation with glycerol at -80°C [49]	<i>Anaeromyces robustus</i> , <i>Neocallimastix californiae</i> , and <i>Piromyces finnis</i>	Up to 23 months with the exception of the <i>Piromyces</i>
Cryopreservation with 5% DMSO followed by centrifugation and freezing the pellet at -80°C [52].	<i>Neocallimastix</i> strains TU1 and TU2, <i>Piromyces</i> strain TU3, an unidentified polycentric fungus strain TU4, and <i>Ceacomyces sp.</i> TU5.	All strains remained viable after storage at -80°C for at least 4 months.
Storage on sisal agar roll tubes followed by incubation at 39°C. Revival by addition of glucose, vigorous shaking and incubation for 2-3 days [6].	Three unidentified anaerobic fungal isolates strains K1, K2, and K3.	Up to 7 months

Table 2-4. Prior methods for spore collection and germination induction for the anaerobic gut fungi.

Method	Fungal species	Reference	Notes
A. Methods for spore collection or release from active sporangia			
Filtering using nylon cloth followed by high speed centrifugation ^a	<i>Neocallimastix frontalis</i> EB188	[46]	Viability of spores questionable, suitable only for nucleic acids extraction
Collection of hyphal-free culture supernatant (spores of monocentric species are abundant in the culture supernatant) ^a .	<i>Neocallimastix</i> sp. R1	[7, 8]	Number of spores released is heavily dependent on the growth phase in monocentric species
	<i>Neocallimastix</i> sp. MC-2 <i>Neocallimastix</i> sp. MC-2, <i>N. frontalis</i> sp. PN1 and PN2, and <i>N. patriciarum</i>	[11, 12]	
	<i>Neocallimastix</i> sp. N1, and <i>Piromyces</i> sp. R1	[10]	
Low speed centrifugation (1000-1500 xg) of actively growing culture to pellet the zoospore present in the culture supernatant ^a .	<i>Piromyces communis</i> strain OTS1 <i>Neocallimastix frontalis</i> MCH3	[45] [47]	Difficulty of maintaining anoxic conditions

Transferring pieces of mycelial mats with abundant sporangia into fresh liquid medium to induce zoosporogenesis and zoospore liberation.	Three polycentric species; <i>Anaeromyces elegans</i> , and <i>Orpinomyces</i> sp. LL and LC2	[5]	While this method allowed collection of spores from polycentric species, the method depends on the stage of sporangial development where only newly developed sporangia differentiated and released spores as opposed to older sporangia.
B. Methods for induction of spore germination			
Incubating spores in the presence of agar cellobiose blocks followed by washing to remove non-adhering spores.	<i>Neocallimastix frontalis</i> MCH3	[47]	The encysted or germinating spores will adhere to the agar blocks and will be difficult to remove.
Addition of glucose to stimulate encystment and germination (within 30 min to 6h of C source introduction).	<i>Neocallimastix frontalis</i>	[9]	The process of germination was not synchronized and so it would be difficult to obtain a germinating spores-only sample.

a: These methods cannot be applied for spore collection from polycentric species due to the scarcity or outright absence of sporangia that has been observed with these species [5].

Discussion

Here, I report on the feasibility of using surface agar cultures for spore collection within the AGF. I show the feasibility of recovery of up to 10^9 spores/50 ml culture under optimal conditions and that the obtained spores are viable and develop in a synchronized fashion from swimming spores to encysted spores to germinating spores within a reasonable timespan. Obtaining viable synchronized spores is a prerequisite for conducting developmental biology and genetic manipulations in fungi. Similar efforts in basal fungal aerobic counterparts have enabled studies on physiology [26, 27, 39], pathogenicity [19, 40], global gene expression patterns during sporulation and germination [21, 28], immunolocalization of proteins [41], as well as *Agrobacterium*-mediated transformations [42, 43]. I hope that this approach opens the door for similar advances in AGF developmental biology and molecular biology research.

Prior Studies on AGF spore collection and cultural synchronization have been relatively sparse (summarized in Table 2-4). Approaches for inducing sporogenesis and spore release have been reported for *Neocallimastix* by adding hemin to the culture media [44]. Most studies that reported spore collection from anaerobic fungi mainly utilized low speed centrifugation or filtration [45, 46]. While straightforward, these approaches would not be suitable for separation of the different developmental stages of spores (i.e. swimming spores, encysted spores, and germinating spores). In addition, most previous reports did not conduct any viability measurements on the released spores. More importantly, the above approaches depend on the presence of free spores in the culture supernatant [7, 8, 10-12, 47]. Such dependency on the presence of free spores could be problematic in monocentric species (e.g. genera *Piromyces*, *Neocallimastix*) where the

number of spores in the culture supernatant greatly depends on the culture age, as well as in polycentric species (e.g. genera *Anaeromyces* and *Orpinomyces*), where the paucity or complete absence of sporangial development and zoosporogenesis with repeated subculturing has been noted [5]. My approach enabled the release and collection of a large number of viable spores under optimal conditions.

I show that the released spores are developmentally synchronized, and, when at the germinating stage, could readily uptake small-interfering RNAs. This is significant, because it opens the door for genetic manipulations in AGF using RNAi approaches, similar to what have previously been reported in *Aspergillus* [13, 15, 18], including *Agrobacterium*-mediated transformation similar to what was reported before for the basal fungal genera *Blastocladiella* and *Batrachochytrium* [42, 43], as well as *Aspergillus* [14]. To my knowledge, apart from a single attempt of biolistic transformation in *Neocallimastix* [48], genetic manipulations of AGF have not been actively pursued so far.

Finally, the proposed protocol builds on the observation that AGF can readily be cultured on solid agar surface under strict anaerobic conditions to allow for aerial sporangial development. My preliminary observation was that AGF isolates could be revived, even after prolonged incubations under anaerobic conditions. Long-term storage of aerobic fungi on solid media at ambient temperatures has long been applied, albeit with the addition of mineral oil or water to prevent culture dryness [36, 37]. This storage technique keeps fungi in a low metabolic state until revived [36]. Addition of mineral oil or other liquids to prevent dryness would be dispensable for AGF since they are cultured in tightly sealed serum bottles. One previous study suggested that AGF cultures on roll tubes in presence of sisal fibers could survive for up to 7 months [6]. I set to examine and

quantify the feasibility of growth of AGF on a solid surface as a method of long-term storage that does not require subjecting cultures to ultra low temperatures. I show that this approach represents an excellent method for storing cultures up to 16 weeks, with easy routine sub-culturing by flooding and re-inoculating fresh media tubes. The simplicity and ease of this growth pattern renders it an interesting alternative and valued addition to prior reported methods for AGF long-term storage that required cryopreservative addition, multiple transfers and manipulations which result in transient air-exposure risks, or the exposure to ultra-low (-80°C) temperatures (Table 2-3).

In conclusion, I show that: 1) under the most optimal conditions, more than 10^9 spores are released from anaerobic fungal colonies growing on a solid agar surface, 2) variation in the incubation time with the flooding solution can be adapted to obtain either swimming or germinating spores, both of which were shown to be viable and resulted in growth when inoculated in fresh media, 3) the released germinating spores can uptake 21-nucleotide small-interfering RNA molecules, and 4) the growth on solid surface can be easily adapted for long-term storage of both polycentric and monocentric anaerobic fungal isolates with no incidental O₂ exposure or a decrease in temperature to the ultra-low values shown before to be detrimental for some isolates [49].

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CHAPTER III

INSIGHTS INTO THE UTILITY OF THE FOCAL ADHESION SCAFFOLDING PROTEINS IN THE ANAEROBIC FUNGUS *ORPINOMYCES* SP. C1A

Abstract

Focal adhesions (FAs) are large eukaryotic multiprotein complexes that are present in all metazoan cells and function as stable sites of tight adhesion between the extracellular matrix and the cell's cytoskeleton. FAs consist of an anchor membrane protein (integrin), scaffolding proteins (α -actinin, talin, paxillin, and vinculin), signaling proteins of the IPP complex (integrin-linked kinase, α -parvin, and PINCH), and two signaling kinases (focal adhesion kinase (FAK) and Src kinase). While genes encoding complete focal adhesion machineries are present in genomes of all multicellular Metazoa; incomplete machineries were identified in the genomes of multiple non-metazoan unicellular Holozoa, basal fungal lineages, and amoebozoan representatives. Since a complete FA machinery is required for functioning, the putative role, if any, of these incomplete FA machineries are currently unclear. I sought to examine the expression patterns of FA-associated genes in the anaerobic basal fungal isolate *Orpinomyces* sp. strain C1A under different growth conditions and at different developmental stages. Strain C1A lacks clear homologues of integrin, and the two signaling kinases FAK and Src, but encodes for all scaffolding

proteins, and the IPP complex proteins. I developed a protocol for synchronizing growth of C1A cultures, allowing for the collection and mRNA extraction from flagellated spores, encysted germinating spores, active zoosporangia, and late inactive sporangia of strain C1A. I demonstrate that the FA scaffolding proteins α -actinin, talin, paxillin, and vinculin are indeed transcribed under all growth conditions, and at all developmental stages of growth. Further, analysis of the observed transcriptional patterns suggests the putative involvement of these components in alternative non-adhesion-specific functions, such as hyphal tip growth during germination, and flagellar assembly during zoosporogenesis. As opposed to previous studies that only documented the genomic presence of components of an incomplete FA machinery in non-metazoan eukaryotes, I show here, for the first time, that these components are indeed transcribed during growth in the absence of an ECM anchor, and propose alternative functions for such proteins in the anaerobic gut fungi. Results highlight the diverse functionalities of FA scaffolding proteins in basal fungi.

Introduction

In eukaryotes, focal adhesions are sites of stable contacts with the extracellular matrix (ECM) and subsequent polymerization of the cell's cytoskeleton. They mediate interaction between the ECM and the cell interior by promoting cell anchorage and mechanical adhesion to the ECM, as well as act as signaling milieu where signaling proteins are concentrated at sites of integrin binding and connect the cell's cytoskeleton to the ECM. FAs are comprised of large multiprotein complexes that are mediated by integrin, a heterodimeric membrane protein that acts as the point of matrix-cytoskeleton connection [1]. The process is initiated in the presence of an extracellular matrix (ECM) protein ligand, e.g. fibronectin that binds to the ECM receptor integrin. This integrin-ECM bond recruits the scaffolding protein talin to the focal adhesion site, which in turn binds actin microfilaments and functions to strengthen the integrin-ECM bond. Integrin-talin-actin complexes recruit additional components such as focal adhesion kinase (FAK), paxillin, and Src-family kinases (SFKs) to integrin tails thereby revealing binding sites for other proteins, such as vinculin. The integrin-cytoskeleton link is further stabilized by the recruitment of the IPP complex, comprising integrin-linked kinase (ILK), parvin, and PINCH, to promote cytoskeleton linkage and integrin signaling. Actin crosslinking occurs via α -actinin, which orchestrates the elongation and growth of focal adhesions. The structure of the integrin adhesome and the mechanism of the focal adhesion process have been extensively studied in metazoan cell culture lines [1, 2, 3].

Focal adhesion is essential for multicellularity since it enables cells to attach to components of the ECM [4]. Accordingly, it was thought until recently, that the integrin adhesome and its function in focal adhesion was metazoan specific [5]. However, this

view was challenged when homologues of FA proteins were identified in the genomes of several unicellular non-metazoan Holozoa; such as the Choanoflagellates *Proterospongia* sp., and *Monosiga brevicollis*, the Filasterea *Capsaspora owczarzaki*, and the Ichthyosporea *Sphaeroforma*, the genome of the Apusozoa (the Opisthokonta sister group) *Amastigomonas* sp., and genomes of several representatives of the Amoebozoa, with (Figure 3-1) [6, 7]. Further, in Fungi, the Holozoa sister group within the Opisthokonta, homologues of FA proteins were also identified in the genomes of various basal fungal phyla, but not the Dikarya (Ascomycota and Basidiomycota). Interestingly, within all basal fungal lineages studied, while the pattern of occurrence of FA components is distinctive (Figure 3-1), all of them invariably lack homologues for integrin and the signaling kinases FAK and Src, but encode for scaffolding proteins. In the absence of integrin and the signaling kinases, the connection between the cytoskeleton and the ECM is lost; hence the known function of focal adhesions might not be realized (Figure 3-2). Therefore, it is currently unclear whether the focal adhesion components in basal fungi are nonfunctional and only represent a remnant of a once complete FA machinery, mediate adhesion with the help of a yet-unidentified integrin functional homologue, or are involved in some hitherto unrecognized function as part of a non-adhesion related machinery.

The anaerobic gut fungal isolate *Orpinomyces* sp. strain C1A encodes a partial FA machinery [8]. The genome contains homologues of the IPP complex and the four scaffolding proteins talin, vinculin, paxillin, and α -actinin, but lacks homologues for integrin, FAK, and Src. Here, I reasoned that transcriptional patterns of genes encoding this partial FA machinery in strain C1A under various growth conditions and

developmental stages could provide preliminary insights into the functionality and putative biological role(s) of these proteins. I show for the first time in basal fungi that the genes encoding scaffolding proteins are indeed transcribed during growth in the absence of the upstream integrin and the signaling kinases. Phylogenetic analyses, conservation of functional domains, and comparative modeling all suggest that the predicted proteins would be functional. Based on transcriptional levels at different stages of the life cycle of this fungus, I discuss the possible cellular roles of scaffolding proteins in C1A.

Materials and Methods

2.1. Organism and culture media. Strain C1A was originally isolated from an Angus steer [8]. Cultures of C1A were regularly transferred twice a week for maintenance in rumen fluid media with cellobiose as the carbon source [9]. Where indicated, cellobiose was replaced with microcrystalline cellulose as the C source. Prior to inoculation, the media were amended with an anaerobic antibiotic mixture of kanamycin, penicillin, streptomycin, and chloramphenicol with final concentrations of 50 µg/mL, 50 µg/mL, 20 µg/mL, and 50 µg/mL, respectively.

2.2. FA components in C1A genomes, phylogenetic analysis, and functional domain prediction. I queried the genome of strain C1A [8, 10] to identify FA-related genes/transcripts. Phylogenetic analysis of the predicted scaffolding proteins was conducted to identify their closest relative and examine whether their topologies are in agreement with the organismal phylogeny. Individual multiple sequence alignments (MSA) were constructed for each of the predicted proteins in Mega [11]. When possible, beside metazoan homologues, predicted proteins from non-metazoan eukaryotic representatives (including basal fungi) were added to the alignment. The resulting MSA was utilized to construct maximum likelihood trees using the best substitution model identified using the calculated values for the Akaike information criterion (AIC), Bayesian information criterion (BIC), and likelihood ratio as tested in Mega [11].

All predicted proteins were checked for functional domain structure and organization by querying against the Pfam database [12]. I used Phyre2 [13] homology modeling to construct pairwise sequence alignments with secondary and tertiary structure predictions for α -actinin, talin, and vinculin. The predicted tertiary structure models were

visualized in PyMol [14], and each of the predicted models were superimposed to the corresponding template structure used for structure predictions. The protein data bank [15] (PDB) ID's for the templates utilized were as follows: α -actinin (PDB ID: 1SJJ), talin (PDB ID's: 1SJ7, 2L10, 2JSW, 2KVP, 2QDQ), and vinculin (PDB ID: 1TR2). When the predicted paxillin sequence was searched against the PDB database, no hits with structural data were identified. Accordingly, for paxillin a secondary and tertiary structure prediction was not possible.

2.3. *Transcriptional studies of genes encoding scaffolding proteins in CIA.* In addition to the mere documentation of transcription of genes encoding scaffolding proteins, I sought to use real time PCR to examine and analyze the transcriptional levels of such genes under various growth conditions and various developmental stages. My aim was to examine whether the transcriptional patterns observed support a putative role for FA scaffolding proteins in an adhesion-related processes, or whether they would be involved in developmental-stage specific function other than adhesion, e.g. in flagellar assembly, as previously suggested for the ciliated cells of *Xenopus tropicalis* [16], or hyphal tip growth as previously speculated in the basal fungus *Allomyces arbuscula* [17].

2.3.1. *Transcriptional patterns in presence and absence of an ECM ligand.* To examine the putative involvement of the scaffolding proteins in adhesion-related processes, I compared the transcription levels of their genes in presence and absence of an extracellular matrix trigger for FA. Fungi differ from animal cells in that they lack a well-defined protein-rich extracellular matrix but possess a polysaccharide-rich cell wall [18]. However, in zoosporic fungi (e.g. members of Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, and Monoblepharidomycota), the zoospore stage was shown to have

some type of polysaccharide-rich [19] extracellular matrix in the form of a cell coat covering the zoospore body and excluding the flagellar axoneme [20]. Therefore, in contrast to in-vitro studies of focal adhesions in Metazoa that used a protein, usually fibronectin, as the extracellular trigger for focal adhesion [21], I opted for using a polysaccharide (microcrystalline cellulose; MCC) as the extracellular trigger for FA in C1A. The hypothesis here is that if the scaffolding proteins are indeed recruited as part of the FA machinery, then their genes are expected to be differentially up-regulated when C1A is grown in the presence of the extracellular polysaccharide MCC as opposed to a soluble substrate such as cellobiose. To this end, C1A cultures were grown in rumen fluid media with cellobiose, as opposed to the insoluble MCC as the carbon source. Cultures of C1A on cellobiose and MCC were sacrificed at mid log phase (51 hours post inoculation, based on biomass determination in preliminary experiments), and the biomass was used for total RNA extraction and for studying the transcriptional levels of the FA scaffolding genes as detailed below.

2.3.2. Transcriptional patterns of scaffolding genes at various developmental stages. As described above, an alternative hypothesis posits that scaffolding proteins might be involved in a non-adhesion-specific process during the life cycle of strain C1A, as shown before in ciliated cells of the metazoan *Xenopus tropicalis* [16], or as previously suggested for the basal fungus *Allomyces arbuscula* [17]. To examine this hypothesis, I quantified the transcriptional levels of talin, α -actinin, vinculin, and paxillin, at four distinct developmental stages in C1A: flagellated spores, encysted non-flagellated germinating spores, active sporangia during zoosporogenesis, and late inactive sporangia samples.

While separation and collection of life cycle stages is a routine practice in aerobic zoosporic fungi, and involves zoospore collection and growth synchronization [22,23], I had repeated difficulties replicating such synchronization starting from flagellated zoospores in the anaerobic fungal representative strain C1A due to the observed wide range of zoospores encystment and germination time following introduction to fresh media. Therefore, I developed alternative approaches for collection of various developmental stages in strain C1A. The procedure (Figure 3-3) was partly described in [9], and involves growing C1A cultures on cellobiose rumen fluid media in presence of 2% agar followed by flooding the agar surface with sterile anoxic water (SAW) to promote spore release from the sporangia. Variation in time between culture flooding and collection of the released spores was used to obtain either 100% swimming spores-only sample (flooding incubation time of 30 minutes), or a >90% non-flagellated germinating spore sample (flooding incubation time of 90 minutes).

To obtain samples representative of active and late sporangia, I started from the swimming spores collected as described above (flooding agar surface followed by 30 minutes incubation). The obtained spores were used to inoculate fresh rumen fluid cellobiose media, and growth was monitored microscopically (using the phase contrast lens of an Olympus BX51 microscope after staining with lactophenol-cotton blue stain solution) on a daily basis for 19 days. Judging by cues from the microscopic examination I sacrificed samples on days 1, 2, 5, 15, and 19 following inoculation as those days coincided with various stages of development as shown in Table 3-1. It has been shown before [24] that the whole basal body carrying the flagella is shed along with the axoneme during encystment of flagellated spores. The basal body and the axoneme are

then re-built during zoosporogenesis in active sporangia. Accordingly, I hypothesized that the transcriptional levels of genes encoding axoneme-specific, as well as basal body-specific proteins should correspond to the level of zoosporogenesis. Therefore, in conjunction with the microscopic examination, I followed the transcriptional levels of RS3 (encoding an axoneme-specific protein) [25], and centrin (encoding basal body and nuclear cap-specific protein) [26] in the samples collected above (days 1-2-5-15-19). Samples with the highest transcriptional levels of RS3 and centrin genes, and microscopic evidence of active zoosporogenesis, were used as representatives of active sporangia; while late samples that showed no microscopic evidence of zoosporogenesis, as well as very low to no transcription of RS3 and centrin, were used as representatives of late sporangia samples.

2.4. RNA extraction, cDNA synthesis, and quantitative RT-PCR. Collected samples were vacuum filtered using sterile 3 μm (for biomass and sporangia samples) or 0.45 μm (for spore-only samples) filters, and the obtained biomass was lysed by crushing with a sterile mortar and pestle upon submersion in liquid nitrogen. Crushed cells were used for total RNA extraction using MasterPure™ Yeast RNA Purification Kit (Epicentre®, Madison, WI) according to manufacturer's instructions. Total RNA reverse transcription (cDNA synthesis) was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen™, Carlsbad, CA) with Oligo(dT)₂₀ according to manufacturer's instructions. Genes' transcriptional levels were investigated using quantitative RT-PCR using a MyIQ thermocycler (Bio-Rad Laboratories, Hercules, CA) and SybrGreenER® qPCR mix (Invitrogen™, Carlsbad, CA). Primers targeting α -actinin, talin, vinculin, paxillin, RS3, and centrin cDNA were designed using the OligoPerfect™ Designer tool

(Invitrogen™, Carlsbad, CA) and their specificity was tested *in-silico* using the standalone NCBI Blastn [27] against all coding sequences of C1A. Primer sequences, their target accession number, as well as amplified regions are shown in Table 3-2. The reactions contained 1µl of C1A cDNA, and 0.5 µM each of the forward and reverse primers. Reactions were heated at 50°C for 2 min, followed by heating at 95°C for 8.5 min. This was followed by 70 cycles, with one cycle consisting of 15 s at 95°C, 60 s at 50°C, and 30 s at 72°C. Using the Δ Ct method, the number of copies of each gene is reported relative to the number of copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the normalizing control.

Results

3.1. Phylogeny, Pfam domain analysis, and structure modeling of FA proteins in C1A.

All four predicted scaffolding proteins from C1A (α -actinin, talin, vinculin, and paxillin), showed a topology consistent with C1A organismal phylogeny. In all maximum likelihood trees, C1A proteins formed a well-supported cluster with proteins from other basal fungi (and Dikarya fungi in case of α -actinin) (Figure 3-4). Likewise, all Metazoan proteins clustered together with strong bootstrap support, with the Choanoflagellates (*Monosiga* and *Proterospongia*) and the Filasterea (*Capsaspora*) proteins forming sister groups. However, the position of proteins from Amoebozoan origin was not consistent across trees (Figure 3-4). When other Neocallimastigomycota genomes/transcriptomes [28] were queried for FA components homologues, a pattern similar to that of C1A was detected, where genes encoding the IPP complex components as well as the scaffolding proteins were identified (Table 3-3) with no homologues for integrin, FAK, or Src.

The results of Pfam domain analysis are shown in Table 3-4 and Figure 3-5. Analysis identified domain organizations that are consistent with functional proteins from Metazoan origin. This includes 2 Calponin homology (CH) (Pfam 00307) domains, one spectrin repeat (Pfam 00435), and one Ca²⁺ insensitive EF hand (EF) domain (Pfam08726) for C1A predicted α -actinin [29]; a talin middle domain (Pfam 09141), 2 vinculin binding site domains (Pfam 08913), and an I/LWEQ domain (Pfam 01608) for C1A predicted talin; and vinculin family domain (Pfam 01044) for C1A predicted vinculin (Figure 3-5). C1A predicted paxillin available sequence was 5' partial and did not span the paxillin domain itself, but comparison against the Pfam database identified 4 LIM domains (Pfam 00412) consistent with Metazoan paxillin C-terminal region [30]

(Figure 3-6). Within the recognized domains, several characteristic residues that were shown before to be conserved and essential for activity [30, 31, 32] were identified (Table 3-4).

Detailed analysis of C1A predicted scaffolding proteins.

1. Alpha-actinin: Comparison against the Pfam database showed that C1A predicted alpha-actinin harbored one actin binding domain comprised of 2 Calponin homology (CH) (Pfam 00307) domains, one spectrin repeat (Pfam 00435), and one Ca^{2+} insensitive EF hand (EF) domain (Pfam08726), a domain organization consistent with alpha-actinin from metazoan origin [29] (Figure 3-5A-I). Due to the partial coverage of C1A genome, the first of the two CH domains was only partial while the second spanned residues 66-169. The presence of two CH domains within the actin-binding domain was shown before to be essential for binding actin [33]. Downstream of the actin-binding domain, the spectrin repeat domain spanned residues 316-408. A closer look at the primary sequence of C1A spectrin repeat in comparison to Pfam00435 HMM profile identified the presence of the aromatic residues Y317 and Y397 and the residue L428, all of which are characteristic of the spectrin repeat. Finally, the Ca^{2+} insensitive EF hand (EF) domain identified downstream of the spectrin repeat spanned residues 505-564. Secondary structure alignments and predicted 3D structure models for each of the alpha-actinin domains are shown in Figure 3-5A. Due to the partial nature of the first CH domain in C1A alpha-actinin, only the second CH domain could be modeled with confidence. I utilized Phyre2 to predict the secondary structure as well as the 3D model of C1A alpha-actinin in comparison to the chicken gizzard smooth muscle alpha-actinin (PDB ID: 1SJJ). C1A CH domain was 60% similar to the corresponding 1SJJ domain and was

modeled with 100% confidence. The predicted model of C1A CH domain displayed the typical structural motif of 4 alpha helices [34], and when superimposed with 1SJJ, it aligned with an RMS value of 0.941 (Figure 3-5A-II). C1A spectrin repeat was 27% similar to the corresponding 1SJJ domain and was modeled with 98.93% confidence. The proposed model of C1A spectrin domain maintained the typical triple-helical coiled-coil motif [35], and superposition with 1SJJ gave an RMS of 0.956 (Figure 3-5A-III). Finally, C1A EF domain was 32% similar to the corresponding 1SJJ domain and was modeled with 99.60% confidence. The predicted model of C1A's alpha-actinin Ca^{2+} insensitive EF hand domain maintained the helix-loop-helix motif typical of EF hands [36]. Superposition of C1A's alpha-actinin EF domain with 1SJJ yields a RMS value of 1.119 (Figure 3-5A-IV).

2. Talin: The Pfam analysis revealed that C1A talin contained 4 domains: a middle domain (Pfam 09141), 2 vinculin binding site domains (VBS1 and VBS2) (Pfam 08913), and an I/LWEQ domain (Pfam 01608) (Figure 2B-I). Talin middle domain spanned residues 100-262. C1A middle domain of talin was modeled with 100% confidence and 30% sequence identity to 1SJ7 (Figure 3-5B-II). The predicted model showed the characteristic bundle comprised of 5 alpha helices [37], and the superimposed structures of the middle domain and 1SJ7 (residues 491-652) yielded an RMS of 0.116 (Figure 3-5B-II). Vinculin binding site domain 1 (VBS1) spanned residues 838-961 and was modeled with 100% confidence and 30% sequence identity to 2L10 (Figure 3-5B-III), while vinculin binding site 2 (VBS2) spanned residues 1455-1578 and was modeled with 97% confidence and 21% sequence identity to 2KVP (Figure 3-5B-IV). Both VBS domain predictions yielded models that were composed of 4 alpha helices characteristic

of vinculin binding sites [38]. Superimposing C1A vinculin binding sites with their templates tertiary structures gave an RMS of 0.902 (VBS1), and 0.653 (VBS2). A closer look at the primary sequence of C1A talin I/LWEQ domain spanning residues 1991-2139 revealed the highly conserved 4-block structure characteristic of this domain [31]. Block 1 (residues 1952-1977) showed several conserved branched chain residues and Q1975, block 2 (residues 1996-2018) showed the conserved W1996 and several non-polar residues, block 3 (residues 2029-2053) showed the conserved E2029, Q2044, and K2052, and block 4 (residues 2109-2128) showed the conserved residues Q2112, R2126, and Y2126. Blocks 1-3 of C1A I/LWEQ domain of talin were modeled with 100% confidence and 53% sequence identity to 2JSW (Figure 3-5B-V), while block 4 was modeled with 96.6% confidence and 45% identity to the dimerization domain 2QDQ. The predicted model showed the characteristic 5-helix bundle [39]. Superimposing C1A I/LWEQ domain on 2JSW, and 2QDQ gave an RMS value of 0.127, and 0.220, respectively (Figure 3-5B-V).

3. Vinculin: Pfam search revealed that C1A vinculin only had 1 domain, the vinculin family domain (Pfam 01044) (Figure 3-5C-I). The available C1A vinculin sequence only spans the C-terminal region of the protein, where it aligned with residues 804-1061 of the human full-length vinculin (PDB: 1TR2) (Figure 3-5C-II). C1A vinculin domain was modeled with x% confidence and 32% sequence identity to 1TR2 (Figure 3-5C-II). The predicted model showed 5 amphipathic helices characteristic of the vinculin tail [40]. C1A vinculin superimposed with 1TR2 tail with an RMS value of 1.224 (Figure 3-5C-II).

4. Paxillin: The C1A predicted paxillin protein was only a partial sequence. Blastp comparison against the nr database identified the paxillin from *Gallus gallus* (Genbank

accession number NP_990315.1) as its first hit with 57% sequence similarity (alignment to NP_990315.1 is shown in Figure 3-6). Comparison against the Pfam database identified 4 LIM domain (Pfam 00412) spanning residues (98-153), (157-213), (217-272), (276-335). A closer look at the primary sequence of each LIM domain and its comparison to the Pfam HMM profile identified several conserved histidine and cysteine residues that are potentially implicated in binding Zn (Figure 3-7). Since the C1A predicted paxillin available sequence did not span the paxillin domain itself, it was not possible to perform any secondary or tertiary structure predictions using the available paxillin proteins in the PDB database.

In summary, predicted 3D model of C1A scaffolding proteins are shown in Figure 3-5. In all cases, typical characteristic structural motifs were predicted for C1A proteins (Table 3-4 and Figure 3-5) [30, 34, 35, 36, 37, 38, 39, 40, 51]. Superimposing the predicted model on template proteins yielded very low RMSD values (Table 3-4), indicative of a high similarity between the superimposed atomic coordinates in C1A versus the template proteins [41]. These results above clearly demonstrate that C1A scaffolding proteins are predicted to be structurally similar to their functional metazoan counterparts.

3.2. Genes encoding scaffolding proteins are transcribed during C1A growth even in the absence of an ECM ligand. I investigated the transcription of genes encoding the 4 scaffolding proteins in C1A cultures grown on cellobiose. C1A total mRNA contained transcripts of genes encoding all 4 scaffolding proteins in levels ranging from 0.6-73 times the level of transcription of the housekeeping gene GAPDH (Figure 3-8). To test the hypothesis that the scaffolding proteins are employed for adhesion in C1A as part of a

FA machinery, I compared the transcriptional levels of genes encoding scaffolding proteins in the presence and absence of an insoluble extracellular polysaccharide matrix (microcrystalline cellulose). The transcriptional levels of genes encoding for all four scaffolding proteins (α -actinin, talin, paxillin, and vinculin) were significantly lower when grown on MCC, as opposed to cellobiose-grown culture (Figure 3-8). These results strongly suggest that adhesion to extracellular matrix components might not be a major physiological function of the scaffolding proteins in C1A.

3.3. Transcripts of scaffolding proteins are up-regulated during zoosporogenesis. Next, I investigated whether the transcriptional patterns of genes encoding scaffolding proteins are dependent on C1A developmental stages. I first quantified transcription levels during active versus inactive sporogenesis to examine the hypothesis that, similar to ciliated cells [16], one of the cellular functions of scaffolding proteins in C1A in absence of integrin or another ECM connection would be association with the basal body during flagellar assembly. Based on microscopic examination (Table 3-1), and the transcriptional levels of centrin and RS3 (Figure 3-6) we chose day 5 as an active zoosporogenesis sample, and day 19 as an inactive late sporangia sample. I studied the transcriptional levels of α -actinin, talin, vinculin, and paxillin in such samples. Results (Figure 3-9) show a significantly higher transcription level in active sporangia for α -actinin, talin, vinculin, and paxillin. I speculate that such significant increase in the transcription levels of genes encoding scaffolding proteins during active zoosporogenesis might be related to their association with basal body during flagellar assembly, as shown before during ciliogenesis [16].

3.4. Flagellated zoospores carry scaffolding proteins mRNAs. I showed above that the genes encoding all 4 scaffolding proteins were highly expressed in the active sporangia sample. Following zoosporogenesis, zoospores are released from the active sporangia, and are motile by means of their posterior flagella. To demonstrate whether the scaffolding genes transcripts are stored with the active zoospores, I collected a swimming spores only sample [9] and used it to study the transcriptional level of α -actinin, talin, vinculin, and paxillin. I show that the zoospores total mRNA contained scaffolding proteins transcripts, albeit with significantly lower transcription levels than what was observed in the active sporangia sample (Figure 3-9A-B) for α -actinin (29,119-fold decrease, Student t-test P-value=0.007), talin (346-fold decrease, Student t-test P-value=1E-09), vinculin (123-fold decrease, Student t-test P-value=0.027), and paxillin (9,734-fold decrease, Student t-test P-value=0.048). Similar results were shown before for *Batrachochytrium dendrobatidis*, where vinculin was found to be differentially expressed in the sporangia as opposed to the zoospore sample [23].

3.5. Scaffolding proteins transcripts are differentially up-regulated in germinating spores as opposed to swimming spores. The presence of scaffolding proteins transcripts as part of C1A swimming spores total mRNA might suggest the utility of these proteins during encystment and germination. To test this hypothesis, I collected a germinating spores-only sample [9] and used it to examine the transcriptional level of α -actinin, talin, vinculin, and paxillin. I show that the transcription level of genes encoding all 4 scaffolding proteins was highest in germinating spores. Levels were significantly higher than in active sporangia undergoing zoosporogenesis, and were also significantly higher than in swimming zoospores for all 4 genes (Figure 3-9). These results suggest that one

of the major cellular functions of scaffolding proteins might be occurring during and post-germination.

Figure 3-1. Genomic evidence for focal adhesion complex components in metazoan and non-metazoan Unikonts. Results shown are from [6, 7, 8, 28], and are based on the genomic analysis of several Metazoa representatives, the Choanoflagellates representatives *Monosiga brevicollis* and *Proterospongia* sp., the Filasterea representative *Capsaspora owczarzaki*, the Ichthyosporea representative *Sphaeroforma*, several representatives of the Dikarya fungi, the basal fungi representatives *Allomyces macrogynus* (Blastocladiomycota), *Spizellomyces punctatus*, and *Batrachochytrium dendrobatidis* (Chytridiomycota), *Orpinomyces* sp. C1A, *Anaeromyces robustus*, *Neocallimastix californiae*, and *Piromyces finnis* (Neocallimastigomycota), the Apusozoa representative *Amastigomonas* sp., and several representatives of the Ameoboza. The dendogram is not drawn to scale and only serves to show the relationships between the different groups. Cells shaded in black denote clear homologues were identified in all representative genomes, cells shaded in grey denote clear homologues were identified in some but not all representative genomes, and cells shaded in white denote no homologues were identified in any of the representative genomes.

Phylogenetic group	Integrins	Scaffolding proteins				IPP complex			Signaling kinases	
		α -Actinin	Paxillin	Vinculin	Talin	ILK	PINCH	Parvin	FAK	Src
Metazoa	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Choanoflagellates	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Filasterea	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Ichthyosporea	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Dikarya fungi	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Basal fungi	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Apusozoa	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
Amoebozoa	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black

Figure 3-2. A simplified schematic of the focal adhesion machinery in Metazoa. Focal adhesion proteins are color coded as follows: Integrins (ECM receptors) are brown, Scaffolding proteins are green, proteins of the IPP complex are blue, signaling kinases are yellow. F-actin polymers are shown in red and proteins of the extracellular matrix are shown in pink.

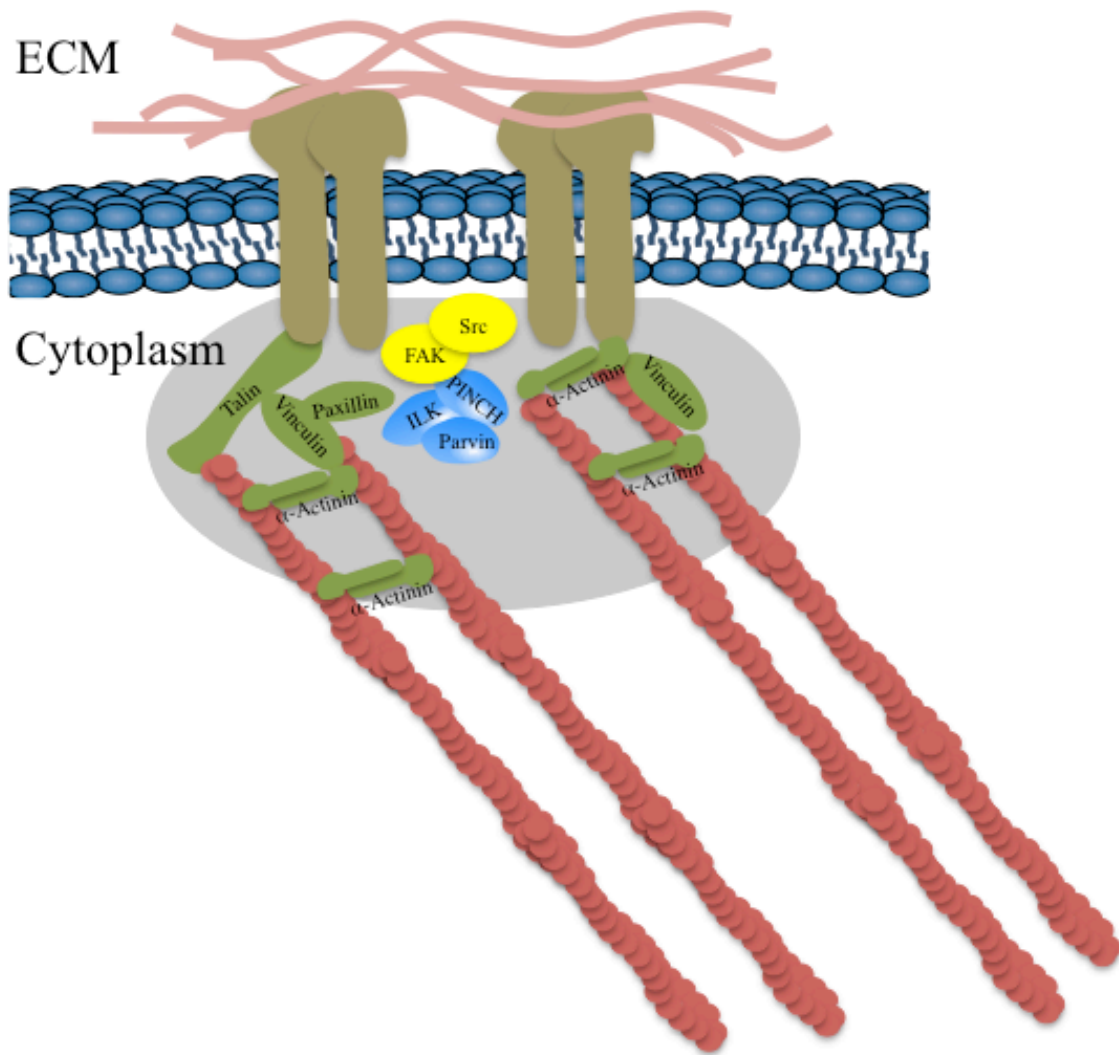


Figure 3-3. Schematic of the protocol used to collect the different developmental stages of C1A employed for the transcriptional study.

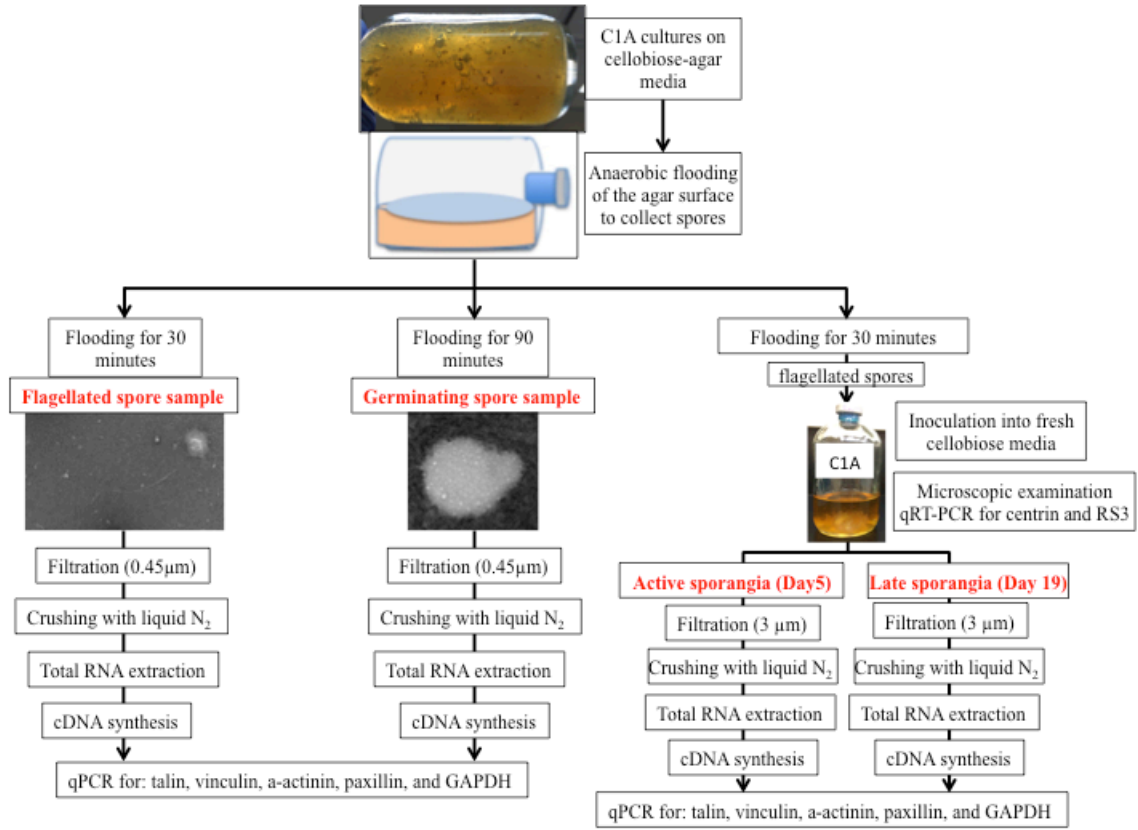


Table 3-1. Microscopy results when C1A was grown in cellobiose (or MCC) media over a period of 19 days.

Sample	Spores		Sporangia	
	Swimming (flagellated)	Resting (not flagellated)	Swimming spores inside	Without swimming spores inside
Day 1	++	+++	-	+
Day 5	+++	++	+++	+++
Day 15	+	++	++	++
Day 19	+	++	+	+++

Scale: (-) none observed, (+) very few observed, (++) some observed, (+++) many observed.

Table 3-2. Quantitative PCR primers used for cDNA amplification.

Transcript/ protein	Accession number	Forward primer sequence	Reverse primer sequence	Amplified region	Function	Use in this study
Talin	KX463728	GCCGCATCTAAAAACGTAGC	CGGTGAAAGCATCGGTATCT	6100–6287 (188 bp)	Scaffolding protein	Transcriptional study
Vinculin	KX463730	CCGCCGAAAAGAATGATTTA	TTCAATGACTGGTGGCTTTG	29–162 (134 bp)	Scaffolding protein	
Paxillin	KX463729	TCCAGCTGGTAGTGGTTTC	TTCAGTTGGGAAGGAGCAAC	569–759 (191 bp)	Scaffolding protein	
α -actinin	KX463732	CACAATTTGCTGCCTTTGAA	CCTTCTGGTGGGTTGTATGG	515–658 (144 bp)	Scaffolding protein	
GAPDH	KX463731	ATTCCACTCACGGACGTTTC	CTTCTTGGCACCACCCTTTA	134–339 (206 bp)	Housekeeping gene	Normalizing control for qPCR
RS3	KX463733	TTTTTGCCATGGCTTACTCA	TGGTTTTCTTCATCGCATT	262–489 (228 bp)	Axoneme component	Zoosporogenesis monitoring
Centrin	KX463734	TTCACAGACAATCACGTTCAAA	TCAGCCATTTTTGCTGACAT	14–285 (272 bp)	Basal body component	

Figure 3-4. Maximum likelihood phylogenetic analysis of C1A predicted scaffolding proteins. All evolutionary analyses and model selections were conducted in MEGA7 [11]. Trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values, in percent, are based on 100 replicates and are shown for branches with >50% bootstrap support. Trees are shown for: (A) α -actinin based on the JTT model with a discrete Gamma distribution (variable site γ shape parameter = 2.0327). Analysis involved 21 amino acid sequences, with a total of 535 positions in the final dataset. (B) Paxillin based on the Dayhoff model with a discrete Gamma distribution (variable site γ shape parameter = 1.7755). Analysis involved 12 amino acid sequences, with a total of 535 positions in the final dataset. (C) Talin based on the Le_Gascuel_2008 model with a discrete Gamma distribution (variable site γ shape parameter = 3.0802). Analysis involved 13 amino acid sequences, with a total of 441 positions in the final dataset. (D) Vinculin based on the Le_Gascuel_2008 model with a discrete Gamma distribution (variable site γ shape parameter = 3.4035). Analysis involved 14 amino acid sequences, with a total of 145 positions in the final dataset.

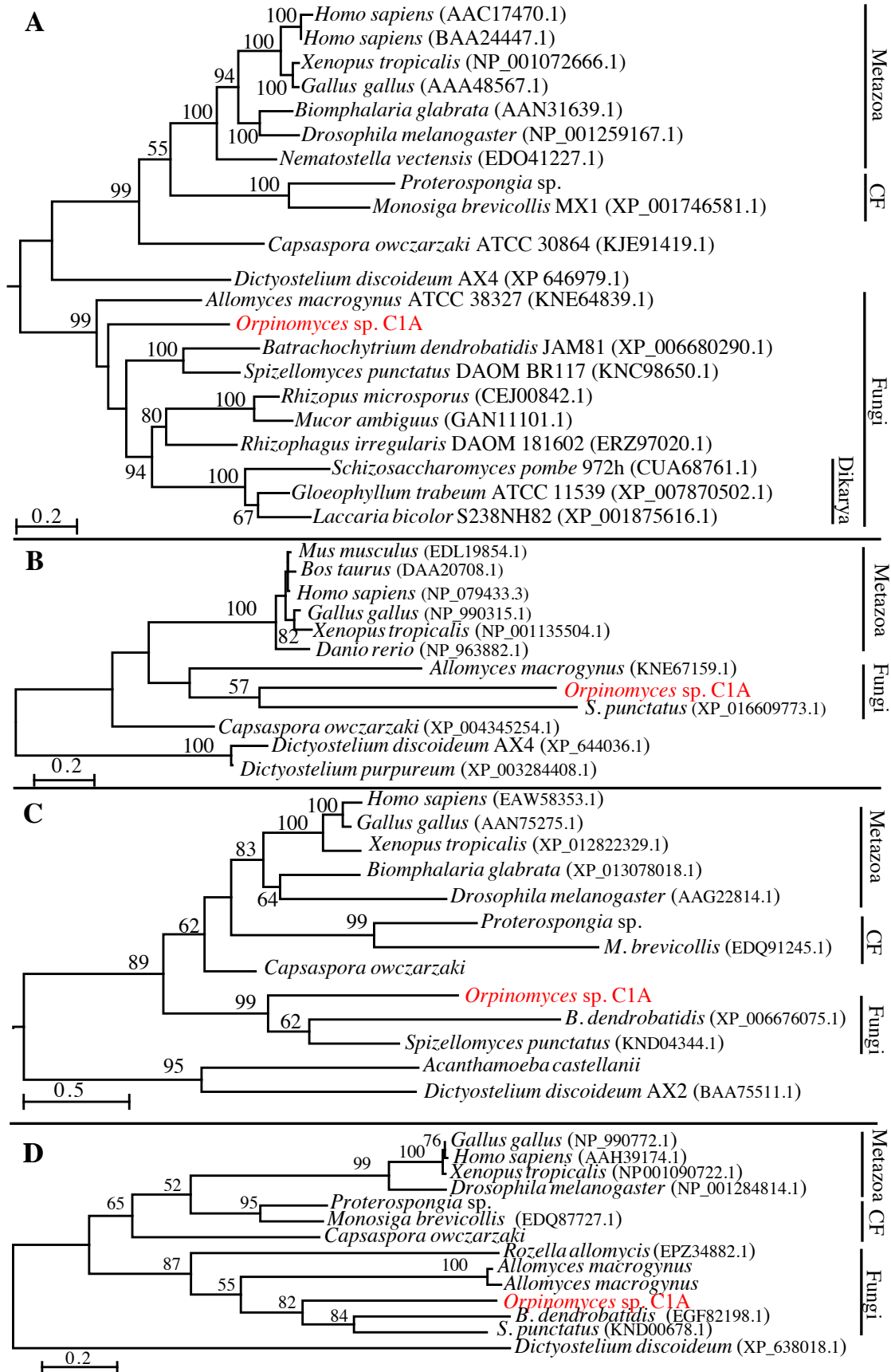


Table 3-3. Blastp results in other Neocallimastigomycota transcriptomes (from (Solomon et al., 2016)).

FA component		Hits in ^a		
		<i>Anaeromyces</i>	<i>Piromyces</i>	<i>Neocallimastix</i>
Anchor	Integrin	N	N	N
Signaling kinases	FAK	N	N	N
	Src	N	N	N
Scaffolding proteins	Talin	Y (g.6332)	Y (g.13026)	Y (g.12367)
	Vinculin	Y (g.7072)	Y (g.15720)	Y (g.8754)
	Paxillin	Y (g.3771)	Y (g.3279)	Y (g.6669)
	α -actinin	Y (g.3928)	Y (g.3512)	Y (g.2424)
IPP complex	PINCH	Y (g.5606)	Y (g.16322)	Y (g.13985)
	Parvin	Y (g.11341)	Y (g.1163)	Y (g.12919)
	ILK	Y (g.9193)	Y (g.2454)	Y (g.9692)

a: Criteria used for Blastp were a minimum of 50% alignment length and > 30%

similarity. N: no homologues identified, Y: a homologue was identified.

Table 3-4. Results of C1A scaffolding proteins comparison to the Pfam database, as well as secondary and tertiary structure predictions.

C1A protein (accession No.)	Blastp First hit Accession No. (organism)	Pfam comparison			Secondary structure prediction and comparative modeling					Reference
		Pfam domain	Residues in C1A protein	Residues important for activity	Template PDB ID (organism)	% similarity	% modeling confidence	Predicted 3' structure	RMSD of superimposition	
α-actinin (KX463732)	KXS11038 (<i>Gonopodya prolifera</i>)	CH (Pfam 00307)	1–55		Partial, not modeled					
		CH (Pfam 00307)	63–171		1SJJ (<i>Gallus gallus</i>)	60%	100%	4 alpha helices	0.941	[39]
		Spectrin repeat (Pfam 00435)	312–413	Aromatic residues Y317 and Y397, and L428	1SJJ (<i>Gallus gallus</i>)	27%	98.93%	triple-helical coiled-coil motif	0.956	[41]
		Ca ²⁺ insensitive EF hand (Pfam 08726)	505–566		1SJJ (<i>Gallus gallus</i>)	32%	99.6%	helix-loop-helix motif	1.119	[35]
Talin (KX463728)	XP_016612383 (<i>Spizellomyces punctatus</i>)	middle domain (Pfam 09141)	100–262		1SJ7 (<i>Mus musculus</i>)	30%	100%	bundle comprised of 5 alpha helices	0.116	[40]
		vinculin binding site (Pfam 08913)	838–961		2L10 (<i>Mus musculus</i>)	30%	100%	4 alpha helices	0.902	[38]
		vinculin binding site (Pfam 08913)	1455–1578		2KVP (<i>Mus musculus</i>)	21%	97%	4 alpha helices	0.653	[38]
		ILWEQ domain (Pfam 01608)	1952–2139. Block 1: 1952–1977, Block 2: 1996–2018, Block 3: 2029–2053, Block 4: 2109–2128	4-block structure: • Block 1: several conserved branched chain residues, Q1975 • Block 2: W1996 and several non-polar residues • Block 3: E2029, Q2044, K2052.	2JSW (<i>Mus musculus</i>)	53%	100%	5-helix bundle	0.127	[33, 37]
				Block 4: Q2112, R2126, Y2126. (dimerization domain)	2QDQ (<i>Mus musculus</i>)	45%	96.6	Antiparallel coiled-coil	0.220	[37]
Vinculin (KX463730)	XP_016608717 (<i>Spizellomyces punctatus</i>)	vinculin family domain (Pfam 01044)	Full length		1TR2 (<i>Homo sapiens</i>)	32%	>90%	5 amphipathic helices	1.224	[36]

Table 2. (Continued)

C1A protein (accession No.)	Blastp First hit Accession No. (organism)	Pfam comparison			Secondary structure prediction and comparative modeling					Reference
		Pfam domain	Residues in C1A protein	Residues important for activity	Template PDB ID (organism)	% similarity	% modeling confidence	Predicted 3' structure	RMSD of superimposition	
Paxillin (KX463729)	NP_990315.1 (<i>Gallus gallus</i>)	LIM domains (Pfam 00412)	98–153, 157–213, 217–272, 276–335	Several conserved Cys and His residues potentially implicated in binding Zn	Partial, not modeled					[51]

Figure 3-5. C1A predicted scaffolding proteins functional domain structure and organization, and predicted protein structure modeling. Results are shown for (A) α -actinin, (B) talin, and (C) vinculin. For each predicted protein, the first row (I) corresponds to the predicted Pfam domain organization. This is followed by 1–5 rows (II–VI) each corresponding to a functional domain. On the left of each of these rows, secondary structure alignments of C1A predicted domain compared to a template’s known and predicted secondary structure are shown. On the right of each row, predicted tertiary structures of C1A domains are shown in pink, compared to the template’s known tertiary structure in cyan. Superimposed structures are also shown. Row (A-II): predicted C1A α -actinin CH domain structure compared to PDB: 1SJJ from *Gallus gallus*. Row (A-III): predicted C1A α -actinin spectrin domain structure compared to PDB: 1SJJ from *Gallus gallus*. Row (A-IV): predicted C1A α -actinin Ca²⁺ insensitive EF hand domain structure compared to PDB: 1SJJ from *Gallus gallus*. Row (B-II): predicted C1A talin middle domain structure compared to PDB: 1SJ7 from *Mus musculus*. Row (B-III): predicted C1A talin VBS1 domain structure compared to PDB: 2L10 from *Mus musculus*. Row (B-IV): predicted C1A talin VBS2 domain structure compared to PDB: 2KVP from *Mus musculus*. Row (B-V): predicted C1A talin I/LWEQ domain (Blocks 1–3) structure compared to PDB: 2JSW from *Mus musculus*. Row (B-VI): predicted C1A talin I/LWEQ domain (Block 4 comprising the dimerization domain) structure compared to PDB: 2QDQ from *Mus musculus*. Row (C-II): predicted C1A vinculin domain structure compared to PDB: 1TR2 from *Homo sapiens*.

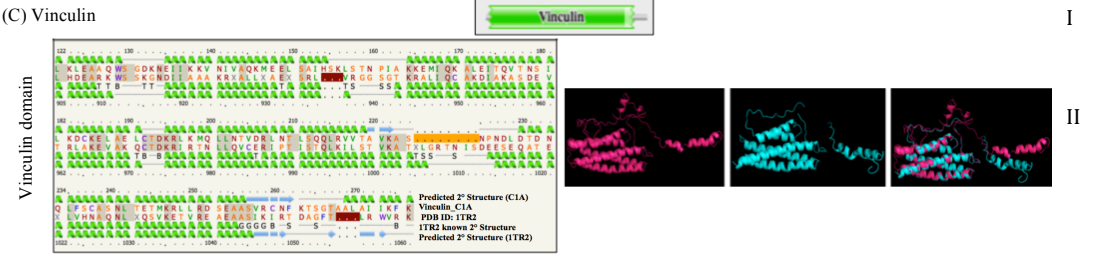
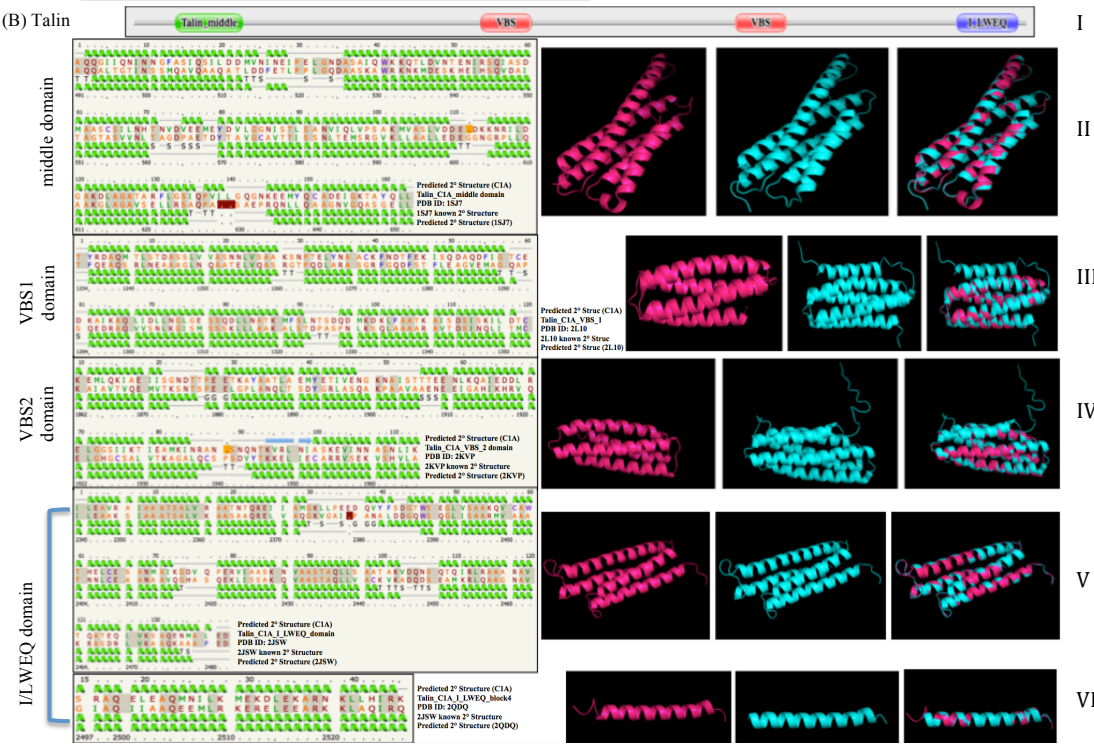
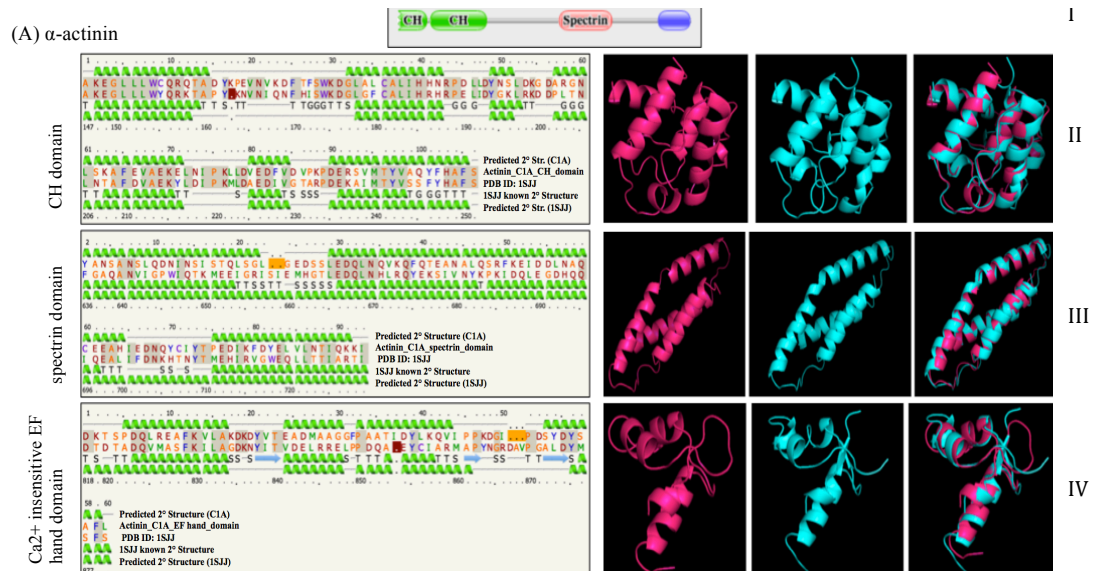


Figure 3-6. C1A predicted paxillin Pfam domain organization (A), and pairwise sequence alignment of to paxillin from *Gallus gallus* (NP_990315).

A



B

paxillin_Gallus_gallus(NP_990315.1)	134	T	S	S	L	G	S	N	L	S	E	L	D	R	L	L	E	L	N	A	V	Q	H	N	P	S	G	F	S	A	D	E	V	S	R	S	P	S	L	P	N	V	T	G	P	H	Y	V	I	184			
Paxillin_C1A	1	M	K	P	N	M	G	-	-	M	N	Q	Y	Q	M	M	S	P	S	L	R	N	P	Q	S	P	G	M	K	Y	Q	N	N	I	P	F	S	P	Q	K	S	N	L	N	-	-	-	-	-	-	-	43	
paxillin_Gallus_gallus(NP_990315.1)	185	P	E	S	S	S	A	G	G	K	A	A	P	P	T	K	E	K	P	K	R	N	G	G	R	G	I	E	D	V	R	P	S	V	E	S	L	L	D	E	L	E	S	S	V	P	S	P	V	P	A	235	
Paxillin_C1A	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	L	N	S	P	S	L	N	S	L	N	S	P	K	-	-	-	-	-	-	-	-	57			
paxillin_Gallus_gallus(NP_990315.1)	236	I	T	V	S	Q	G	E	V	S	S	P	Q	R	V	N	A	S	Q	Q	T	R	I	S	A	S	A	T	R	E	L	D	E	L	M	A	S	L	S	D	F	K	F	M	A	Q	G	K	A	G	286		
Paxillin_C1A	58	-	-	-	-	-	-	-	-	-	-	-	I	E	D	K	L	A	T	K	-	-	-	-	-	-	-	S	Q	S	T	P	E	L	S	P	Q	I	S	P	-	-	-	-	-	-	-	-	-	79			
paxillin_Gallus_gallus(NP_990315.1)	287	G	S	S	P	P	S	T	T	P	K	P	G	S	Q	L	D	T	M	L	G	S	L	Q	S	D	L	N	K	L	G	V	A	T	V	A	K	C	V	C	A	C	K	K	P	I	A	G	Q	V	337		
Paxillin_C1A	80	-	-	-	-	-	-	-	-	-	T	A	N	P	T	Q	K	S	-	-	-	-	-	-	-	-	-	N	P	Q	-	-	K	Q	V	R	K	I	I	C	A	T	C	K	G	Q	I	K	G	K	V	109	
paxillin_Gallus_gallus(NP_990315.1)	338	V	T	A	M	G	K	T	W	H	P	E	H	F	V	C	T	H	C	Q	E	E	I	G	S	R	N	F	F	E	R	D	G	Q	P	Y	C	E	K	D	Y	H	N	L	F	S	P	R	C	Y	Y	C	388
Paxillin_C1A	110	V	S	C	L	G	K	T	W	H	P	E	H	F	I	C	S	G	C	K	K	L	D	P	N	R	F	F	E	K	N	N	L	P	C	C	E	N	C	H	R	E	S	F	V	P	R	C	A	Y	C	160	
paxillin_Gallus_gallus(NP_990315.1)	389	N	G	P	I	L	D	K	V	V	T	A	L	D	R	T	W	H	P	E	H	F	F	C	A	D	C	G	V	F	G	P	-	E	G	F	H	E	K	D	G	K	A	Y	C	R	K	D	Y	F	D	438	
Paxillin_C1A	161	G	D	V	I	T	K	C	I	T	A	L	G	T	W	H	T	D	H	F	F	C	C	Q	C	A	M	Q	F	P	A	G	S	G	F	L	E	K	D	G	K	A	Y	C	E	R	D	Y	R	211			
paxillin_Gallus_gallus(NP_990315.1)	439	M	F	A	P	K	C	G	G	C	A	R	A	L	E	N	Y	I	S	A	L	N	T	L	W	H	P	E	C	F	V	C	R	E	C	F	T	P	F	I	N	G	S	F	F	E	H	D	G	Q	P	489	
Paxillin_C1A	212	L	F	A	T	R	C	G	K	C	G	E	I	V	G	E	Y	I	S	A	L	G	K	E	W	H	N	K	C	F	T	C	K	E	C	G	C	S	F	P	T	E	S	F	Y	E	Y	K	G	M	P	262	
paxillin_Gallus_gallus(NP_990315.1)	490	Y	C	E	V	H	Y	H	E	R	R	G	S	L	C	S	G	C	Q	K	P	I	T	G	R	C	I	T	A	M	G	K	K	F	H	P	E	H	F	V	C	A	F	C	L	Q	L	N	K	G	-	539	
Paxillin_C1A	263	Y	C	E	T	H	Y	H	L	K	R	G	S	L	C	S	R	C	N	K	P	I	V	G	R	C	I	S	A	S	G	K	K	Y	H	P	D	H	T	C	V	N	C	N	K	L	L	A	T	G	N	313	
paxillin_Gallus_gallus(NP_990315.1)	540	-	-	-	T	F	K	E	Q	N	D	K	P	Y	C	Q	N	C	F	L	K	L	F	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	559		
Paxillin_C1A	314	M	D	D	E	Y	K	E	V	N	G	H	P	F	C	G	S	C	A	S	K	I	R	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	336		

Figure 3-7. Transcriptional levels of genes encoding RS3 and centrin in C1A. The number of transcript copies of RS3 (□) and centrin (■) relative to the number of transcript copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were followed over a period of 19 days. Error bars are standard deviations from at least two replicates.

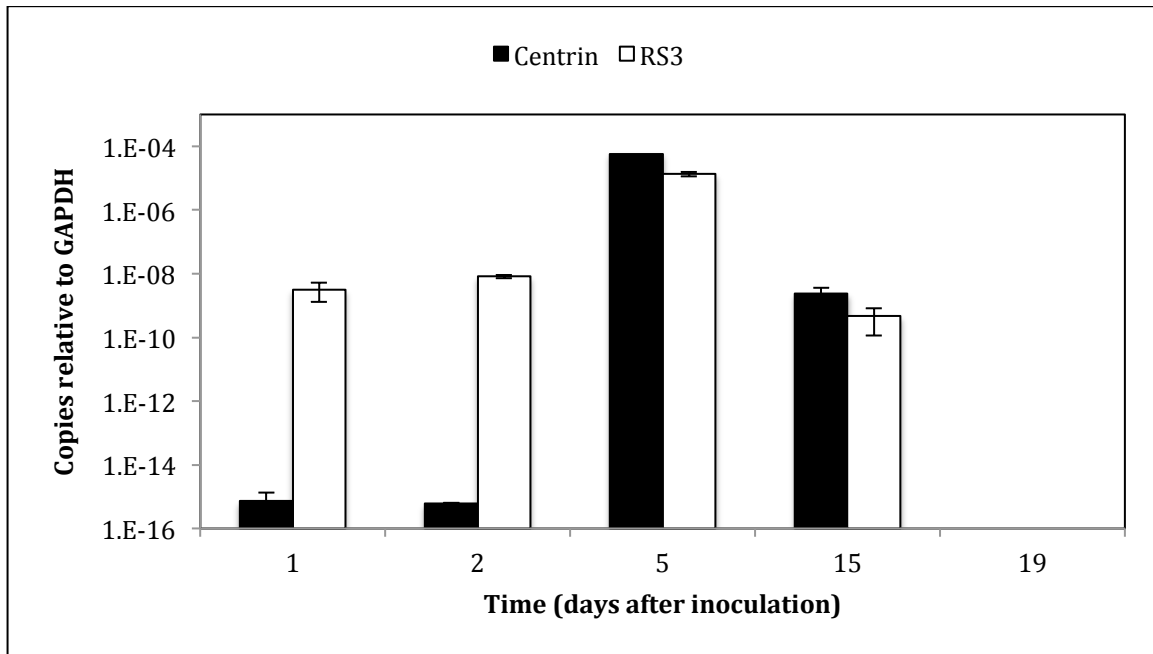


Figure 3-8. Transcriptional levels of genes encoding scaffolding proteins in the presence and absence of an extracellular matrix polysaccharide. The number of transcript copies of talin, paxillin, vinculin, and α -actinin relative to the number of transcript copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown when C1A was grown on soluble cellobiose media (\square) (i.e. in absence of an ECM) versus when grown on a MCC media (\blacksquare) (i.e. in presence of an ECM). Error bars are standard deviations from two experiments (each with 2 replicates) for paxillin, vinculin, and α -actinin, and four experiments (each with two replicates) for talin. Values were significantly higher in absence of ECM for talin (5.7-fold increase, Student t-test P-value = 0.001), α -actinin (13.1-fold increase, Student t-test P-value = 0.009), vinculin (8.7-fold increase, Student t-test P-value = 0.008), and paxillin (5.7-fold increase, Student t-test P-value = 0.07).

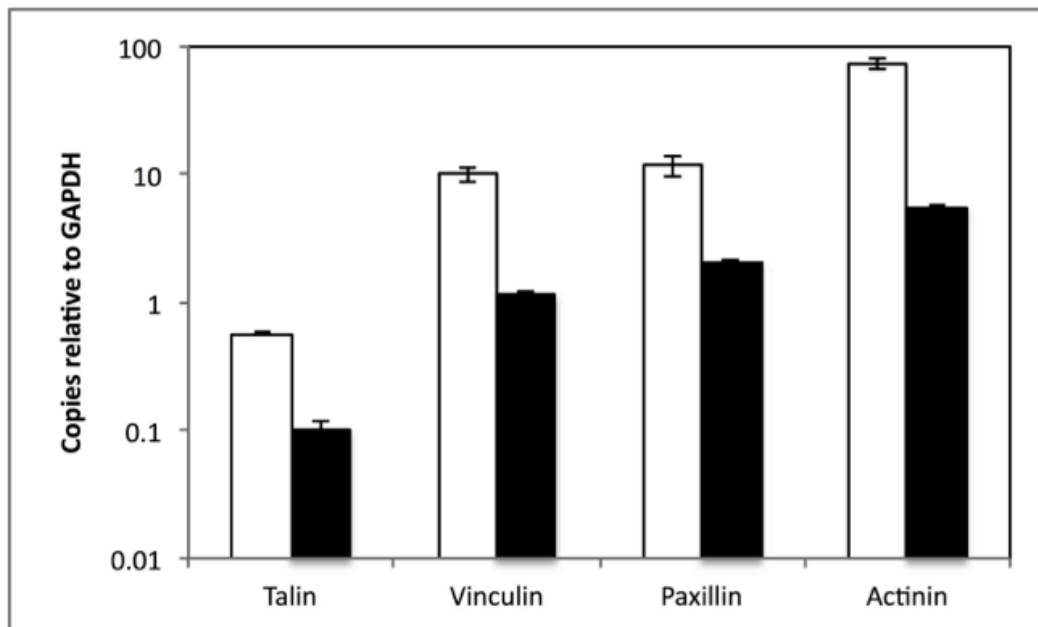
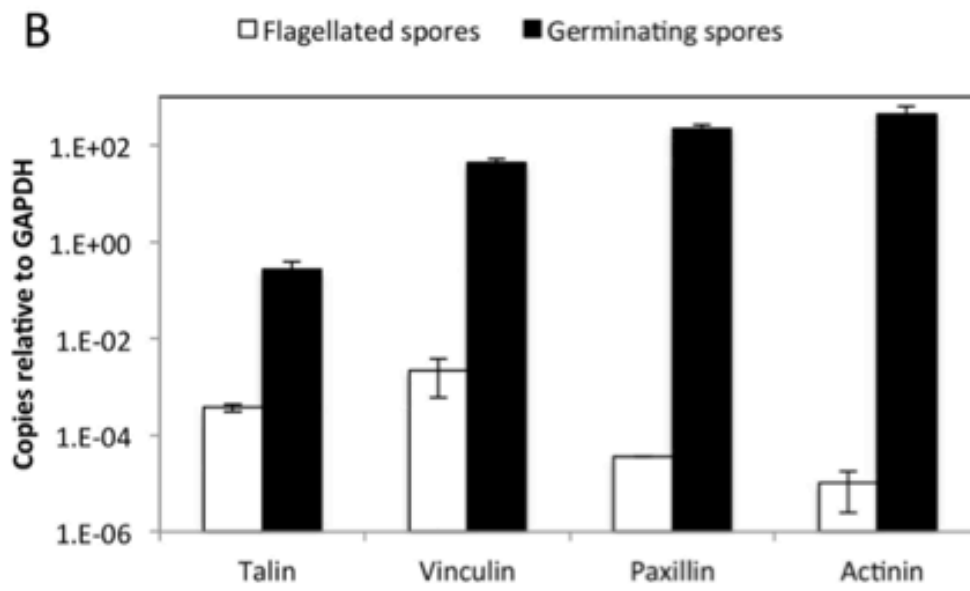
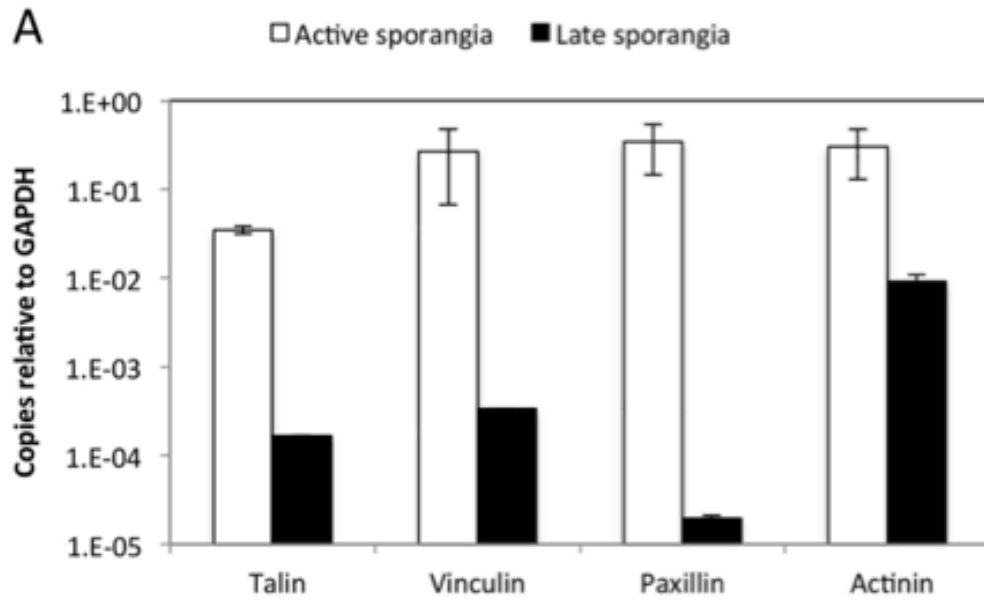


Figure 3-9. Transcriptional levels of genes encoding scaffolding proteins in various life cycle stages of C1A. The number of transcript copies of talin, paxillin, vinculin, and α -actinin relative to the number of transcript copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown for the active sporangia (\square) versus the late sporangia (&) samples (A), as well as for the flagellated spores (\square) versus germinating spores (&) samples (B). Error bars are standard deviations from two experiments (each with 2 replicates) for paxillin, vinculin, and α -actinin, and four experiments (each with two replicates) for talin. Transcriptional levels were significantly higher in active sporangia compared to late sporangia [for talin (773-fold increase, Student t-test P-value = 0.005), vinculin (807-fold increase, Student t-test P-value = 0.03), paxillin (17,463-fold increase, Student t-test P-value = 0.049), and α -actinin (33-fold increase, Student t-test P-value = 0.007)]. Likewise, transcriptional levels were significantly higher in germinating spores compared to flagellated spores [for talin (730-fold increase, Student T t-test P-value = 0.002), vinculin (19,589-fold increase, Student t-test P-value = 0.022), paxillin (6,300,742-fold increase, Student T-test p-value = 0.018), and alpha-actinin (43,513,108-fold increase, Student t-test P-value = 0.048)]. Comparing active sporangia to germinating spores, transcriptional levels were also significantly higher in germinating spores [for talin (7.8-fold increase, Student t-test P-value = 0.005), vinculin (160-fold increase, Student t-test P-value = 0.027), paxillin (647-fold increase, Student t-test P-value = 0.048), and alpha-actinin (1,494-fold increase, Student t-test P-value = 0.007)].



Discussion

In this study, I investigated the transcriptional levels and the possible cellular functions of α -actinin, paxillin, vinculin, and talin in the anaerobic fungus strain C1A in the absence of homologues for extracellular matrix anchors (e.g. integrin). I show that α -actinin, talin, vinculin, and paxillin are actively transcribed, and their proteins are predicted to be structurally similar to their functional metazoan counterparts. Analysis of their transcriptional patterns at different stages of fungal development further demonstrated that the scaffolding proteins transcripts were detectable in mRNA from swimming zoospores, that their transcriptional levels were higher during active zoosporogenesis and highest in germinating spores.

Based on these results, I speculate on the putative functions of scaffolding FA proteins in the anaerobic gut fungi. As explained above, the absence of integrin and the presence of scaffolding proteins in basal fungal lineages (Figure 3-1) is notable, since integrin represents the anchor for stable contact between the extracellular matrix and the cell's cytoskeleton. Since the scaffolding proteins are interacting with integrin (directly or indirectly) in metazoan FA systems, it is unclear what the cellular functions of the scaffolding proteins would be in basal fungi. It is possible that other proteins (non-homologous to integrin) could replace the cellular functions of the missing ECM anchor. This has been shown before in the non-metazoan *Dictyostelium discoideum* whose genome lacks clear homologues of integrin. However, cell substratum adhesion during early development was found to occur via the membrane proteins SibA and SadA [42], both of which were shown to interact with the FA scaffolding protein talin [43]. However, when C1A was grown in the presence of an extracellular polysaccharide (the

insoluble microcrystalline cellulose), which theoretically would provide the ECM trigger needed for FA induction and attachment, genes for scaffolding proteins were not differentially up-regulated (Figure 3-8).

Therefore, I reason that scaffolding proteins transcription in C1A reflects their involvement in non-adhesion associated functions. One possible function could be postulated based on the observed structural similarities between the ciliates basal bodies and the fungal spores flagella. In ciliated eukaryotes, the scaffolding proteins paxillin and vinculin were shown to localize to ciliary adhesion complexes during ciliogenesis, linking the basal bodies to the actin cytoskeleton [16]. This cellular function of scaffolding proteins did not require the presence of integrin. Interestingly, all basal fungi with sequenced genomes with clear homologues for FA scaffolding proteins have a flagellated zoospore stage. Flagella and cilia are similar in that they are both attached to a basal body [44]. It is therefore plausible that the scaffolding proteins in basal fungi could have a function in basal body formation during zoosporogenesis, and that they interact with actin similarly to what was shown in ciliated cells during ciliogenesis. Indeed, my transcriptional study showed that the genes encoding all 4 scaffolding proteins in C1A were differentially up-regulated during active zoosporogenesis compared to late sporangia. The function of scaffolding proteins during ciliogenesis [16] is similar to their function in FA [1, 7]; being interaction with the actin cytoskeleton. Based on the results shown here, I hypothesize that one of the cellular functions of scaffolding proteins in C1A could potentially be interaction with the microfilaments during flagellar assembly on the basal body.

Previous research in the chytrid fungi *Blastocladiella emersonii* and *Batrachochytrium dendrobatidis* argue that zoospores stored transcripts for proteins that are possibly required upon encystment and germination [22, 23, 45]. For example, zoospores of *Batrachochytrium dendrobatidis* contained transcripts of several chitin synthases and chitin binding genes that would be required during encystment for cell wall synthesis [23]. I showed here that C1A zoospores carry scaffolding protein transcripts during swimming, which would suggest a cellular function for scaffolding proteins during and/or post encystment and germination. One possible function is the involvement of FA scaffolding proteins in C1A hyphal tip growth. Currently, there is much debate on the driving force behind hyphal tip growth, with studies suggesting that turgor pressure might not be the only driver for hyphal expansion [46, 47]. Mechanistic similarity between hyphal tip growth and animal cell amoeboid movement via focal adhesion to solid surfaces has been suggested [47, 48]. Microfilaments were shown to be essential for hyphal tip growth [47], where they were found to be axially oriented in the apical regions of fungal hyphae [17]. Scaffolding proteins might be functional in the hyphal tip for linking the microfilaments to the plasma membrane. Evidence to support this speculation comes from research on the chytrid fungus *Allomyces arbuscula*, where a focal adhesion-specific protease (similar to calpains in animal cells) was co-localized with microfilaments to the hyphal apical tip [49]. The preferred substrates for calpains in animal cells are focal adhesion scaffolding proteins [50]. The presence of such calpain in the fungal apical tip suggests that its substrates, the scaffolding proteins, might also be co-localized to perform a specific function during hyphal tip elongation. Here, I provide further evidence that one of the major cellular functions for the scaffolding proteins in

basal filamentous fungi could potentially be post-germination during hyphal tip growth, where they would act as the point of attachment of the microfilament and the cell membrane providing a driving force for the hyphal tip growth.

Results shown here are essential from an evolutionary standpoint. As shown before [7, 16], homologues for all focal adhesion components were identified in the genomes of representatives of Apusozoa, the Opisthokonta sister group, suggesting an ancient origin of focal adhesion that seems to predate the divergence of Opisthokonta. Components of the FA machinery were independently lost during evolution [7], but the presence of scaffolding proteins homologs among all unikonts (Opisthokonts and Amoebozoa) examined (Figure 3-1) [7], prompted [16] to suggest that the scaffolding proteins might have evolved first to perform a non-adhesion-related function (e.g. flagella- or cilia-related, or hyphal tip growth in filamentous fungi) and then were later co-opted for FA. Regardless of whether scaffolding proteins assumed a non-adhesion related function following an integrin loss event [7], or were originally performing a non-adhesion-related function then were coopted for FA following an integrin gain event [16], the current study suggests that scaffolding proteins could have more diverse functionalities than originally understood.

Conflict of Interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER IV

DEVELOPMENT OF AN RNA INTERFERENCE (RNAI) GENE KNOCKDOWN PROTOCOL IN THE ANAEROBIC GUT FUNGUS *PECORAMYCES* *RUMINANTIUM* STRAIN C1A

Abstract

Members of the anaerobic gut fungi (AGF) reside in rumen, hindgut, and feces of ruminant and non-ruminant herbivorous mammals and reptilian herbivores. No protocols for gene insertion, deletion, silencing, or mutation are currently available for the AGF, rendering gene-targeted molecular biological manipulations unfeasible. Here, I developed and optimized an RNA interference (RNAi)-based protocol for targeted gene silencing in the anaerobic gut fungus *Pecoramyces ruminantium* strain C1A. Analysis of the C1A genome identified genes encoding enzymes required for RNA silencing in fungi (Dicer, Argonaute, *Neurospora crassa* QDE-3 homolog DNA helicase, Argonaute-interacting protein, and *Neurospora crassa* QIP homolog exonuclease); and the competency of C1A germinating spores for RNA uptake was confirmed using fluorescently labeled small interfering RNAs (siRNA). Addition of chemically-synthesized siRNAs targeting D-lactate dehydrogenase (*ldhD*) gene to C1A germinating spores resulted in marked

target gene silencing; as evident by significantly lower *ldhD* transcriptional levels, a marked reduction in the D-LDH specific enzymatic activity in intracellular protein extracts, and a reduction in D-lactate levels accumulating in the culture supernatant. Comparative transcriptomic analysis of untreated versus siRNA-treated cultures identified a few off-target siRNA-mediated gene silencing effects. As well, significant differential up-regulation of the gene encoding NAD-dependent 2-hydroxyacid dehydrogenase (Pfam00389) in siRNA-treated C1A cultures was observed, which could possibly compensate for loss of D-LDH as an electron sink mechanism in C1A. The results demonstrate the feasibility of RNAi in anaerobic fungi, and opens the door for gene silencing-based studies in this fungal clade.

Introduction

The role played by non-coding RNA (ncRNA) molecules in epigenetic modulation of gene expression at the transcriptional and post-transcriptional levels is now well recognized (1). Small interfering RNAs (siRNA) are short (20-24 nt) double stranded RNA molecules that mediate post-transcriptional regulation of gene expression and gene silencing by binding to mRNA in a sequence-specific manner (2). The process of RNA interference (RNAi) has been independently documented in fungi (3-5), animals and human cell lines (6, 7), as well as plants (8). The fungal RNAi machinery has been investigated in several model fungi, e.g. *Neurospora crassa* (5), *Mucor circinelloides* (9), and *Magnaporthe oryzae* (10), and encompasses: 1. Dicer (Dic) enzyme(s): RNaseIII dsRNA-specific ribonucleases that cleave double stranded RNA (dsRNA) to short (20-25 bp) double stranded siRNA entities, 2. Argonaute (Ago) protein(s), the core component of the RNA-induced silencing complex (RISC) which binds to the dicer-generated siRNAs and other proteins and cleaves the target mRNA, 3. RNA-dependent RNA polymerase (RdRP) enzyme (present in the majority, but not all fungi) that aids in amplifying the silencing signal through the production of secondary double stranded siRNA molecules from single stranded mRNAs generated by the RISC complex, 4. DNA helicase, *Neurospora crassa* QDE-3 homolog (11), that aids in the production of the aberrant RNA to be targeted by RdRP, and 5. Argonaute-interacting protein, *Neurospora crassa* QIP homolog (12), an exonuclease that cleaves and removes the passenger strand from the siRNA duplex.

The phenomenon of RNA interference could induce gene silencing due to the action of endogenously produced microRNA (miRNA), or could be triggered due to the

introduction of foreign siRNA (e.g. due to viral infection or genetic manipulation). Under normal physiological conditions, RNAi is thought to play a role in endogenous regulation of gene expression (13), development of resistance to viruses (14-17), and silencing the expression of transposons (18, 19). On the other hand, the introduction of foreign siRNA could be utilized for targeted, sequence-specific, gene knockdown in fungi (2, 3, 5). Indeed, demonstration of the feasibility of RNAi approaches for targeted gene silencing has been shown in Ascomycota (5, 20-30), Basidiomycota (31-35), and Mucoromycota (36, 37); and RNAi-based protocols were used to infer the putative roles of several genes or simply as a proof of principle.

The anaerobic gut fungi (AGF) represent a basal fungal phylum (Neocallimastigomycota) that resides in the herbivorous gut and plays an important role in enhancing plant biomass metabolism by the host animals (38). The AGF have multiple potential biotechnological applications such as a source of lignocellulolytic enzymes (39-45), direct utilization of AGF strains for sugar extraction from plant biomass in enzyme-free biofuel production schemes (46), additives to biogas production reactors (47, 48), and feed additives for livestock (49-55). However, the strict anaerobic nature of AGF renders genetic manipulation procedures involving plating and colony selection extremely cumbersome. Consequently, there are currently no protocols for transformation, gene insertion, gene deletion, or sequence-specific homologous recombination-based genetic manipulation in AGF, hindering in-depth investigation of their biotechnological potential.

I here report on the development of an RNAi-based protocol for targeted gene knockdown in the anaerobic gut fungal isolate *Pecoramyces ruminantium* strain C1A.

The protocol does not involve transformation, and does not require homologous recombination, or colony selection. I demonstrate the uptake of chemically synthesized short double stranded siRNA by germinating spores of *P. ruminantium* strain C1A, and subsequently demonstrate the feasibility of using this approach for silencing D-lactate dehydrogenase (*ldhD*) gene. I finally examine the off-target effects of *ldhD* gene knockdown, as well as the impact of inhibiting D-lactate production on the glycolytic and fermentation pathways in C1A.

Materials and Methods

Microorganism and culture maintenance. *Pecoramyces ruminantium* strain C1A was isolated previously in our laboratory (56) and maintained by biweekly transfers into an antibiotic-supplemented rumen-fluid-cellobiose medium (RFC) as described previously (57).

Identification and phylogeny of RNAi complex in anaerobic fungi. The occurrence of genes encoding Dic, Ago, RdRP, QIP, and QDE3 proteins was examined in the genome of *P. ruminantium* C1A (58) (Genbank accession number ASRE00000000.1), as well as in three additional publicly available Neocallimastigomycota genomes (59) (Genbank accession numbers: MCOG00000000.1, MCFG00000000.1, MCFH00000000.1). The phylogeny of the translated amino acid sequences of identified homologues was compared to fungal and eukaryotic homologues in MEGA7. Representative sequences were aligned using ClustalW and the aligned sequences were manually refined and used to construct Neighbor Joining trees in Mega7 (60) with bootstrap values calculated based on 100 replicates.

RNAi experimental design.

Choice of delivery procedure. Delivery of the inhibitory RNA molecules to fungal cultures is commonly achieved using appropriate vectors that either express short hairpin RNA (61-63), or individual sense and antisense RNA strands that will subsequently be annealed into dsRNA (64, 65). The process involves transformation (PEG-CaCl₂-mediated into protoplasts, Li acetate-mediated, *Agrobacterium*-mediated, or via

electroporation) and necessitates transformants' selection on marker (usually hygromycin) plates. Alternatively, direct delivery of exogenous, chemically synthesized short double stranded RNA (siRNA) has also been utilized for targeted gene silencing in fungi (22-24, 28, 66). This approach exploits the machinery for nucleic acids uptake, and the natural competence of the germinating spore stage observed in the filamentous fungus *Aspergillus* (23). Due to the strict anaerobic nature of AGF which would hinder the process of transformation and selection on plates, we opted for direct addition of chemically synthesized siRNA to C1A germinating spores, in spite of its reported lower efficacy (24).

dsRNA synthesis. I targeted D-lactate dehydrogenase (*ldhD*) gene encoding D-LDH enzyme (EC 1.1.1.28). D-LDH is an NAD-dependent oxidoreductase that reduces pyruvate to D-lactate, a major fermentation end product in C1A (46). Only a single copy of *ldhD* (996 bp in length) was identified in C1A genome (IMG accession number: 2511055262). A 21-mer siRNA targeting positions 279-298 in the *ldhD* gene transcript (henceforth *ldhD*-siRNA) was designed using Dharmacon® siDesign center (<http://dharmacon.gelifesciences.com/design-center/>) with the sense strand being 5'-CGUUAGAGUUCCAGCCUAUUU-3', and the antisense strand being 5'-AUAGGCUGGAACUCUAACGUU-3'. Included within the designed siRNAs were 3' overhanging UU dinucleotides to increase the efficiency of target RNA degradation as suggested before (67). The siRNA was ordered from Dharmacon® (LaFayette, CO) as 21-mer duplex (double stranded) with a central 19-bp duplex region and symmetric UU dinucleotide 3' overhangs on each end. The 5' end of the antisense strand was modified

with a phosphate group required for siRNA activity (68), while the 5' end of the sense strand was modified with a Cy-3 fluorescent dye to facilitate visualization of the siRNA uptake by C1A germinating spores. In addition, a 21-mer duplex that should not anneal to any of C1A's mRNA transcripts (henceforth unrelated-siRNA) was also designed and used as a negative control with the sense strand being 5'-UCGUUGGCGUGAGCUUCCAUU-3', and the antisense strand being 5'-UGGAAGCUCACGCCAACGAUU-3'. The unrelated-siRNA was modified in the same way as the *ldhD* siRNA.

RNAi protocol. The basic protocol employed is shown in Figure 4-1. Strain C1A was grown on RFC-agar medium in serum bottles at 39⁰C in the dark as described previously (57) until visible surface colonies are observed (usually 4-7 days). Surface growth was then flooded by adding 10 ml sterile anoxic water followed by incubation at 39⁰C (57). During this incubation period, spores are released from surface sporangia into the anoxic water. Previous work has shown that the duration of incubation with the flooding solution has a major impact on the spore developmental stage, where exclusively active flagellated spores were observed in incubations shorter than 30 minutes, while 90-100-minute incubation exclusively produced germinating spores. The onset of spore germination was observed at 75-80 minutes during incubation with the flooding solution (57). Germinating spores were previously shown to be most amenable for accumulating the highest amount of exogenously added nucleic acids (23). I, therefore, reasoned that addition of chemically synthesized siRNA to the sterile anoxic flooding water at the onset of spore germination (at around 75 minutes from the onset of flooding) followed by re-

incubation at 39°C for 15 additional minutes (for a total of 90-minute incubation period) would allow for uptake of the siRNA by the germinating spores. Chemically synthesized siRNA was added from a stock solution constituted in a sterile anoxic RNase-free siRNA buffer (60 mM KCl, 6 mM HEPES-pH 7.5, and 0.2 mM MgCl₂) to the desired final concentration. Initial experiments were conducted using Cy3-labeled *ldhD*-siRNA molecules to test the uptake of siRNA by the germinating spores. Subsequent experiments were conducted using unlabeled siRNA. Following siRNA addition and incubation, spores were gently recovered from the serum bottle using a 16G needle and used to inoculate fresh RFC media bottles (57), and the impact of silencing *ldhD* gene on gene expression, enzyme activities, and D-lactate concentrations was assessed in these cultures. Controls included treatments with unrelated-siRNA, as well as cultures with no siRNA addition.

Impact of *ldhD* gene knockdown on transcriptional levels, D- LDH enzyme activity, and D-lactate production in strain C1A. The supernatant of both siRNA-treated and control C1A cultures was periodically sampled (0.5 ml) and used for D-lactate quantification. The fungal biomass was vacuum filtered on 0.45 µm filters, and immediately crushed in a bath of liquid nitrogen using a mortar and pestle as described previously (69). The crushed cells were then poured into 2 separate 15-mL plastic falcon tubes, and stored at -80°C for subsequent RNA, and protein extraction, respectively.

D-Lactate quantification. D-lactate was determined in the culture supernatant using the D-Lactate Assay Kit (BioAssay Systems, Hayward, CA) following the manufacturer's instructions.

RNA extraction, qRT-PCR, and RNA-seq. RNA was extracted following the protocol in Epicentre® MasterPure™ Yeast RNA Purification Kit, with few modifications as detailed previously (69). RNA concentrations were measured using the Qubit® RNA HS Assay Kit (Life Technologies®). Total RNA was utilized for both transcriptional studies using qRT-PCR, as well as for transcriptomic analysis using RNA-seq.

For transcriptional studies, replicate samples were chosen to cover a range of fungal biomass ranging from 6-22 mgs corresponding to various growth stages. Reverse transcription (cDNA synthesis) was performed using the Superscript IV First-Strand Synthesis System kit for RT-PCR (Life Technologies®), following the manufacturer's protocols. Quantitative reverse transcription PCR (qRT-PCR) was conducted on a MyIQ thermocycler (Bio-Rad Laboratories, Hercules, CA). *ldhD*, as well as the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), were amplified using primers designed by the OligoPerfect™ Designer tool (Life Technologies, Carlsbad, CA) (*ldhD*-forward primer: AGACCATGGGTGTCATTGGT, *ldhD*-reverse primer TTCATCGGTTAATGGGCAGT; *GAPDH*-forward primer: ATTCCACTCACGGACGTTTC, *GAPDH*-reverse primer: CTTCTTGGCACCACCCTTTA). The reactions contained 1µl of C1A cDNA, and 0.5 µM each of the forward and reverse primers. Reactions were heated at 50°C for 2 min, followed by heating at 95°C for 8.5 min. This was followed by 50 cycles, with one cycle consisting of 15 s at 95°C, 60 s at 50°C, and 30 s at 72°C. Using the ΔC_t method, the number of copies of *ldhD* is reported relative to the number of copies of *GAPDH* used as the normalizing control.

Transcriptomic analysis was used both to evaluate off-target effects of the chemically synthesized *ldhD* siRNA (transcripts that will be down-regulated in siRNA-treated versus untreated cultures), and to examine the effect of *ldhD* knockdown on other NADH-oxidizing mechanisms to compensate for loss of D-LDH as an electron sink in C1A (transcripts that will be up-regulated in siRNA-treated versus untreated cultures). For transcriptomic analysis, RNA from untreated (2 biological replicates) as well as siRNA-treated (2 biological replicates) cultures was sequenced using Illumina-HiSeq. RNA sequencing as well as sequence processing were as described previously (70). Briefly, de novo assembly of the generated RNA-Seq reads was accomplished using Trinity (71), and quantitative levels of assembled transcripts were obtained using Bowtie2 (72). Quantitative values in Fragments Per Kilobase of transcripts per Million mapped reads (FPKM) were calculated in RSEM. edgeR (73) was used to determine the transcripts that were significantly up- or down-regulated based on the Benjamini-Hochberg adjusted p-value (False discovery rate, FDR). I used a threshold of 10% FDR as the cutoff for determining significantly differentially expressed transcripts.

Total protein extraction and D-Lactate dehydrogenase enzyme assay. For total protein extraction, replicate samples were chosen to cover a range of fungal biomass ranging from 6-22 mgs corresponding to various growth stages. C1A cells crushed in liquid nitrogen were suspended in 0.5mL of Tris-Gly buffer (3g Tris base, 14.4g Glycine, H₂O up to 1L, pH 8.3), and mixed briefly. Cell debris were pelleted by centrifugation (12,500x g for 2 min at 4°C) and the sample supernatant containing the total protein extract was carefully transferred into a sterile microfuge tube. Protein concentrations were quantified

in cellular extracts using QubitTM Protein assay kit (Life Technologies). D-LDH enzyme activity was quantified in the cell extracts using the AmpliteTM Colorimetric D-Lactate Dehydrogenase Assay Kit (ATT Bioquest[®], Sunnyvale, CA), following the manufacturer's protocols.

Nucleotide Accession. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFSU00000000. The version described in this paper is the first version, GFSU01000000.

Results

RNAi machinery in the Neocallimastigomycota. The four examined Neocallimastigomycota genomes harbored most of the genes constituting the backbone of the RNAi machinery: ribonuclease III dicer, argonaute, QDE3-homolog DNA helicase, and QIP-homolog exonuclease. Phylogenetically, these genes were closely related to representatives from basal fungal lineages (Figure 4-2). Gene copies in various genomes ranged between 1 to 4 (Figure 4-2). However, it is notable that all four examined genomes lacked a clear homolog of RNA-dependent RNA polymerase (RdRP) gene. RdRP has been identified in the genomes of diverse organisms including *Caenorhabditis elegans* (74), plants, and the majority of examined fungi (75) but is absent in the genomes of vertebrates and flies; in spite of their possession of a robust RNAi machinery that mediates sequence-specific gene silencing in response to exogenously added dsRNAs.

Uptake of synthetic siRNA by C1A germinating spores and effect on growth. The addition of fluorescently labeled siRNA targeting *ldhD* transcript to C1A spores at the onset of germination followed by a 15-minute incubation at 39°C resulted in the uptake of the siRNA by the germinating spores as evident by their fluorescence (Figure 4-3). Under the examined conditions, the majority of the germinating spores picked up the siRNA since 80-90% of spores stained with the nuclear stain DAPI also exhibited Cy3-fluorescence. *ldhD*-siRNA-treated spores were collected and used to inoculate fresh RFC liquid media, and the growth rate of these cultures were compared to siRNA-untreated controls. As shown in Figure 4-3, *ldhD*-siRNA treatment had no significant effect on either the rate of fungal growth or the final fungal biomass yield.

3.3 Knockdown of *ldhD*-gene by exogenously added *ldhD*-siRNA.

Inhibition at the mRNA level. Table 4-1 shows the effect of adding exogenous *ldhD*-siRNA on *ldhD* transcriptional level relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Results from qRT-PCR revealed that there was an observable decrease in *ldhD* transcription levels in samples treated with *ldhD*-specific siRNA compared to siRNA-untreated samples or unrelated siRNA-treated samples. The inhibitory effect increased with the concentration of *ldhD*-specific siRNA added. At 100 nM, a four-fold decrease in transcription was observed.

Inhibition at the protein level. Similar to the effect of treatment on the mRNA level, *ldhD*-siRNA-treated samples exhibited a marked decrease in the specific D-LDH activity (Table 4-2). This decrease was dependent on the concentration of siRNA added and ranged from 70-93% reduction compared to siRNA-untreated samples.

*Effect of *ldhD* gene knockdown on the extracellular levels of D-lactate in culture supernatants.* D-lactate production in C1A culture supernatant is non-linear, with higher amounts of D-lactate produced at later stages of growth (Figure 4-4A). D-lactate production in *ldhD*-siRNA-treated cultures was invariably lower when compared to controls, with the difference especially pronounced at later stages of growth. The level of reduction was dependent on the siRNA concentration added and ranged from 42-86% in the early log phase, 49-67% in the mid log phase, and 57-86% in the late log-early stationary growth phase (Figure 4-4B).

3.4 Transcriptomic analysis. Differential gene expression patterns between *ldhD*-siRNA-treated and siRNA-untreated samples were analyzed to identify possible off-target effects of siRNA treatment, i.e. transcripts that were significantly down-regulated

in the siRNA-treated cultures. Only 29 transcripts were significantly (FDR < 0.1) down-regulated (Figure 4-5). Predicted functions of these transcripts are shown in Table 4-3 and included hypothetical proteins (n=11), several glycosyl hydrolases (n=5), and other non-fermentation related functions. Comparison of the siRNA sequence to these 29 transcripts revealed matches to the first 7 bases of the *ldhD*-siRNA sequence to only 3 of the down-regulated transcripts indicating that the off-target effect was mainly not sequence-specific.

In an attempt to decipher the impact of inhibiting the D-lactate dehydrogenase enzyme (one of the major electron sinks in C1A) on the glycolytic and fermentation pathways in C1A, I investigated the significantly up-regulated transcripts in the siRNA-treated cultures. A total of 53 transcripts were significantly upregulated in the siRNA-treated cultures (FDR < 0.1) (Figure 4-5). Predicted functions of these transcripts are shown in Table 4-3. One transcript encoding NAD-dependent 2-hydroxyacid dehydrogenase (Pfam 00389) was significantly upregulated (1542-fold) in the siRNA-treated cultures (P-value = 0.02). Enzymes belonging to this family act specifically on the D-isomer of their substrates (76). In case of D-LDH inhibition in the siRNA-treated cultures, the Pfam 00389 enzyme might act to compensate for the loss of NADH oxidation by acting on an alternate substrate (e.g. hydroxypyruvate, 2-oxoisocaproate, or other 2-oxo carboxylic acids) and reducing it as a sink of electrons to regenerate NAD. However, it is difficult to know the actual substrate based on sequence data alone. Transcripts of other glycolytic and fermentative enzymes of C1A were not differentially expressed in siRNA-treated cultures (Table 4-3).

Figure 4-1. A cartoon depicting the RNAi gene knockdown protocol used in this study.

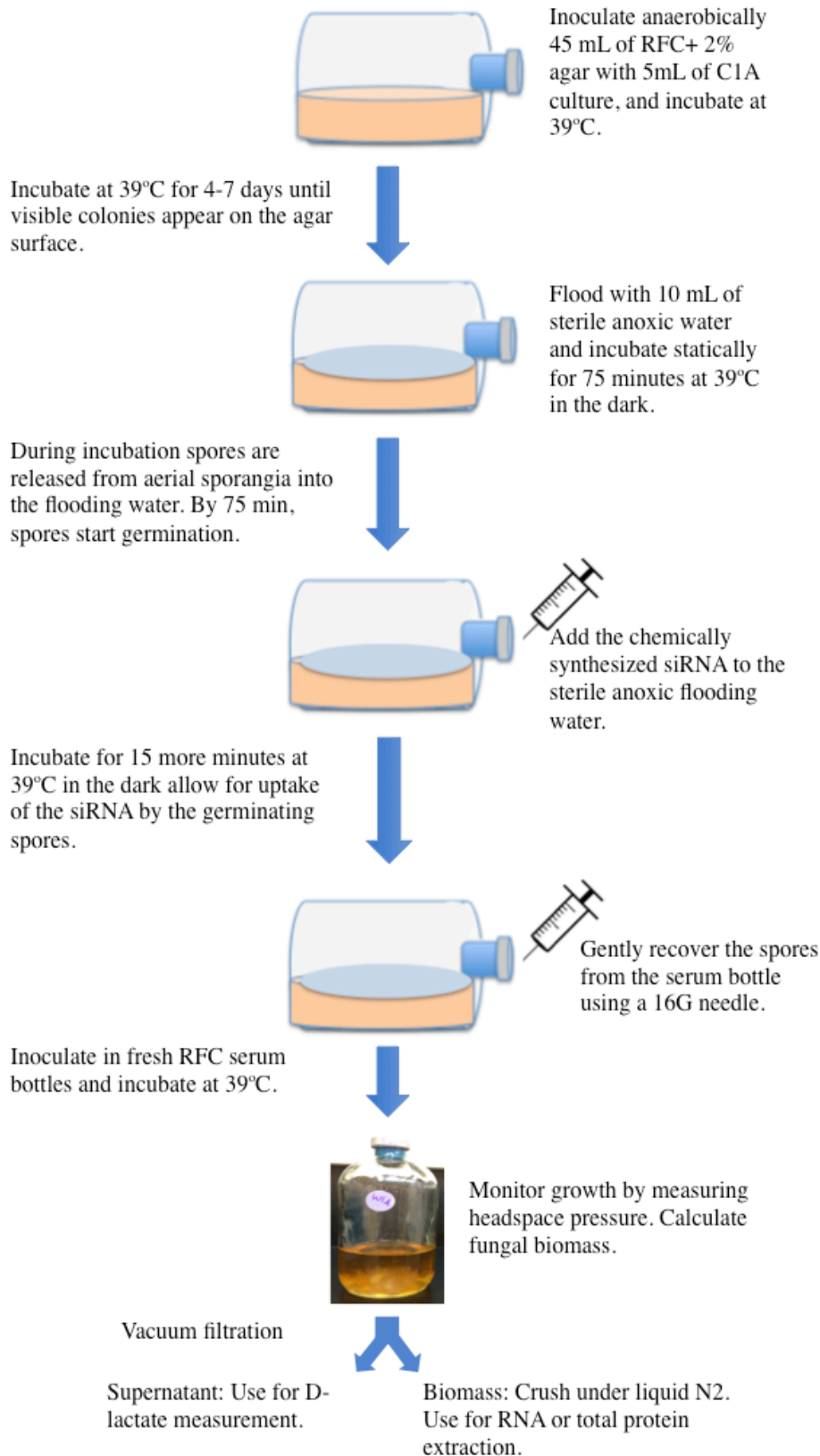
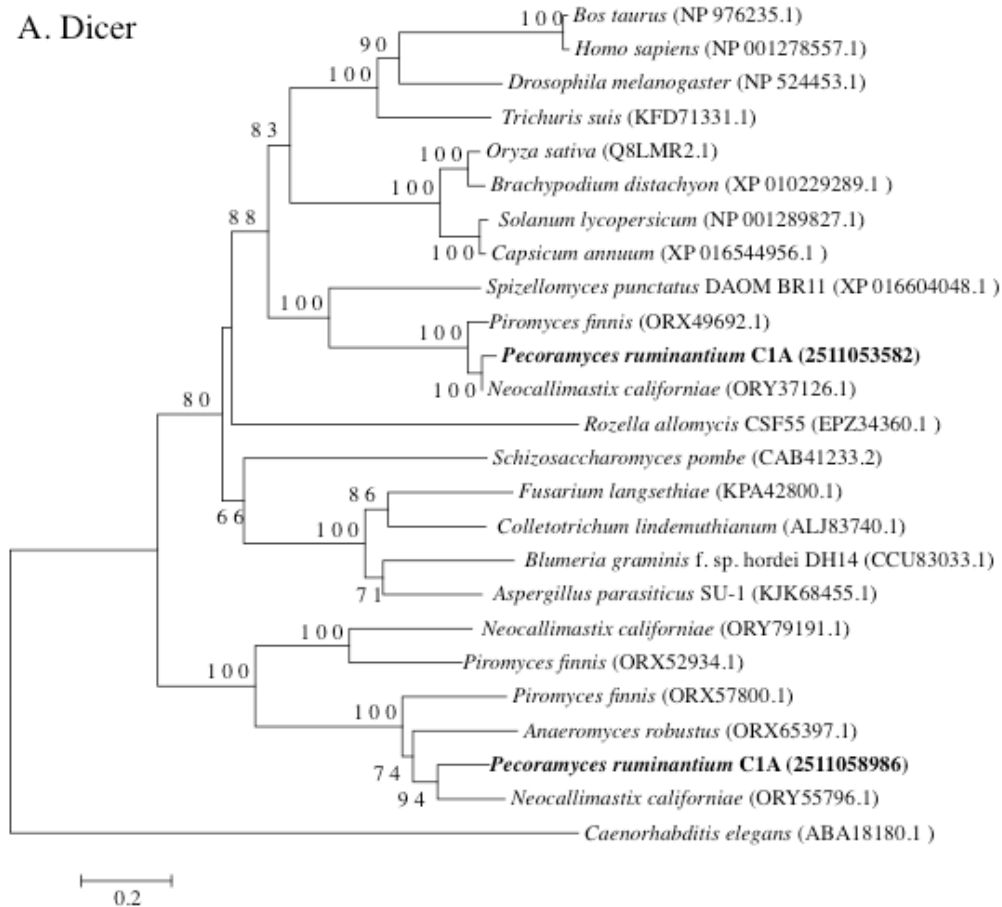
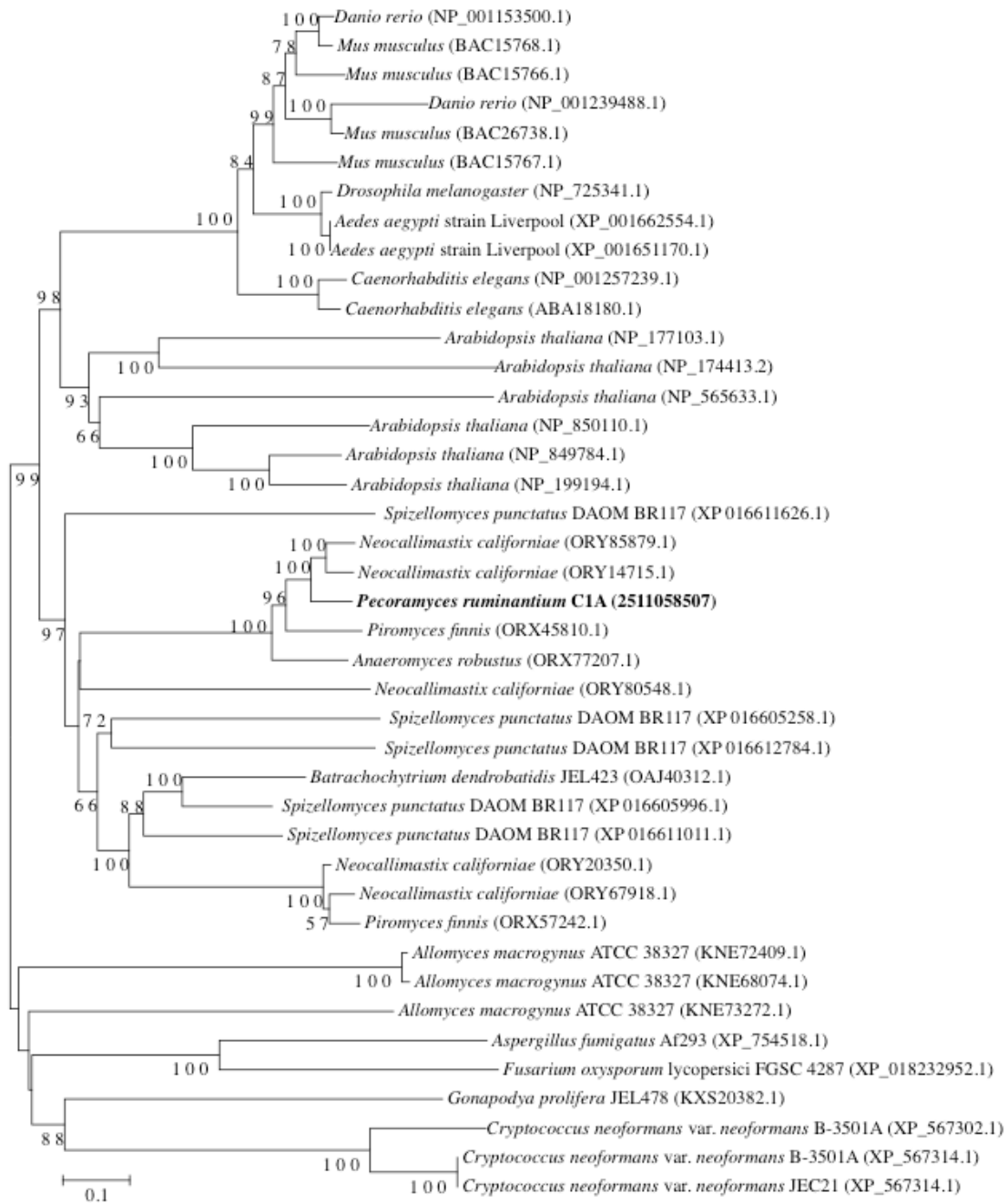


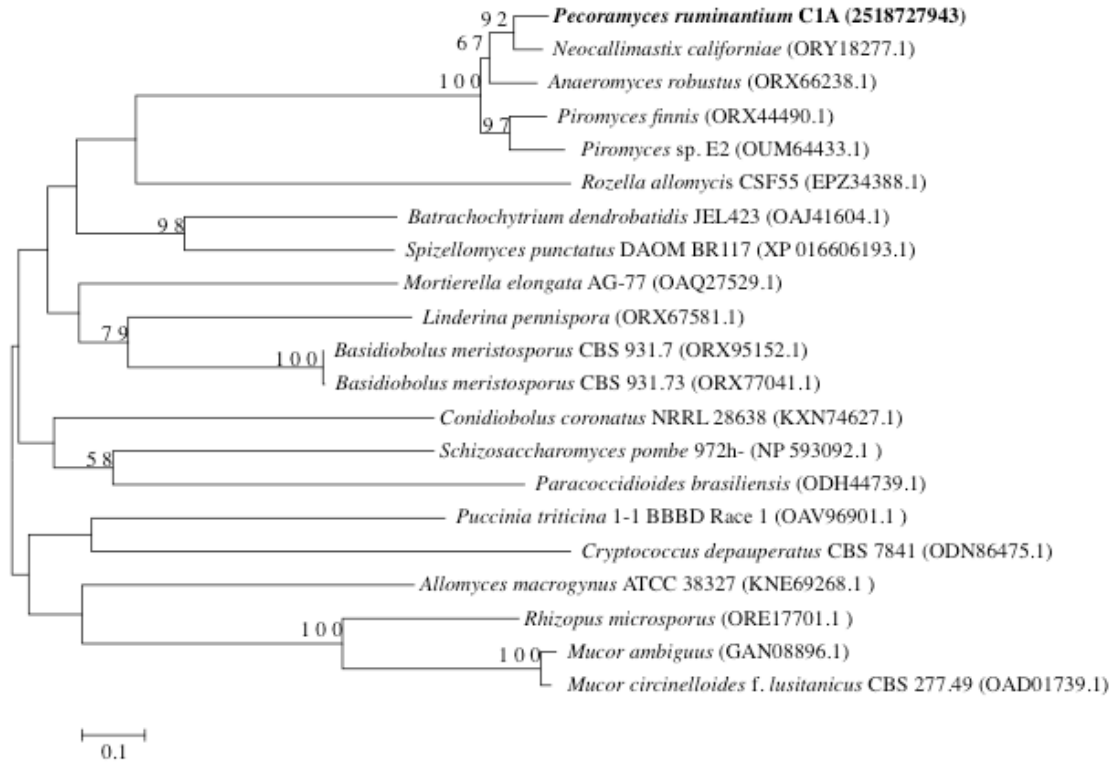
Figure 4-2. Neighbor joining phylogenetic tree depicting the phylogenetic relationship between *Pecoramyces ruminantium* strain C1A predicted Dicer (A), Argonaute (B), QDE-3 helicase (C), and QIP exonuclease (D) sequences and those from other fungal and eukaryotic species. Trees were constructed in Mega7 with bootstrap support based on 100 replicates. Bootstrap values are shown for branches with >50 bootstrap support.



B. Argonaute



C. QDE-3 homolog



D. QIP homolog

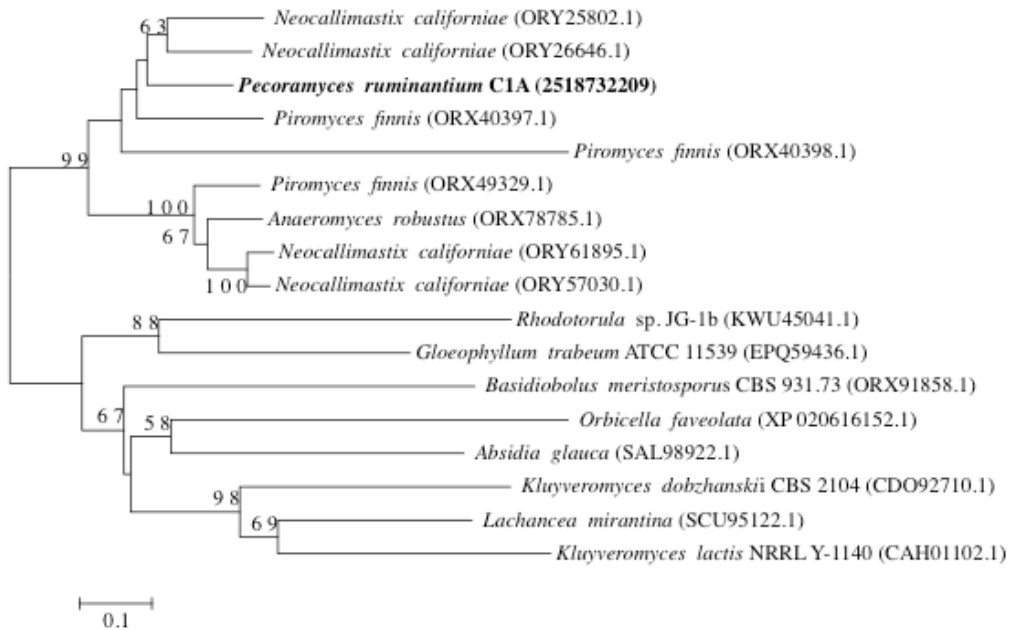


Figure 4-3. Uptake of fluorescently (Cy3) tagged siRNA by C1A spores. (A) The *ldhD*-specific siRNA was added to the flooding solution 75 minutes after the onset of flooding followed by incubation for 15 more minutes at 39°C. Samples (a few microliters) were taken at regular intervals for visualization. The same field is shown for DAPI-, and Cy3-labeled germinating spores (Note that the spores were concurrently stained with DAPI and fluorescing green indicating the uptake of the Cy3-labeled siRNA) (bar=20 µm). (B) Effect of the siRNA treatment on fungal growth rate. siRNA-treated spores were collected and used to inoculate fresh RFC medium. Control cultures were started at the same time using siRNA-untreated spores. Headspace pressure was measured daily and used to calculate fungal biomass as described previously (1). Error bars are standard deviations from at least three replicate cultures for each condition.

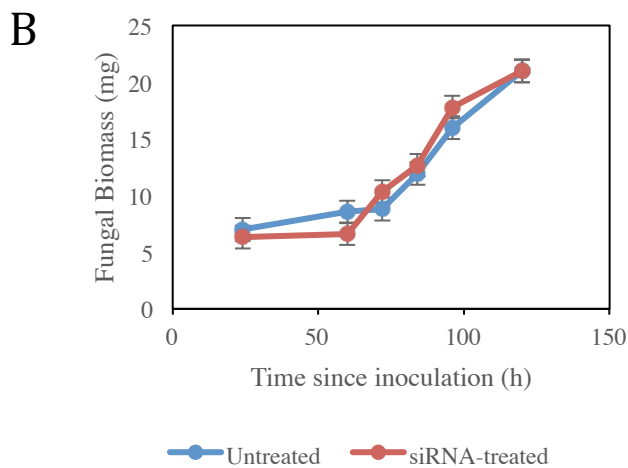
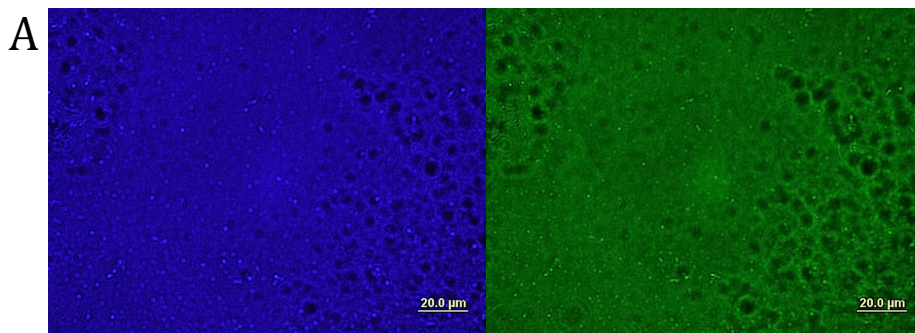


Table 4-1. Effect of the uptake of exogenous *ldhD*-siRNA by C1A germinating spores on the transcriptional level of *ldhD* relative to the housekeeping gene *gapdh*.

Treatment	Final siRNA concentration (nM)	Copies of <i>ldhD</i> relative to <i>gapdh</i> ¹	Fold change in transcription level ($\Delta\Delta C_t$) compared to untreated samples	Number of biological replicates	Fungal biomass yield (mg) at the time of sacrificing ¹
<i>ldhD</i> -siRNA	20	4.2E-03±3E-03	0.02	4	12.3±5
	50	4.4E-03±2E-03	0.02	5	9.3±5.2
	75	3.6E-04±1.8E-04	0.0017	4	15.4±3.7
	100	6.1E-05±2.4E-05	0.0003	4	15.9±6
	150	7.3E-04±3.6E-04	0.003	2	7.2±0.7
Untreated	NA	0.21±0.04		5	9.6±2
unrelated-siRNA	50	0.26±0.07	1.29	2	13.5±3.8

¹. Values are average±standard deviation

Table 4-2. Effect of the uptake of *ldhD*-siRNA by C1A germinating spores on the D-LDH specific activity.

Treatment	siRNA concentration (nM)	D-LDH specific activity (U/ mg protein) ¹	Fold change in D-LDH specific activity compared to untreated samples	Total number of biological replicates	Fungal biomass yield (mg) at the time of sacrificing ¹
<i>ldhD</i> -siRNA	20	332.2±90	0.29	6	16.5±5.8
	50	331.9±144.5	0.29	17	10±4.3
	75	194.2±79	0.17	6	12.8±5.3
	100	180.6±131	0.16	6	12.7±7.4
	150	85.4±32	0.07	2	7.2±0.7
Untreated	NA	1157.6±308.6		13	10.9±2.9
unrelated-siRNA	50	926.4±69	0.8	2	13.5±3.8

¹. Values shown are average±SD.

Figure 4-4. (A) Pattern of D-lactate production in C1A culture supernatant as a factor of fungal biomass. The majority of the D-lactate production occurs at the late log-early stationary phase. Data is shown for both siRNA-untreated cultures (green), as well as *ldhD*-specific siRNA-treated cultures with final concentration 20 nM (dark blue), 50 nM (orange), 75 nM (grey), 100 nM (yellow), and 150 nM (light blue). (B) A bar-chart depicting average \pm standard deviation (from at least two replicates) of D-lactate levels in C1A culture supernatant during early log (6-13 mg biomass), mid-log (14-17 mg biomass), and late log/early stationary (18-23 mg) phases. Data is shown for both siRNA-untreated cultures (green), as well as *ldhD*-specific siRNA-treated cultures with final concentration 20 nM (dark blue), 50 nM (orange), 75 nM (grey), 100 nM (yellow), and 150 nM (light blue).

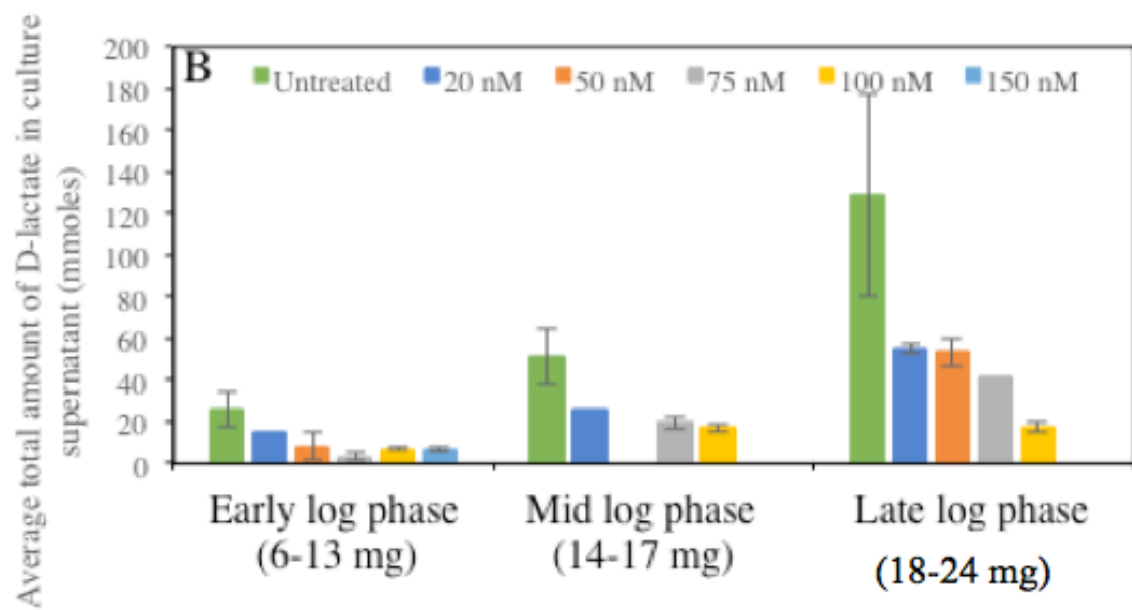
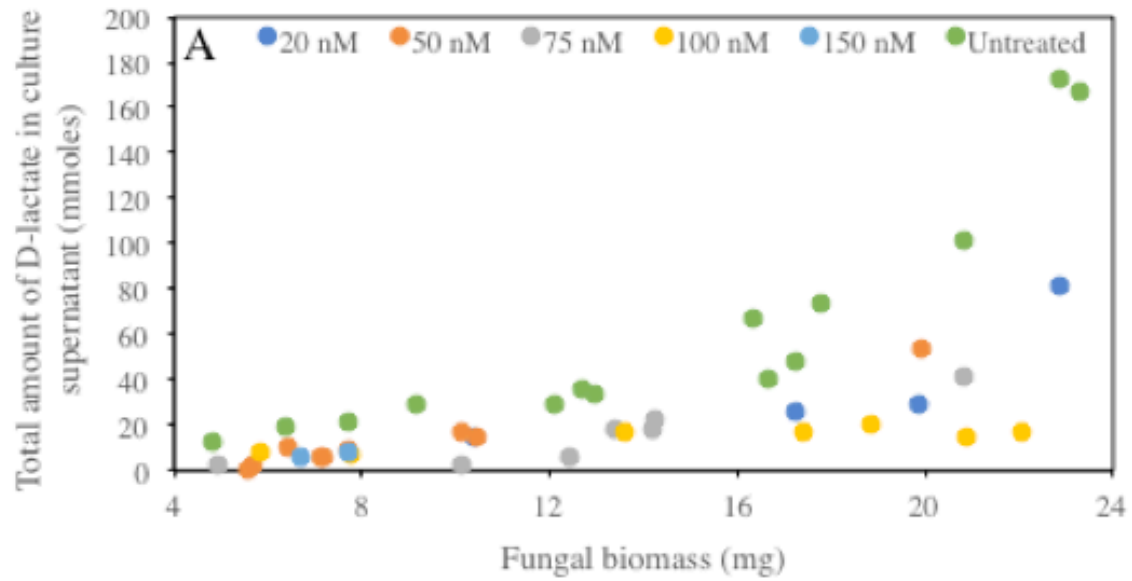


Figure 4-5. Volcano plot of the distribution of gene expression for C1A cultures when treated with *ldhD*-specific siRNA (50 nM) versus untreated cultures. The fold change [\log_2 (average FPKM in siRNA-treated cultures/ average FPKM in control cultures)] is shown on the X-axis, while the significance of the change [$-\log_{10}$ (false discovery rate)] is shown on the Y-axis. Red data points are those transcripts that were significantly down-regulated (n=29), while green data points are those transcripts that were significantly up-regulated (n=53). The corresponding IMG gene accession numbers and the predicted functions for these genes are shown in Table 4-3. The orange data point corresponds to the D-lactate dehydrogenase transcript (targeted in the RNAi experiment) with 2.5-fold decrease in FPKM compared to the untreated control, while the purple data point corresponds to the NAD-dependent 2-hydroxyacid dehydrogenase (Pfam 00389) transcript (possibly acting to compensate for the loss of NADH oxidation that occurred as a result of *ldhD* knockdown) with 1542-fold increase in FPKM compared to the untreated control.

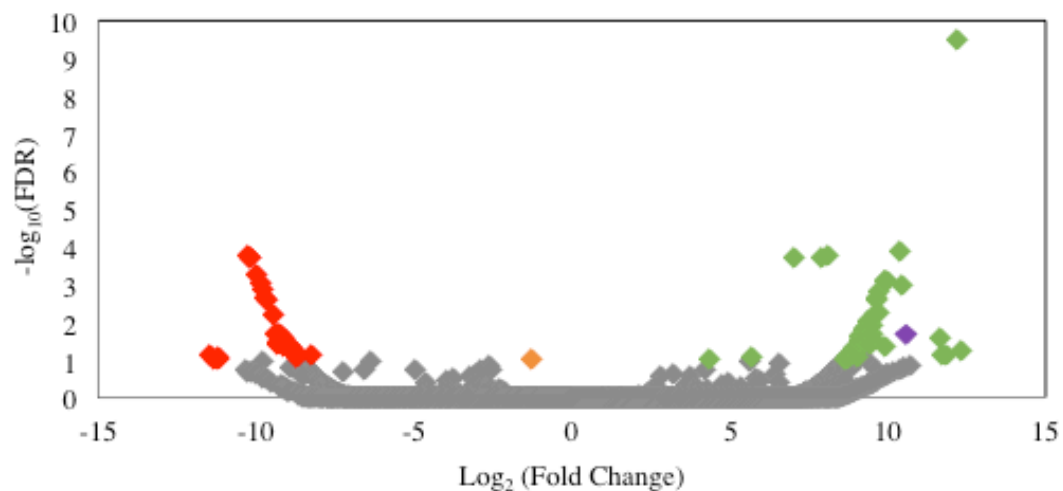


Table 4-3. Transcripts with a significant (False Discovery Rate (FDR) < 0.1) fold change in the *ldhD* siRNA-treated cultures.

Transcript ID	IMG Gene accession number	Predicted function	Log ₂ fold change [TPM in siRNA-treated cultures/TPM in untreated cultures]	-log ₁₀ FDR
Significantly down-regulated transcripts				
TRINITY_DN25404_c0_g1_i1	2511056874	Actin and related proteins; ACTR8, ARP8, INO80N actin-related protein 8	-9.89	3.03
TRINITY_DN27647_c0_g2_i1	2511052130	Ankyrin repeat	-8.89	1.22
TRINITY_DN26914_c3_g1_i10	2511052359	Aspartyl aminopeptidase	-11.31	1.04
TRINITY_DN27318_c0_g1_i15	2511054689	Beta-1,4-xylanase	-10.27	3.79
TRINITY_DN27318_c0_g1_i3	2511054689	Beta-1,4-xylanase	-9.47	2.21
TRINITY_DN26767_c0_g1_i2	2518731831	chloride channel 3/4/5	-8.94	1.37
TRINITY_DN27688_c5_g4_i3	2511053193	DNA methylase	-9.97	3.24
TRINITY_DN26958_c2_g1_i9	2511057393	DnaJ family protein C member 3	-8.83	1.22
TRINITY_DN27149_c6_g1_i13	2511052023	Endoglucanase	-11.50	1.13
TRINITY_DN26597_c3_g6_i4	2511059612	Endoglucanase	-9.65	2.58
TRINITY_DN24006_c1_g2_i3	2511053004	Fumarase	-9.13	1.34
TRINITY_DN27371_c3_g1_i1	2511061203	Glycosyl hydrolase family 9	-9.31	1.47

c7_g1_i12				
TRINITY_DN23827_c0_g1_i1	2511061779	Hypothetical protein	-11.23	1.03
TRINITY_DN22650_c0_g1_i1	2511057661	Hypothetical protein	-11.21	1.08
TRINITY_DN26878_c4_g5_i1	2518722908	Hypothetical protein	-10.19	3.71
TRINITY_DN72241_c0_g1_i1	2511055911	Hypothetical protein	-9.79	2.88
TRINITY_DN25954_c3_g3_i7	2511052693	Hypothetical protein	-9.34	1.39
TRINITY_DN27282_c5_g1_i4	2511054952	Hypothetical protein	-9.32	1.70
TRINITY_DN20666_c0_g1_i2	2511055315	Hypothetical protein	-9.24	1.71
TRINITY_DN24836_c0_g1_i3	2511055352	Hypothetical protein	-9.10	1.57
TRINITY_DN27173_c0_g2_i1	2511052832	Hypothetical protein	-8.95	1.37
TRINITY_DN26709_c2_g3_i8	2511053925	Hypothetical protein	-8.83	1.18
TRINITY_DN22602_c0_g1_i7	2511058339	Hypothetical protein	-8.23	1.10
TRINITY_DN61803_c0_g1_i2	2511052980	Permeases of the drug/metabolite transporter (DMT)	-10.17	3.71
TRINITY_DN25216_c1_g1_i1	2511061481	Serine/threonine protein kinase	-8.74	1.10
TRINITY_DN26990_c2_g2_i2	2511049217	Uncharacterized conserved protein	-8.76	1.08
TRINITY_DN23344_c0_g2_i1	2511048410	V8-like Glu-specific endopeptidase	-9.42	1.67

TRINITY_ DN26795_ c1_g3_i15	251106253 7	Vacuolar sorting protein 9	-9.72	2.64
TRINITY_ DN27051_ c5_g9_i6	251105907 0	WD40-repeat- containing domain	-9.17	1.55
TRINITY_ DN27737_ c3_g9_i1	251105526 2	d-Lactate dehydrogenase	-1.31	1.02
Significantly up-regulated transcripts				
TRINITY_ DN23455_ c1_g3_i1	251105344 2	NAD-dependent 2- hydroxyacid dehydrogenase (Pfam00389)	10.59	1.67
TRINITY_ DN26116_ c0_g1_i2	251873220 4	ABC-type multidrug transport system, ATPase component	9.03	1.08
TRINITY_ DN27116_ c8_g3_i1	251872044 3	ABC-type multidrug transport system, ATPase component	9.04	1.22
TRINITY_ DN25404_ c0_g1_i6	251871633 3	Actin and related proteins; ACTR8, ARP8, INO80N actin- related protein 8	9.23	1.67
TRINITY_ DN25945_ c5_g1_i1	251872411 3	Ankyrin repeat	11.62	1.55
TRINITY_ DN27367_ c4_g7_i1	251871784 0	Beta-ketoacyl synthase, N-terminal domain/AMP-binding	9.08	1.53
TRINITY_ DN26659_ c2_g1_i6	251872469 3	Calponin	10.43	2.98
TRINITY_ DN27260_ c3_g1_i2	251871720 4	Cell division protein	9.28	1.85
TRINITY_ DN25040_ c0_g1_i2	251872384 7	Aminopeptidase	10.37	3.88
TRINITY_ DN84844_ c0_g1_i1	251872416 5	Cyclin	9.38	2.05
TRINITY_ DN27688_ c5_g4_i4	251873184 3	Zinc finger, PHD-type	8.13	3.79

TRINITY_ DN27658_ c7_g1_i9	251872741 4	Enterochelin esterase and related enzymes	9.41	1.38
TRINITY_ DN27456_ c0_g1_i1	251873221 3	Transposase	8.89	1.30
TRINITY_ DN27284_ c8_g3_i8	251872284 5	Galactose binding lectin domain	9.56	1.53
TRINITY_ DN27371_ c7_g1_i8	251105091 9	Glycosyl hydrolase family 9	8.94	1.26
TRINITY_ DN27514_ c12_g7_i3	251105831 4	Glycosyl hydrolase family 9	9.10	1.50
TRINITY_ DN26559_ c2_g2_i3	251106159 1	Hypothetical protein	4.35	1.04
TRINITY_ DN26871_ c0_g1_i6	251104872 6	Hypothetical protein	5.66	1.08
TRINITY_ DN26577_ c7_g3_i6	251106002 9	Hypothetical protein	7.06	3.71
TRINITY_ DN27338_ c2_g6_i9	251105027 9	Hypothetical protein	7.91	3.71
TRINITY_ DN20198_ c0_g1_i1	251105406 3	Hypothetical protein	8.64	1.04
TRINITY_ DN59842_ c0_g2_i1	251105300 0	Hypothetical protein	8.67	1.08
TRINITY_ DN26705_ c9_g11_i5	251106142 9	Hypothetical protein	8.79	1.10
TRINITY_ DN24643_ c0_g3_i3	251106191 1	Hypothetical protein	9.24	1.78
TRINITY_ DN26861_ c6_g1_i1	251872296 6	Hypothetical protein	9.36	1.68
TRINITY_ DN26789_ c7_g2_i2	251106035 3	Hypothetical protein	9.63	2.59
TRINITY	251871670	CAP-Gly domain	9.74	2.81

DN25732_c3_g1_i2	4			
TRINITY_DN20193_c0_g1_i1	2511049097	Hypothetical protein	9.74	2.27
TRINITY_DN27291_c5_g1_i27	2511062706	Hypothetical protein	9.92	1.34
TRINITY_DN26268_c0_g6_i2	2511056562	Hypothetical protein	9.94	3.10
TRINITY_DN72241_c0_g4_i1	2511055911	Hypothetical protein	9.99	3.10
TRINITY_DN23108_c0_g1_i1	2511055897	Leucine-rich repeat (LRR) protein	8.67	1.04
TRINITY_DN26891_c1_g2_i3	2518723924	Long-chain acyl-CoA synthetases (AMP-forming)	9.22	1.59
TRINITY_DN27245_c11_g4_i1	2511055785	Metal-dependent hydrolase	11.86	1.14
TRINITY_DN26212_c0_g1_i6	2511051817	Mismatch repair ATPase (MutS family)	9.29	1.68
TRINITY_DN26388_c1_g1_i3	2511051700	Nucleosome-binding factor SPN, POB3 subunit	8.96	1.26
TRINITY_DN25459_c0_g1_i3	2518723044	Nucleotide-sugar transporter.	8.72	1.08
TRINITY_DN26193_c4_g1_i3	2518729432	Phosphatidylinositol-4-phosphate 5-Kinase	9.92	3.10
TRINITY_DN18878_c0_g1_i3	2511061142	Predicted Rossmann fold nucleotide-binding protein	12.17	9.52
TRINITY_DN27738_c2_g1_i2	2518732026	Reverse transcriptase (RNA-dependent DNA polymerase)/Integrase	9.48	1.71
TRINITY_DN26725_c0_g1_i2	2511052637	RhoGEF domain.	9.24	1.78
TRINITY	251105618	Ribosomal protein	9.49	1.90

DN25303_c1_g1_i1	7	L11 methylase		
TRINITY_DN26960_c5_g2_i8	2518725849	Superfamily II DNA/RNA helicases, SNF2 family	9.01	1.35
TRINITY_DN11227_c0_g2_i1	2511055978	Trehalose-6-phosphate synthase	12.34	1.26
TRINITY_DN27057_c7_g3_i8	2511061879	WD40-repeat-containing domain	9.13	1.62
TRINITY_DN24706_c0_g1_i2	2518725042	WD40-repeat-containing domain	9.31	1.55
TRINITY_DN22853_c0_g1_i4	2518720070	WD40-repeat-containing domain	9.32	1.56
TRINITY_DN27087_c6_g1_i1	2511051329	WD40-repeat-containing domain	9.36	1.84
TRINITY_DN17665_c0_g1_i1	2518731672	Zinc metalloprotease (elastase)	9.56	2.22
TRINITY_DN26802_c4_g9_i1	NA ^a	1,4-alpha-glucan branching enzyme	9.21	1.55
TRINITY_DN27699_c2_g2_i5	NA	Hypothetical protein	9.65	2.62
TRINITY_DN27163_c4_g2_i1	NA	Hypothetical protein	11.75	1.13
TRINITY_DN19604_c0_g3_i1	NA	SH3 domain	8.89	1.26

a: NA Blastx comparison of the transcript sequence against C1A proteins showed no hits.

Discussion

Here, I explored the feasibility of RNA interference for targeted gene silencing in the anaerobic gut fungi (phylum Neocallimastigomycota) via the exogenous addition of synthetic double stranded siRNAs targeting the *ldhD* gene to *Pecoramyces ruminantium* strain C1A germinating spores. I show that ds-siRNA was uptaken by germinating spores, and, as a consequence, the transcription of the target gene (*ldhD*) was down-regulated (Table 4-1), leading to lower D-LDH enzymatic activity (Table 4-2) and lower D-lactate concentration in the culture supernatant (Figure 4-4).

In general, the fungal RNAi machinery encompasses Dicer (Dic) enzyme(s), Argonaute (Ago) protein(s), RNA-dependent RNA polymerase (RdRP) enzyme, QDE3-like DNA helicase, and Argonaute-interacting exonuclease (QIP-like). Genomes of Neocallimastigomycota representatives belonging to four genera (*Pecoramyces*, *Neocallimastix*, *Piromyces*, and *Anaeromyces*) encode at least one copy of Dic, Ago, QDE3-like helicase, and QIP exonuclease. However, all genomes lacked a clear homolog of RdRP. The absence of an RdRP homolog is not uncommon. While present in almost all studied fungi, RdRP seems to be missing from the genomes of other basal fungal phyla (Chytridiomycota and Blastocladiomycota) representatives (77, 78). The absence of clear RdRP homologues in the Neocallimastigomycota and related basal fungal phyla despite their presence in other fungi could suggest that either an RdRP is not involved in dsRNA-mediated mRNA silencing as shown before in mammals (79). Alternatively, RNA-dependent RNA polymerase activity could be mediated through a non-canonical RdRP in basal fungi, e.g. the RNA polymerase II core elongator complex subunit Elp1

shown to have RdRP activity in *Drosophila*, as well as *Caenorhabditis elegans*, *Schizosaccharomyces pombe*, and human (80, 81).

I chose as a gene knockdown target the D-Lactate dehydrogenase gene (*ldhD*) that mediates NADH-dependent pyruvate reduction to D-lactate, for several reasons. First, the gene is present as a single copy in the genome. Second, quantification of the impact of *ldhD* gene knockdown is readily achievable in liquid media at the RNA (using RT-PCR and transcriptomics), and protein (using specific enzyme activity assays) levels, as well as phenotypically (by measuring D-lactate accumulation in the culture media); providing multiple lines of evidence for the efficacy of the process. Finally, D-lactate dehydrogenase is part of the complex mixed acid fermentation pathway in *P. ruminantium* (46, 58) and other anaerobic gut fungi, and I sought to determine how blocking one route of electron disposal could lead to changes in C1A fermentation end products.

ldhD-siRNA-treated cultures showed a significant reduction in *ldhD* gene transcription and D-LDH enzyme activity. Both of these effects were dependent on the concentration of siRNA added (Tables 4-1 and 4-2) similar to previous reports in filamentous fungi (22-24, 28). I show that the addition of 100 nM of *ldhD*-siRNA resulted in a four-fold reduction in *ldhD* transcription, 84% reduction in D-LDH specific activity, and 86% reduction in D-lactate concentration in culture supernatant. The fact that targeted gene silencing using exogenously added gene-specific siRNA results in reducing rather than completely abolishing gene function is an important advantage of RNAi approaches allowing functional studies of housekeeping or survival-essential genes.

While initial studies of gene silencing using exogenously added siRNAs suggested that the process was highly sequence-specific (67, 82), subsequent studies showed silencing of off-target genes based on less than perfect complementarity between the siRNA and the off-target gene (83). Here, I used RNA-seq to quantify the off-target effects of *ldhD*-siRNA. In contrast to previous studies that used similar approaches to quantify RNAi off-targets (84), I show here that the off-target effects of *ldhD* silencing were minimal (only 29 transcripts out of 55,167 total transcripts were differentially down-regulated as a result of siRNA treatment) and appeared to be not sequence-specific.

Currently, and due to their strict anaerobic nature, there are no established procedures for genetic manipulations (e.g. gene silencing, insertion, deletion, and mutation) of AGF leading to a paucity of molecular biological studies of the phylum. This is in stark contrast to the rich body of knowledge available on genetic manipulations of various aerobic fungal lineages (22, 24, 28, 66, 85, 86). My work here represents a proof of principal of the feasibility of the RNAi approach in AGF, and opens the door for genetic manipulation and gene function studies in this important group of fungi.

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CONCLUSIONS

The studies I have conducted for this dissertation resulted in the development and optimization of a novel anaerobic flooding technique for collection of spores from anaerobic gut fungi, such as *Pecoramyces ruminantium* sp. C1A. The ability to collect spores from C1A using anaerobic flooding has proven to be invaluable in the following ways: allowed for reliable long-term culture storage for AGF isolates for evasion of senescence; provided a means of spore separation from other life cycle stages for collection of spore-only samples; and allowed for collection of viable, developmentally synchronized spores.

Therefore, the ability of AGF spore collection that this multifaceted technique has provided is a promising piece of the puzzle that had been missing, which was necessary to further broaden the scope of knowledge in this understudied fungal phylum. Moving forward, further transcriptional studies similar to that of the focal adhesion study conducted here, can be conducted on other evolutionarily unique pathways using C1A or other AGF members. Moreover, the ability of C1A's germinating spores to readily uptake synthetic siRNAs for successful RNAi-based gene silencing will be one avenue to explore in future studies. Alternatively, spores collected using the anaerobic flooding technique could also be used as a starting material for use in *Agrobacterium*-mediated transformation type studies for gene function analysis. These approaches could be

utilized on other genes involved in ethanol production in C1A, which could potentially be significant from a biofuels perspective.

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