

**ROLE OF *UV90* GENE IN THE FRQ-LESS OSCILLATOR OF THE
NEUROSPORA CRASSA CIRCADIAN SYSTEM**

LALANTHI K.R. RATNAYAKE MUDIYANSELAGE

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Abstract

Circadian rhythms are found in all domains of life and control many physiological processes. Transcription/translation feedback loops (TTFLs) are important molecular mechanisms in circadian systems. In the model eukaryote *Neurospora crassa*, TTFL includes FRQ (frequency) and WCC (white-collar complex). Rhythms have been observed in the absence of a functional FRQ/WCC TTFL. These are called FRQ-less rhythms which are assumed to be driven by one or more FRQ-less oscillators (FLOs).

Through UV mutagenesis former lab members have found a mutation (*uv90*) that compromises FRQ-less rhythms and also affects rhythmicity when FRQ is functional, demonstrating the close integration between the TTFL and FLO(s). My PhD dissertation research is focused on the *uv90* mutation. The gene it affects has now been identified as gene number NCU05950. The *uv90* mutation was characterized as a deletion of 35,128 nucleotides in chromosome VI.

NCU05950 was identified as a homologue of the conserved nutrient-sensing TOR pathway proteins EGO1 in yeast and LAMTOR1 in mammals. We have named the *N. crassa* protein as VTA (Vacuolar-TOR-Associated protein). GFP-tagging of VTA showed it is anchored to the outer vacuolar membrane. Deletion of putative acylation sites destroys this localization as well as the protein's function in rhythmicity, demonstrating that vacuolar localization of VTA is required for clock function. The NCU05950 deletion mutant is defective in its growth response to different amino acids and different concentrations of glucose, confirming its function in nutritional sensing.

A FLAG fusion of NCU05950 exhibited nearly constant levels of protein across two circadian cycles. Northern blot results using a probe for the

wild type VTA showed arrhythmic transcript levels. IP-MS analysis showed interaction of VTA with other TOR pathway proteins. Deletion mutants of other *N. crassa* TOR pathway associated genes are defective in their response to different nutritional conditions and showed altered conidiation rhythms.

My findings establish a connection between the TOR pathway and circadian rhythms. I conclude that VTA is a key protein in the complex that anchors TOR to the vacuole. It plays a role in maintaining circadian rhythmicity by integrating networks of metabolism and TTFLs to construct a complete circadian system.

Dedication

I dedicate this dissertation to my grandparents, parents, husband, daughter and all the family members for the love and support they have provided throughout my education and life.

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List of Abbreviations

4E-BP: 4E-binding protein
aa: amino acids
AGC: Kinase group AGC which includes protein kinase A (PKA), G (PKG) and C (PKC) families
AMP: Adenosine monophosphate
AMPK: AMP kinase
bd: band mutation
bHLH: basic-helix-loop-helix
BLAST: Basic Local Alignment Search Tool
BMAL1: Brain and muscle Arnt-like protein-1
BSA: Bovine Serum Albumin
C7orf59: Chromosome 7 open reading frame 59
CAMK-1: calcium/calmodulin-dependent protein kinase I
CAPS: Cleaved Amplified Polymorphic Sequences
CCA1: Circadian Clock Associated 1
ccgs: clock-controlled-genes
Chk2: checkpoint kinase 2
chol-1: choline mutant
CK: casein kinase
CK2: Casein Kinase 2
CKI: casein kinase I
Clk: Clock
CLK: CLOCK
CO: Constans
CRY: cryptochrome

esp-1: Conidial separation mutant
CWO: Clock Work Orange
CYC: CYCLE
DAG: diacylglycerol
DBT: DOUBLETIME
DD: constant darkness
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
EGO: Exit from rapamycin induced growth arrest
ELF3: Early Flowering 3
ELF4: early Flowering 4
FFC: FRQ-FRH Complex
FGSC: Fungal Genetics Stock Center
FKBPs: FK506 binding proteins
FKR-2: FK506 resistant -2
FKR-3: FK506 resistant -3
FKR-5: FK506 resistant -5
FLO: FRQ-less oscillator
FPR2: Peptidylprolyl isomerase family protein FPR2
FRAP: FKBP12 rapamycin associated protein
FRH: FRQ-interacting RNA helicase
FRQ: Frequency
Frq¹⁰: frequency null mutation
FWO: FRQ-White collar Oscillator
GAP: GTPase activating protein
GEF: Guanine nucleotide exchange factor
GFP: green fluorescent protein

GI: Gigantea
GMP: Guanosine monophosphate
GTP: Guanosine triphosphate
GTPase: Guanosine triphosphatase
GTR1: GTP binding protein resemblance 1
GTR2: GTP binding protein resemblance 2
HBXIP: Hepatitis B virus X-interacting protein
his-3: Histidine requiring mutant
HPLC: High performance liquid chromatography
Hyg^R: Hygromycin B resistance
IP-MS: Immunoprecipitation – Mass spectrometry
ipRGC: Intrinsically Photosensitive Retinal Ganglion Cells
KOG1: Kontroller of growth
LAMTOR: Late endosomal / lysosomal adapter and MAPK and mTOR activator
LAMTOR1: Late endosomal / lysosomal adapter and MAPK and mTOR activator 1
LAMTOR2: Late endosomal / lysosomal adapter and MAPK and mTOR activator 2
LAMTOR3: Late endosomal / lysosomal adapter and MAPK and mTOR activator 3
LAMTOR4: Late endosomal / lysosomal adapter and MAPK and mTOR activator 4
LAMTOR5: Late endosomal / lysosomal adapter and MAPK and mTOR activator 5
LB: Luria-Bertani
LD: Light-dark

LHY: Late Elongated Hypocotyl
LL: Constant light
LNd: lateral neurons located dorsally
LNs: Lateral Neurons
LNv: lateral neurons located ventrally
LRE: light response element
LUX: LUX Arrhythmo
MA: Maltose-arginine
MAPK: Mitogen activated protein kinase
Mat A: Mating type 'A'
Mat a: Mating type 'a'
mCry: mammalian cryptochrome
Meh1: Multicopy suppressor of Ers1 Hygromycin B sensitivity
MP1: Mitogen activated protein kinase scaffold protein 1
mPer: mammalian period gene
mTOR: Mechanistic / Mammalian target of rapamycin
mTORC1: Mammalian target of rapamycin complex 1
MV: Mauriceville
NR: Nitrate reductase
OR : Oak Ridge
p14: Endosomal adapter protein p14
p18: Lipid raft adapter protein p18
PAS: PER-ARNT-SIM
PCR: Polymerase chain reaction
PER: period
PHYs: phytochromes
PI3K: Phosphoinositide 3-kinase

PKA: cyclic AMP dependent kinase
PKC: protein kinase C
PKG: cyclic GMP dependent kinase
PP: protein phosphatase
PP1: protein phosphatase 1
PP2A: protein phosphatase 2 A
PPH-1: protein phosphatase 1
prd-1: period mutation
PRR: Pseudo Response Regulator
PTO: Post-translational Oscillator
RAFT: Rapamycin and FKBP12 target
RagA: Ras related GTP binding protein A
RagB: Ras related GTP binding protein B
RagC: Ras related GTP binding protein C
RagD: Ras related GTP binding protein D
RAPT: Rapamycin target
ras-1^{bd} : Band mutant
Rheb: Ras homolog enriched in brain
RpaA: regulator of phycobilisome-associated
S6K: S6 kinase
SasA: Synechococcus adaptive sensor A
SCH9: Serine/threonine protein kinase SCH9
SCM: Synthetic crossing medium
SCN: suprachiasmatic nucleus
Ser: Serine
SG: Silica gel
SIT4: Suppressor of initiation of transcription 4

SNPs: Single Nucleotide polymorphisms
Ssg: Shaggy
STK-10: Serine/threonine kinase 10
Tap42: Two A phosphatase associated protein 42
TE: Tris-EDTA
Thr: Threonine
TIM: Timeless
TOC1: timing of CAB 1 (chlorophyll A/B binding protein 1)
TOR: Target of Rapamycin
TOR1: Target of Rapamycin 1
TOR2: Target of Rapamycin 2
TORC1: Target of Rapamycin complex 1
TORC2: Target of Rapamycin complex 2
TSC1: Tuberous sclerosis complex 1
TSC2: Tuberous sclerosis complex 2
TTFL: Transcriptional/Translational feedback loop
TTO: Transcription/Translation feedback oscillator
UV: Ultra violet
VAM6: Vacuolar morphogenesis 6
Vri: Vrille
VTA: vacuolar TOR-associated protein
WC-1: White Collar 1
WC-2: White Collar 2
WC: white collar
WCC: White collar complex
WT: wild type
Ypk1: Yeast protein kinase 1

Ypk2: Yeast protein kinase 2

ZTL: ZEITLUPE

CHAPTER 01: Introduction

1.1: Circadian rhythms

Circadian rhythms are biological fluctuations found in almost all eukaryotic and some prokaryotic organisms in the absence of physical environmental stimuli imposed by the daily rotation of the Earth on its axis (Bennett *et al.*, 2013). These rhythms are generated by an endogenous biological clock at the cellular level with a period of about 24 hours and they are referred to as “circadian” (i.e., circa - about; diem - a day) (Harmer *et al.*, 2001; Dunlap 1999; Lakin-Thomas 2006b; Harmer 2009; Mellow and Roenneberg 2007; Gyöngyösi *et al.*, 2017; Mendoza-Viveros *et al.*, 2017; Honma 2018; Montenegro-Montero *et al.*, 2015). Organisms and cells utilize this characteristic internal timekeeping mechanism to adjust their molecular, physiological and behavioral or social activities according to anticipated environmental changes (Jinhu and Yi 2010; Harmer 2009; Mohawk *et al.*, 2012). Single celled organisms such as the photosynthetic bacterium *Synechococcus*, marine alga *Gonyaulax* and individual cells in small, semitransparent organisms such as *Drosophila* and zebrafish contain an entire circadian clock (Ditty *et al.*, 2003; Mittag 2001; Plautz *et al.*, 1997; Whitmore *et al.*, 2000). Furthermore, circadian oscillators are found in many tissues in multicellular organisms and isolated cells from multicellular organisms have shown rhythmicity under *in vitro* conditions (Fan *et al.*, 2007; Welsh *et al.*, 1995). Therefore, circadian rhythmicity is a property of individual cells and it is a fundamental property of cellular physiology. Circadian clocks are programmed to optimize and regulate processes such as the sleep-wake cycle (basis of jet lag), migration patterns (in birds and butterflies), hibernation, metabolism, gene expression, body temperature, blood pressure, hormone

activity, development, cell cycle, plant flowering, leaf movement, pollen and seed germination, stress response, growth, enzyme activity, stomatal movement, gas exchange, photosynthetic activity, flower opening, fragrance emission from plants and developmental transitions in filamentous fungi (Harmer 2009; de Paula *et al.*, 2008; McClung 2006a; Cumming and Wagner 1968).

Circadian clocks control many aspects of physiology, cellular and molecular processes, including metabolism, gene / protein expression, etc. Therefore, disruption of normal circadian rhythms impacts on health and multiple disorders in mammals and humans. Shiftwork, rapid travel across time zones, illnesses, advanced age are some of the reasons to acquire a defective endogenous clock mechanism or misalignment between internal circadian rhythms with the external environment. Disturbed circadian rhythmicity in humans has been linked to multiple mental and physical disorders including metabolic syndrome, depression, cognitive impairment, cardiovascular abnormalities, gastrointestinal and genitourinary dysfunctions, and cancer (Bass 2012; Sancar and Brunner 2014; Zhu and Zee 2012). Understanding the precise molecular mechanisms that link the circadian clock and diseases may lead to novel treatments like chronotherapy for diseases and conditions linked to circadian pathophysiology (Çalıyurt 2017) as well as valuable information on how to adjust human lifestyle to have a healthy and high-quality life.

All circadian rhythms have some basic properties, such as: the rhythms are endogenous and persisting with approximately 24 hour periods under constant conditions i.e. free-running; circadian rhythms continue even when the environmental signals, such as light, temperature, humidity, salinity, etc. are removed i.e. they are self-sustained ; the period of their rhythmicity is almost constant with temperature fluctuations i.e. rhythms are temperature

compensated; and they are entrained by environmental factors like light/dark or temperature cycles to keep the physiological functions synchronized with the environment (Dunlap 1999; Harmer *et al.*, 2001; Lakin-Thomas 2006a ; Harmer 2009). The period of the free-running oscillator is called the free-running period (FRP) which is close to, but not exactly 24 hours. Biological rhythms with significantly shorter or longer periods exist. The shorter period rhythms are called ultradian and longer period rhythms are called infradian. Circalunar (monthly) and circannual (yearly) are among the infradian rhythms.

1.2: The central oscillator and transcriptional-translational feedback loop

The circadian system consists of three components: entrainment input pathways, internal oscillator and output pathways. The “entrainment input pathways” receive and transmit environmental signals such as light, pH and temperature to the time keeping apparatus in order to reset or entrain the oscillator. The self-sustained core component of the circadian system “internal oscillator or rhythm generator”, interprets the time-cues and operates in the absence of environmental cues. The “output pathways” are activated at specific times of the circadian cycle by the circadian oscillator to generate the observed rhythms in clock-controlled gene (ccg) expression (molecular activities) and other biochemical, physiological, behavioral processes (Figure 1.1) (Hardin 2000; Harmer *et al.*, 2001; Lakin-Thomas and Brody 2004; de Paula *et al.*, 2006). The entrainment input variables can give information to the central oscillator and those do not necessarily determine the action of the central oscillator. The environmental cues are also referred to as “zeitgebers” (from German meaning “time givers”).

A transcriptional-translational feedback loop (TTFL) is the current model for the core of the circadian oscillatory process (Harmer *et al.*, 2001;

Egli and Johnson 2015). In this model, a set of clock genes is rhythmically expressed / transcribed and their protein products construct the positive arm with a heterodimeric transcription complex that behaves as the activator to promote the transcription of one or more components of the negative arm, which then directly or indirectly feedback to negatively regulate their own transcription by repressing the activity of the positive arm (Figure 1.2) (Lakin-Thomas 2006b; Hurley *et al.* 2016). The elements in the negative arm are post-translationally regulated by phosphorylation and those are subjected to step-wise phosphorylation followed by degradation via the ubiquitin / proteasome pathway (Lakin-Thomas *et al.*, 2011). Rhythmic transcription gives rise to rhythmic levels of RNA, rhythmic mRNA causes rhythmic levels of proteins and rhythmic levels of proteins cause rhythmic transcription (Lakin-Thomas 2006b). Rhythmic transcription of clock genes is essential for rhythmicity of organisms in this model (Lakin-Thomas 2006b).

Circadian rhythm generating clock genes have been identified in diverse organisms such as the cyanobacterium *Synechococcus elongatus*; a small flowering weed plant that belongs to the mustard family Brassicacea, *Arabidopsis thaliana*; the filamentous fungus *Neurospora crassa*; the fruit fly *Drosophila melanogaster*; and mammals. The clock components and their organization into TTFL in different eukaryotes are conserved. However, these clock proteins are not homologous. In cyanobacterium *Synechococcus elongatus*, the positive element that activates transcription is the *kaiA* gene product and in eukaryotes PAS-domain containing transcription activators form a heterodimeric transcription complex that binds to the E-box element in clock gene promoters (Dunlap 1999). The clock proteins in different organisms exhibit multiple interactions to make the circadian system more complex. Therefore, it is important to understand how these circadian systems function in

diverse model organisms at the structural, functional and mechanistic level for the betterment of all living organisms.

1.3: Molecular mechanism of circadian rhythms in Prokaryotes

Circadian rhythms are not a property exclusive to eukaryotes. Cyanobacteria are the simplest organism and the only prokaryote that has a rigorously studied circadian clock, which consists of a post-translational oscillator (PTO) (Cohen and Golden 2015; Egli and Johnson 2015).

1.3.1: Cyanobacteria clock

Circadian rhythms in cyanobacteria were first reported in cyanobacterium *Oscillatoria* during the nitrogen fixation process (Grobbelaar *et al.*, 1986; Iwasaki and Kondo 2000; Johnson *et al.*, 2008). Furthermore, global rhythms in gene transcription, compaction of the chromosome, photosynthesis and the timing of cell division are some of the observed rhythms in cyanobacteria (Johnson *et al.*, 2008, Cohen and Golden 2015). Most studies have been done with the fresh water, unicellular, mesophilic cyanobacterium *Synechococcus elongatus* and the core oscillator of the cyanobacterial clock is composed of three proteins, KaiA, KaiB and KaiC (Kai in Japanese means “cycle”), and a set of mutually antagonistic output/input signaling proteins, SasA, CikA and RpaA (Figure 1.3) (Ishiura *et al.*, 1998; Dong *et al.*, 2010; Tseng *et al.*, 2017; Swan *et al.*, 2018). The three *kai* genes are located as a tandem cluster on the chromosome and *kaiABC* locus expressed rhythmically. KaiB and *kaiC* are expressed as an operon, share a single promoter and are co-transcribed (Ishiura *et al.*, 1998).

Cyanobacteria are unique in the fact that the transcriptional-translational feedback loop (TTFL) is not essential for the cyanobacterial circadian system and the master timer in cyanobacteria is the KaiABC post-translational oscillator (PTO) (Tomita *et al.*, 2005; Brunner and Schafmeier 2006; Zhang and Kay 2010; Cohen and Golden 2015; Egli and Johnson 2015). PTOs comprise a cycle of biochemical processes and they don't depend on changes in expression levels (Swan *et al.*, 2018). The behavior of PTOs is controlled by post translational modifications, conformational changes, protein-protein interactions and / or subcellular localization (Swan *et al.*, 2018). These post-translational steps link dynamic molecular processes to biological phenotype (Swan *et al.*, 2018).

KaiC is an autokinase, autophosphatase and ATPase (Dong *et al.*, 2010). The abundance and the phosphorylation states of KaiC cycle under *in vivo* conditions (Dong *et al.*, 2010). Three Kai proteins and adenosine triphosphate (ATP) generate the circadian oscillation of KaiC phosphorylation and ATP hydrolysis under *in vitro* conditions (Dong *et al.*, 2010). Mutations in KaiC alter the circadian period *in vivo* and also alter the post-translational oscillator *in vitro* (Johnson *et al.*, 2008). Therefore, KaiC phosphorylation in the presence of adenosine triphosphate (ATP) is the basic molecular timer for the circadian system of *Synechococcus elongatus* (Johnson *et al.*, 2008; Nakajima *et al.*, 2005; Tseng *et al.*, 2017).

Upon stimulation by KaiA, KaiC autophosphorylates and then it will undergo dephosphorylation (Dong *et al.*, 2010; Brunner and Schafmeier 2006). KaiB binds to dephosphorylated KaiC and this KaiB-KaiC complex traps KaiA to inactivate KaiA and allow further dephosphorylation of KaiC (Dong *et al.*, 2010). The phosphorylation status of KaiC and the degradation rate of KaiC determine the circadian period in cyanobacteria (Xu *et al.*, 2000; Xu *et al.*,

2003; Xu *et al.*, 2004; Nakajima *et al.*, 2005). Apart from that, in a simple mixture that contains the KaiA, KaiB and KaiC proteins, the change in fluorescence polarization of a tagged component as it associates and dissociates from the larger complex shows a 24-hour oscillation of protein (Golden 2019; Leypunskiy *et al.*, 2017). The phosphorylation cycle causes changes to the conformation of KaiC protein (Dong *et al.*, 2010).

During phosphorylation states and interactions with other clock proteins, KaiC functions as a hexamer (Dong *et al.*, 2010). This homo-hexameric repeat domain structure of KaiC consists of two ring shaped hexamers that stack one on the other (Golden 2019; Cohen and Golden 2015). The C-terminal domain of KaiC is the CII ring and N-terminal domain is the CI ring (Golden 2019; Chang *et al.*, 2012; Tseng *et al.*, 2017). The tightness of these two hexamer rings depend on the phosphorylation state of two residues in CII ring and the ATP hydrolysis ability of CI domain (Golden 2019; Chang *et al.*, 2012; Tseng *et al.*, 2017). The accessibility of binding sites of KaiA and KaiB on KaiC is determined by the conformation changes and tightness of the two hexamer rings (Golden 2019). During the subjective day, the active form of KaiA binds to the C-terminal tail of KaiC and stimulates KaiC to autophosphorylate (Golden 2019). KaiA is trapped by KaiB in its inactive form (Golden 2019; Tseng *et al.*, 2017). The structure of KaiA itself oscillates between an active and an inactive form (Golden 2019; Tseng *et al.*, 2017). KaiB spends most of its time in an inactive tetrameric state which does not interact with KaiA or KaiC (Golden 2019). To become the active form of KaiB, the tetramer dissociates, polypeptides loosened and refold to give an entirely different tertiary structure (Golden 2019). The “fold switched” active form of KaiB is unstable and if it doesn’t find its binding site on N-terminal of KaiC, it rapidly switches to the inactive ground state (Golden 2019). When KaiB binds

to KaiC, it forms a third ring on the complex and it functions as the center of the “night time complex” (Golden 2019; Tseng *et al.*, 2017). The KaiB ring captures and sequesters KaiA, which is in its inactive form and initiates dephosphorylation phase of KaiC to prepare for the dawn phase of the cycle (Golden 2019).

Two histidine kinases SasA (*Synechococcus* adaptive sensor A) and CikA (Circadian input kinase A), and potential DNA binding protein which acts as a cognate response regulator and a transcription factor, RpaA (Regulator of phycobilisome-associated proteins), are the clock output components acting downstream of KaiC to drive rhythms in biological activities such as timing of cell division and gene expression (Dong *et al.*, 2010; Cohen and Golden 2015; Tseng *et al.*, 2017). SasA interacts with Kai proteins and rhythmically phosphorylates RpaA (Figure 1.4) (Pattanayak and Rust 2014; Takai *et al.*, 2006b). SasA binds to KaiC and RpaA, but SasA is not necessary to control the basic oscillation (Takai *et al.*, 2006b). Phosphorylated RpaA binds directly to the regulon of genes to cause rhythms in transcription (Pattanayak and Rust 2014; Markson *et al.*, 2013). RpaA controls expression of multiple sigma factors, a modulator subunit of RNA polymerase that is required for generation of global rhythms of transcription (Pattanayak and Rust 2014). The oscillatory transcription of the RpaA regulon gives rise to rhythms of the metabolic state of the cell that influence DNA supercoiling via regulating DNA gyrase activity, which is sensitive to the ATP/ADP ratio (Pattanayak and Rust 2014; van Workum *et al.*, 1996). Therefore, SasA-RpaA communicates the signals from the KaiC oscillator to control the genome wide transcription rhythms in cyanobacteria (Takai *et al.*, 2006a; Takai *et al.*, 2006b). Apart from the above-mentioned circadian output components, *crm* (circadian rhythm modulator) and

rpaB (regulator of phycobilisome association B) have recently been identified as novel elements of the output pathway (Cohen and Golden 2015).

Even though circadian clocks are self-sustained biological oscillators, they are highly sensitive to their environment and can be reset by environmental stimuli. In cyanobacteria, light signals are not sensed directly by photoreceptors as in eukaryotic systems. The external light signals change the redox state of electron carriers such as quinone in the cyanobacterial cell to modulate the phosphorylation state of KaiC and thereby coordinate the internal circadian system with the external light/dark cycle (Dong *et al.* 2010; Cohen and Golden 2015). Redox sensitive bacterio-phytochrome CikA (Circadian input kinase A) and LdpA (light dependent period A) are major components of the input pathway (Cohen and Golden 2015). Apart from that, the major clock components KaiA and KaiC directly sense metabolites of photosynthesis such as ATP/ADP ratio and amount of glycogen in order to orchestrate the internal circadian oscillator with the external environment (Cohen and Golden 2015). CikA binds to quinone and represses the KaiC phosphorylation (Dong *et al.* 2010; Wood *et al.* 2010). Only the oxidized forms of quinone bind directly to KaiA and thereby destabilize KaiA (Dong *et al.* 2010). Furthermore, recent genetic and biochemical analysis have indicated that CikA modulates RpaA phosphorylation (Pattanayak and Rust 2014; Gutu and O'Shea 2013; Taniguchi *et al.*, 2010). The other input component LdpA is an iron-sulfur center-building redox active protein and it is responsible for regulating the period of circadian rhythms by sensing the changes in electron transport based on external environmental signals (Cohen and Golden 2015).

In cyanobacteria, rhythmic gene expression has been observed even when KaiC is locked into a specific phosphorylation state (Dong *et al.*, 2010; Kitayama *et al.*, 2008). This observation suggests the transcriptional

translational feedback loop (TTFL) exists independent of the Kai protein oscillator (Dong *et al.*, 2010; Lakin-Thomas 2006b). Johnson and colleagues found that when KaiC phosphorylation is clamped, there is damping of gene expression rhythms (Dong *et al.*, 2010; Johnson *et al.*, 2008). It indicates the involvement of another oscillator, which interacts with the master pacemaker Kai protein oscillator (Dong *et al.*, 2010; Johnson *et al.*, 2008). Mathematical modeling and other experiments have shown that enhancement of the strength of the circadian clock is based on the integration of TTFL with the protein oscillator and TTFL can provide entraining signals to the post translational Kai based pacemaker (Dong *et al.*, 2010; Qin *et al.*, 2010).

Most recent investigations demonstrated that nearly all genes in *Synechococcus elongatus* genome are rhythmically expressed and the gene expression profiles have been categorized into different groups based on phasing of peak expression time (Cohen and Golden 2015). *Synechococcus elongatus* circadian oscillator maintains approximately 24 hr period and this periodicity is independent of the cell cycle (Cohen and Golden 2015). Even though cell division is not synchronous with the circadian system, the circadian clock regulates the timing of cell division and it is forbidden in the early night of the 24 hr cycle (Cohen and Golden 2015). Furthermore, the *Synechococcus elongatus* circadian oscillator regulates super-helical status, compaction of the chromosome and the topological status of the chromosome, regulating circadian gene expression patterns (Cohen and Golden 2015).

1.4: Molecular mechanisms of circadian rhythms in Eukaryotes

1.4.1: *Drosophila* clock

Drosophila melanogaster, the fruit fly, has been used as a model organism to study the circadian system at genetic, molecular and neural circuit levels (Tataroglu and Emery 2014; Hardin 2009). The period of rest and activity, the timing of feeding and temperature preferences, eclosion and courtship, locomotor activity, sleep pattern, learning and memory, feeding behavior, chemo-sensation and immune responses are some of the physiological and behavioral processes determined by the *Drosophila* circadian clock (Tataroglu and Emery 2014; Zhang and Emery 2012; Kaneko *et al.*, 2012; Nobrega and Lyons 2017). Apart from control of various behaviors, the *Drosophila* circadian system coordinates many rhythms in peripheral organs, such as olfactory and gustatory sensitivity rhythms and the mitotic response of gut stem cells to damage (Tataroglu and Emery 2014; Chatterjee *et al.*, 2010; Krishnan *et al.*, 1999; Karpowicz *et al.*, 2013, Nobrega and Lyons 2017).

The genetic identification and isolation of the first clock gene, period (*per*) in *Drosophila* by a mutagenesis screen using *Drosophila* eclosion and subsequent studies of its expression laid the foundation for the first molecular model of the circadian oscillator, which is an auto-regulatory transcriptional-translational feedback loop (TTFL) in gene expression (Hardin 2009; Konopka and Benzer 1971; Hardin *et al.*, 1990; Nobrega and Lyons 2017). The 2017 Nobel Prize in Physiology and Medicine was awarded to Jeffrey Hall, Michael Rosbash and Michael W. Young, for their discovery of the TTFL. The discovery of additional clock genes *timeless*, *clock*, *cycle*, *doubletime*, *shaggy*, *casein kinase 2* subunits and *cryptochrome* suggested there are two interlocked feedback loops (Hardin 2009; Nobrega and Lyons 2017).

The central pacemaker of the *Drosophila* circadian system is located in the brain and is composed of approximately 150 clock neurons, which are organized into a network of oscillators (Nobrega and Lyons 2017; Mendoza-Viveros *et al.*, 2017). These clock neurons are anatomically divided into seven major clusters: the dorsal neurons DN1, DN2 and DN3; the lateral posterior neurons (LPNs); the dorsolateral neurons (LNDs); the large ventrolateral neurons (l-LN_{vs}) and the small ventrolateral neurons (s-LN_{vs}) (Mendoza-Viveros *et al.*, 2017). The small and large ventrolateral neurons control the morning peak of activity and the evening peaks of activity are control by dorsolateral and dorsal neurons (Nobrega and Lyons 2017; Mendoza-Viveros *et al.*, 2017).

The positive regulatory elements in the *Drosophila* core molecular oscillator are the basic-helix-loop-helix (bHLH) containing circadian transcription factors CLOCK (CLK) and CYCLE (CYC). CLOCK (CLK) gets phosphorylated and forms a heterodimeric complex with CYCLE (CYC) to promote transcription of *period* (*per*), *timeless* (*tim*), *vrille* (*vri*), *clockwork orange* (*cwo*) and hundreds of clock-controlled output genes by binding to E1 E-boxes on the promoter (Figure 1.5 & Figure 1.6) (Hardin 2009; Tataroglu and Emery 2014; Ceriani *et al.*, 1999; van Gelder *et al.*, 2003; Nobrega and Lyons 2017). In the *per/tim* loop, the mRNA and protein levels of *per* and *tim* cycle every 24-hours (Ceriani *et al.*, 1999; Plautz *et al.*, 1997; Leloup *et al.*, 1999). PER and TIM accumulate during the night and form a heterodimer (Tataroglu and Emery 2014; Zeng *et al.*, 1996; Gekakis *et al.*, 1995). PER forms a complex with DOUBLETIME (DBT) kinase, a homolog of mammalian casein kinase 1 ϵ (Hardin 2009; Price *et al.*, 1998; Kloss *et al.*, 1998; Kloss *et al.*, 2001). PER-DBT then binds TIM to form a complex DBT-PER-TIM and accumulate in the cytoplasm (Hardin 2009; Curtin *et al.*, 1995; Gekakis *et al.*, 1995; Zeng *et al.*,

1996). Phosphorylation of PER by casein kinase 2 (CK2) and TIM by Shaggy (SGG), a homolog of mammalian glycogen synthase kinase 3, promotes nuclear localization of PER-DBT and TIM (Hardin 2009; Tataroglu and Emery 2014; Akten *et al.*, 2003; Lin *et al.*, 2002; Martinek *et al.*, 2001). DBT-PER-TIM complex then binds to the CLK-CYC heterodimer and promotes DBT-dependent CLK phosphorylation, which inhibits transcription initiation activity of CLK-CYC by reducing its affinity for DNA and releasing it from E1 E-box on the promoter (Menet *et al.*, 2010; Yu *et al.*, 2009; Hardin 2009; Tataroglu and Emery 2014). This forms a negative feedback loop to repress transcription of *per* and *tim*. PER-mediated repression is strengthened by binding of CWO to the E1 E-box and ultimate displacement of CLK-CYC from the promoter (Figure 1.6) (Hardin 2009). Therefore, the pace of the *per/tim* loop is strictly coordinated by the activity of kinases [such as DOUBLETIME (DBT), SHAGGY and casein kinase 2 (CK2)] and phosphatases (such as PP1 and PP2A), which ultimately regulate PER/TIM stability and sub-cellular localization (Franco *et al.*, 2018). Furthermore, VRI has also been seen to repress activation of *clk* and *tim* (van Gelder *et al.*, 2003; Zhang and Kay 2010). Repression of transcription by the CLK-CYC complex is also controlled by TIM (Hardin 2009). During the daytime PER and TIM are gradually modified by phosphorylation and this ultimately leads to their degradation to release CLK-CYC to start a new cycle by activating transcription (Tataroglu and Emery 2014; Hardin 2009).

The PAR (**P**roline and **A**cidic amino acid-**R**ich) transcription factor *vrille* (*vri*) and PAR domain protein 1 ϵ and δ (Pdp11 ϵ/δ) are the components of the *Clk* feedback loop (Figure 1.6) (Hardin 2011). In the *Clk* feedback loop, CLK-CYC binds to E-boxes to activate *vri* transcription (Hardin 2011). As VRI protein accumulates, VRI binds to VRI/PDP1-boxes (V/P-boxes) in the *Clk*

promoter and thereby represses *Clk* transcription (Hardin 2011). As PER-TIM-DBT complex feeds back to inhibit CLK-CYC dependent transcription, *vri* mRNA and protein levels decline and it permits the activation of *Clk* transcription (Hardin 2011). PAR domain protein 1 ϵ and δ (*Pdp11 ϵ / δ*) is also important for *Clk* activation (Hardin 2011). CLK-CYC binds to the E-box of *Pdp11 ϵ / δ* and activates its transcription (Hardin 2011). When the *Pdp11 ϵ / δ* levels rise, *Pdp11 ϵ / δ* binds to V/P-boxes to activate *Clk* transcription (Hardin 2011). The next cycle of *vri* transcription and VRI-dependent repression get started with the degradation of PER complexes during the mid-day (Hardin 2011).

In addition to *per* and *Clk* feedback loops, there is another feedback loop in *Drosophila*, which is controlled by bHLH-orange transcription inhibitor CLOCKWORK ORANGE (CWO) (Hardin 2011). In this feedback loop, CLK-CYC binds E-boxes to activate *cwo* transcription and CWO then feeds back to inhibit CLK-CYC transcription (Hardin 2011).

The *per* and *Clk* feedback loops control rhythmic transcription in opposite phases in the circadian cycle (Hardin 2011). CLK-CYC dependent activation and PER-TIM-DBT dependent repression control the rhythms in *vri* and *Pdp11 ϵ / δ* transcription and therefore the *per* feedback loop is required for the function of the *Clk* feedback loop (Hardin 2011). On the other hand, the *Clk* loop is not necessary for the *per* feedback loop (Hardin 2011).

The *Drosophila* circadian system receives light information via three independent pathways: through synaptic connections from (1) the compound eye and ocelli and (2) the Hofbauer-Buchner eyelets and (3) through the CRYPTOCHROME (CRY) photoreceptor (Mendoza-Viveros *et al.*, 2017). But the molecular clock receives light signals from the environment only through the blue light photoreceptor CRYPTOCHROME (CRY) (Tataroglu and Emery

2014; Emery *et al.*, 2000; Stanewsky *et al.*, 1998; Emery *et al.*, 1998, Mendoza-Viveros *et al.*, 2017). When exposed to light, CRY absorbs photons and undergoes a conformational change, which allows it to bind to TIM (Hardin 2009; Tataroglu and Emery 2014). This promotes TIM ubiquitination and proteasome-dependent degradation and thereby it resets the oscillator to local time (Hardin 2009; Tataroglu and Emery 2014). This indicates that light regulates the TIM protein levels and CRY protein interacts with TIM to stop formation of the PER-TIM complex (Ceriani *et al.*, 1999; Leloup *et al.*, 1999; Cashmore *et al.*, 1999).

1.4.2: Mammalian clock

The intrinsic molecular clock coordinates external signals with physiology and behavioral patterns of mammals (Partch *et al.*, 2014). Circadian synchronization permits anticipation of regular environmental changes and these influences molecular as well as behavioral patterns important for fitness and survival, such as food intake and metabolism, predator/prey interactions, activity-rest or sleep –wake cycles, body temperature, blood pressure, hormone levels and the evasion of DNA damage from environmental insults (Mohawk *et al.*, 2012; Partch *et al.*, 2014; Lowrey and Takahashi 2004). A hierarchy of oscillators functions at the cellular, tissue and system levels in mammals to form the circadian system and it includes a central clock located at the suprachiasmatic nucleus (SCN) of the hypothalamus as well as peripheral clocks found throughout the body (Mohawk *et al.*, 2012; Mendoza-Viveros *et al.*, 2017; Honma 2018).

A population of neuronal circadian oscillators present in the central suprachiasmatic nucleus (SCN) of the hypothalamus acts as a master pacemaker to coordinate with the rest of the body and thereby to drive

behavioral and physiological rhythms (Mohawk *et al.*, 2012; Robinson and Reddy 2014; Mendoza-Viveros *et al.*, 2017). The SCN is made up of approximately 20000 neurons and each have individual circadian oscillators that are coupled together to form a highly unified synchronous circadian network (Partch *et al.*, 2014; Mohawk *et al.*, 2012; Zhang and Kay 2010; Mohawk and Takahashi 2011, Mendoza-Viveros *et al.*, 2017). The master/central clock in the SCN is the only molecular clock that receives light input (photic signals) from the retina through photoreceptor cell type ipRGC (intrinsically photoreceptive retinal ganglion cell) via the retino-hypothalamic-tract (RHT) and it synchronizes internal peripheral clock timing to the external solar day (Partch *et al.*, 2014, Honma 2018). ipRGC acts as an optimal circadian photoreceptor, but rod and cone photoreceptors also participate to provide photic inputs to the central SCN based oscillator (Mohawk *et al.*, 2012). In addition to light, circadian clocks can be entrained via non-photic entrainment pathways which includes temporal cues such as temperature, food intake, stress and exercise (Mendoza-Viveros *et al.*, 2017). These external signals are then passed to peripheral clocks via endocrine and systemic cues (Partch *et al.*, 2014; Buhr *et al.* 2010; Dibner 2010). The central oscillator is coupled with individual peripheral oscillators to ensure that peripheral clocks are appropriately synchronized, but oscillators in the peripheral tissues function independently of the central oscillator (Robinson and Reddy 2014; Mohawk *et al.*, 2012; Zhang and Kay 2010). Molecular clocks located in the neurons of the SCN (Central circadian clocks) and throughout the peripheral tissues (Peripheral circadian clocks) have the equivalent molecular architecture and capacity to generate continuous circadian rhythms (Partch *et al.*, 2014; Yoo *et al.*, 2004). There is a key difference between the master and peripheral clocks in the degree of their intercellular coupling (Partch *et al.*, 2014; Liu *et al.*, 2007).

The peripheral oscillators found in the fibroblast, liver, kidneys, lungs and other tissues are not robust and can be easily entrained by systemic changes in low-amplitude body temperature cycles, circulating hormones and other metabolic cues, whereas in the central oscillator, a high degree of intercellular coupling among neurons in the SCN forms a neuronal network, which makes it more resistant to entrainment from internal cues (Partch *et al.*, 2014; Mohawk *et al.*, 2012; Buhr *et al.*, 2010; Ralph *et al.*, 1990; Brown *et al.*, 2002; Saini *et al.*, 2012).

Two interlocking transcription translation feedback loops (TTFL) that function together form the cell-autonomous molecular clock in mammals to produce robust circadian rhythms (Partch *et al.*, 2014; Robinson and Reddy 2014). In addition to that, the cycling clock genes of the TTFL induce numerous other output genes to facilitate timing of cellular processes (Robinson and Reddy 2014). The mammalian core TTFL is comprised of the master transcription factors CLOCK (Circadian locomotor output cycle kaput) or the paralog of CLOCK, NPAS2 (Neuronal PAS domain-containing protein) and BMAL1/2 (brain and muscle Arnt-like protein) which act as transcriptional activators and the two repressors, PER (Period: *Per1-3*) and CRY (Cryptochrome: *Cry1/2*) (Figure 1.7) (Partch *et al.*, 2014; Robinson and Reddy 2014; Mohawk *et al.*, 2012 ; Mendoza-Viveros *et al.*, 2017). Kinases (CKI α , CKI δ , CKI ϵ) and phosphatases (PP1, PP5) regulate the phosphorylation and thereby mediate the cellular localization as well as the stability of the integral clock proteins that form the core TTFL (Partch *et al.*, 2014). CLOCK and BMAL1 form a heterodimeric complex by interaction between basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) that act as a transcription factor CLOCK: BMAL1 (Mendoza-Viveros *et al.*, 2017). With the help of other transcriptional co-factors, CLOCK: BMAL1 activate transcription of the

repressor *Per1/2/3* and *Cry1/2* genes as well as the other clock-controlled genes via interaction with E-box promoter elements (Partch *et al.*, 2014; Robinson and Reddy 2014; Mohawk *et al.*, 2012; Mendoza-Viveros *et al.*, 2017). PER and CRY form heterodimeric complexes in the cytoplasm and PER-CRY complexes are subsequently translocated to the nucleus where they interact with CLOCK: BMAL1 to inhibit further transcriptional activation and thus repress the PER and CRY transcription (Partch *et al.*, 2014; Robinson and Reddy 2014; Mohawk *et al.*, 2012; Mendoza-Viveros *et al.*, 2017). The inhibitory PER-CRY complexes are degraded in the proteasome through an ubiquitin (Ub)-dependent pathway following phosphorylation by casein kinase I ϵ (CKI ϵ) and other kinases are also involved in degradation of PER and CRY (Partch *et al.*, 2014; Robinson and Reddy 2014; Mohawk *et al.*, 2012). The ubiquitination removes the repression on CLOCK: BMAL1 and the feedback loop begins again with a ~24 hours periodicity (Partch *et al.*, 2014; Robinson and Reddy 2014; Mohawk *et al.*, 2012).

In addition to the core molecular loop, secondary interlocking feedback loops have been identified, that add more complexity to the circadian rhythm regulation mechanism (Honma 2018; Allada *et al.*, 2015). The retinoic acid receptor-related orphan receptors, REV-ERB α/β and ROR α bind to enhancer elements on the *Bmal1* promoter to inhibit or promote transcription, respectively (Robinson and Reddy 2014; Mohawk *et al.*, 2012; Tsuchiya *et al.*, 2009; Zhang and Kay 2010; Preitner *et al.*, 2002). Oscillation in transcription of REV-ERB α/β and ROR α determines the rhythmic expression of BMAL1 and the CLOCK: BMAL1 complex acts directly on REV-ERB α/β gene to form an ‘accessory’ loop (Robinson and Reddy 2014).

1.4.2.1: Circadian rhythms and human health

Synchronization of organisms' physiological and behavioral patterns with 24-hour day-night cycles in the natural environment has a huge impact on human health. The circadian clock in cells of organisms is the internal regulator that coordinates physiological and behavioral activities with daily environmental variations within 24-hour cycles. In humans, almost all physiological and behavioral processes are rhythmic and these include body temperature, food intake and metabolism, hormone levels (melatonin, cortisol, gonadal steroids, prolactin, thyroid hormone, growth hormone, insulin, leptin, ghrelin and adiponectin), respiration, blood pressure, activity-rest or sleep-wake cycles, predator/prey interactions, heart rate and the evasion of DNA damage from environmental insults (Mohawk *et al.*, 2012; Partch *et al.*, 2014; Lowrey and Takahashi 2004; Gnocchi and Bruscalupi 2017). Misalignment of the circadian clock with environmental cues disrupts the rhythmic physiological and behavioral activities. Therefore, disruption of the circadian clock has been linked to multiple disorders in humans including metabolic syndrome (too much fat around the waist, elevated blood pressure, high triglycerides, elevated blood sugar, low HDL cholesterol, heart attack, stroke, and diabetes), sleep disorders, cancer, cardiovascular disease and age-related diseases (Sancar and Brunner 2014; Bass 2012; Hower *et al.*, 2018). Not getting enough sun or natural light during the day, exposure to bright light at night, continuously shifting light – dark schedule (Shift workers), consumption of caffeine at night, not having a consistent sleep schedule and traveling between different time zones (repeated jet-lag) are some of the well known reasons for misalignment of the circadian clock with the environment, which leads to disruption of clock gene expression patterns. Human shift-workers, who are subject to circadian rhythm disruption, have a higher incidence of serious long-term health risks

such as increased risk of cancer (breast, colorectal, endometrial and prostate cancers), diabetes, obesity, strokes and cardiovascular disease (Bass 2012; O'Neill and Reddy 2011; Reddy and O'Neill 2010; Portaluppi *et al.*, 2012; Roenneberg and Merrow 2016; Hower *et al.*, 2018).

Alzheimer's disease, Huntington's disease and dementia are also associated with disrupted sleep patterns in humans (Reddy and O'Neill 2010). Children with autism have exhibited severely disrupted sleep - wake cycles and sleep disorders have been linked to substance abuse and addiction, which in turn lead to disruption of the circadian rhythm (Glickman 2010; Hasler *et al.*, 2012). Therefore, therapy implicating sleep-wake cycles, feeding –fasting cycles and light-dark entrainment of the circadian system by using artificial zeitgebers can be used as treatment of these diseases and addictions (Hasler *et al.*, 2012; Roenneberg and Merrow 2016). Furthermore, awareness of the link between human health, circadian rhythms, behavior and physiology of the human body and environmental factors such as light, food, temperature, etc. is beneficial for human welfare.

1.4.3: Plant clock

Circadian clocks synchronize numerous biological events with the environment to enhance the growth and fitness of plants (Hsu and Harmer 2014; Harmer 2009). The rhythms in stomatal conductance were described almost 100 years ago by Francis Darwin (Kim *et al.* 2017). In many plant species, the circadian system controls many essential processes, such as photosynthetic activity, hypocotyl elongation, leaf movement, hormone signaling, metabolism, chlorophyll accumulation, CO₂ fixation, floral transition, cold/freezing tolerance, drought tolerance, pathogen response, plant growth and fragrance emission (Seo and Mas 2014; Harmer 2009; Nagel and

Kay 2012; Oakenfull and Davis 2017; Kim *et al.*, 2017). The plant transcriptome contains a large fraction of clock-controlled genes to modulate diverse signals and metabolic pathways for regulating development and environment adaptation (Seo and Mas 2014; Nagel and Kay 2012). *Arabidopsis thaliana* is one of the best-studied model plants in circadian research (Salome and McClung 2004; Nagel and Kay 2012). Studies via genomic, transcriptomic and proteomic approaches were able to identify clock components and downstream targets to construct the molecular architecture of the *Arabidopsis* circadian clock, which consist of three interlocked transcriptional-translational feedback loops (Figure 1.8) (Hsu and Harmer 2014; Nagel and Kay 2012; Harmer 2009).

In *Arabidopsis*, the first identified core negative feedback loop of the circadian oscillator consists of three components (Harmer 2009). These components are two MYB-like transcription factors CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) along with TIMING OF CAB EXPRESSION 1 (TOC1), which is also known as PSEUDO-RESPONSE REGULATOR 1 (PRR1) (Figure 1.8) (Hsu and Harmer 2014; Harmer 2009; Oakenfull and Davis 2017; Nohales and Kay 2016). CCA1 and LHY are acting as morning phase components and when their transcript and protein levels are highly abundant, they physically interact with each other to form a heterodimeric complex (Lu *et al.*, 2009; Yakiri *et al.*, 2009; Hsu and Harmer 2014; Nohales and Kay 2016). CCA1 and LHY repress the expression of evening-phase component TOC1 via binding to the *cis*-regulatory promoter motif called the evening element (EE) in the TOC1 promoter and this makes up the negative arm of the *Arabidopsis* core oscillator (Hsu and Harmer 2014; Harmer *et al.*, 2000; Alabadi *et al.*, 2001; Wang and Ma 2013; Oakenfull and Davis 2017; Nohales and Kay 2016). During the

evening, the nuclear localized TOC1 promotes the expression of CCA1 and LHY to form a transcriptional feedback loop to repress each other's expression (Hsu and Harmer 2014; Harmer 2009; Nohales and Kay 2016). The regulation mechanism of the expression of CCA1 and LHY by TOC1 remains unknown (Wang and Ma 2013).

In the morning-phased loop, CCA1 and LHY function as an activator for the day phase clock genes such as PSEUDO-RESPONSE REGULATOR 7 (PRR7), PSEUDO-RESPONSE REGULATOR 9 (PRR9) and PSEUDO-RESPONSE REGULATOR 5/NIGHT INHIBITOR (PRR5/NI), which subsequently downregulate the expression of CCA1 and LHY by binding to their promoters (Wang and Ma 2013; Hsu and Harmer 2014). By forming the evening-phase loop, CCA1 and LHY have also been shown to repress the expression of several evening genes such as GIGANTEA (GI), a large plant-specific protein, LUX ARRHYTHMO (LUX, also called PHYTOCLOCK1), EARLY FLOWERING 3 and 4 (ELF3 and ELF4), BROTHER OF LUX ARRHYTHMO (BOA, also known as NOX, meaning “night” in Latin) (Wang and Ma 2013; Hsu and Harmer 2014). DEETIOLATED1 (DET1), a key repressor in photomorphogenesis controls the repression of evening genes by CCA1 and LHY (Hsu and Harmer 2014; Nagel and Kay 2012). Furthermore, LUX, ELF3 and ELF4 promote the expression of CCA1 and LHY through an indirect mechanism (Nagel and Kay 2012). Deletion of even one component of the evening-loop can result in photoperiod insensitive plants which show early flowering, long hypocotyl growth and arrhythmicity in the free-running circadian period (Oakenfull and Davis 2017).

Light and temperature are the major entrainment signals for plants. Light is perceived by multiple photoreceptors such as the blue light photoreceptor ZETLUPE (ZTL), phytochromes, cryptochromes and UV

RESISTANCE LOCUS 8 (UVR8) (Oakenfull and Davis 2017; Nohales and Kay 2016).

The plant circadian clock consists of a central loop interlocked with the “morning” and “evening” loops (Wang and Ma 2013). In addition to transcription regulation, many post-transcriptional regulatory mechanisms such as post-translational regulation and chromatin remodeling control the circadian rhythmicity (Nagel and Kay 2012; Hsu and Harmer 2014).

Understanding how the plant circadian oscillator regulates biological processes is useful for agronomic issues. Correct coordination of circadian period with anticipated external environmental cues may increase the plant vegetative growth and ultimately enhance the productivity of crops. With the increasing population and availability of cultivable land, the development of methods that can be used to enhance the crop yield is an advantageous approach.

1.4.4: *Neurospora crassa* clock

1.4.4.1: *Neurospora crassa* life cycle

Neurospora crassa is a heterothallic, non-pathogenic fungus belonging to the Ascomycete genus (Galagan *et al.*, 2003; Davis 2004). As a saprophytic organism, *Neurospora crassa* can grow on carbohydrate rich substrates such as wood, bread, and the residues of sugar processing plants (Davis 2004). It is a coenocytic organism, which produces true hyphae as tubular structures consisting of cellular compartments that are usually defined by incomplete septa and thereby share several nuclei in the same cytoplasm (Galagan *et al.*, 2003; Davis 2000).

The lifecycle of *N. crassa* involves a sexual phase and an asexual or vegetative phase. The haploid asexual phase is simple. During this asexual life cycle, multinucleated mycelia that originate from sexual (ascospore) or asexual (conidium) spores grow radially and form aerial hyphae and these aerial hyphae produce multinucleate asexual spores (macroconidia) through branching, budding at the tips and segmenting (Davis 2004; Davis 2000). The ascospore germination requires heat activation for germination and conidial cells germinate spontaneously.

The sexual phase requires the participation of mycelia of different mating types, designated as A and a (Davis 2004). The two mating types are morphologically indistinguishable from each other and strains of either mating type can act as “females” or “males” (Aramayo and Selker 2013). Under nutrient limiting conditions, *N. crassa* activates its sexual phase. The female structures (protoperithecia) contain the female gamete, the ascogonial cell, and it is fertilized by a macro- or microconidium of the opposite mating type (Davis 2004; Davis 2000). Cell fusion is followed by many mitotic divisions of the haploid nuclei of the two mating types, nuclear fusion, meiosis of the diploid nucleus, mitosis and the formation of the ascus, which yields 8 haploid ascospores (Davis 2004; Davis 2000).

1.4.4.2: *Neurospora crassa* as a model organism for circadian research

Neurospora crassa is one of the well-established model organisms for investigating several aspects of biochemistry, ecology, evolutionary genetics, photobiology, population biology, fungal morphogenesis, mitochondrial import, DNA repair as well as recombination mechanisms, DNA methylation, epigenetic processes, cellular processes (polarized growth, exocytosis, endocytosis, cytoskeleton-based organelle traffic) and the molecular mechanism

of circadian rhythmicity (Lakin-Thomas 2006a; Koritala and Lee 2017 ; Aramayo and Selker 2013). Compared to prokaryotic and other eukaryotic model organisms, *Neurospora crassa* provides several advantages as a model organism for circadian research (Galagan *et al.*, 2003; Roche *et al.*, 2014; Lakin-Thomas 2006a; Zamborszky *et al.*, 2014). As a microorganism, it can be easily grown in cultures and due to its fast growth rate and short life cycle, large amounts of material can be harvested to make biochemical analysis easy. *Neurospora crassa* can generate a high number of progeny via sexual as well as asexual reproduction, which make it an excellent model organism for population genetics studies (Lakin-Thomas *et al.*, 2011; Roche *et al.*, 2014; Koritala and Lee 2017). The maintenance of *Neurospora crassa* strains in the laboratory is easy and economical (Koritala and Lee 2017).

Neurospora crassa is a haploid organism and this has given it advantages for genetic analysis in the ease of isolation of new mutations and the expression of mutant phenotypes in the haploid (Lakin-Thomas *et al.* 2011). There is a huge collection of mutant strains including an almost complete catalogue of knockout strains and all the details are available online through the Fungal Genetics Stock Center (FGSC). These strains are useful for understanding the gene functions and associated morphology (Lakin-Thomas *et al.* 2011; Koritala and Lee 2017).

The approximately 40-megabase size of the *Neurospora* genome consist of 7 chromosomes (Linkage groups) and about 10000 protein-coding genes (Galagan *et al.* 2003; Roche *et al.* 2014). The *Neurospora crassa* genome has been sequenced and is available at the FungiDB database (Stajich *et al.*, 2012; Galagan *et al.*, 2003; Lakin-Thomas *et al.*, 2011). Well established transformation methods are routinely used for *N. crassa* and most molecular genetics methods, such as use of inducible and constitutive promoters for

dosage control, RNAi based gene silencing mechanisms operating in mitotic and meiotic cells, selectable markers, methods of targeted gene disruption, CRISPR-Cas 9 (Clustered Regularly Interspaced Short Palindromic Repeats) genome editing system and integration of recombinant DNA are available for *Neurospora* (Lakin-Thomas *et al.*, 2011; Roche *et al.*, 2014; Hurley *et al.*, 2016). Modern imaging techniques such as use of luciferase, GFP and mCherry can be applied to *Neurospora* (Lakin-Thomas *et al.*, 2011). Signaling pathways, metabolic pathways, and organelles such as mitochondria have been extensively studied in *Neurospora* (Davis 2000; Lakin-Thomas *et al.*, 2011).

Easily observable and measurable circadian regulated behavior is important for circadian rhythms associated studies. The primary clock output, the conidiation developmental pathway, has been extensively studied and strains carrying the *bd* (band) mutation can produce easily observed visible rhythms of asexual spore formation (conidiation) as a circadian output on petri dishes or long glass “race tubes” containing solid agar medium (Lakin-Thomas *et al.* 2011; Roche *et al.* 2014). A race tube is a long glass tube that is bent up at both ends to hold the solid growth medium (Figure 1.9). At one end of the race tube a few spores are inoculated and the fungus grows towards the other end of the tube. The cultures are allowed to germinate and grow in constant light for a day to synchronize the cells to the same time of the day and then transferred to constant darkness to develop free running rhythms. Under these conditions, *Neurospora* demonstrates a free running period around 22.5 hours for asexual spore development (Loros and Dunlap 2001; Montenegro-Montero *et al.*, 2015). The conidiation rhythm is seen as a pattern of thick bands of orange colored spore production alternating with “interbands” with few spores and primary apical extension of filamentous mycelium (Lakin-Thomas *et al.*, 2011; Montenegro-Montero *et al.*, 2015). The *bd* (band) mutation was critical for

Neurospora to become a pioneer genetic model system for circadian clocks, which allowed ultimately the investigation of the genetic, molecular and biochemical foundation of rhythmicity in eukaryotes (Roche *et al.*, 2014). In the strains that do not carry the *bd* mutation, the rhythmic asexual spore formation in race tubes is suppressed by the accumulated CO₂, which is a by-product of respiration.

Molecular rhythms can be assayed either in liquid medium or in the same solid agar cultures in which the conidiation rhythms are observed (Lakin-Thomas *et al.*, 2011). Several clock-controlled genes (ccgs), genes that affect clock function, and output pathways through which the clock controls cellular functions are being identified and characterized (Lakin-Thomas *et al.*, 2011; Correa *et al.*, 2003; Vitalini *et al.*, 2007).

1.4.4.3: The transcriptional-translational feedback loop

In *Neurospora* the core FRQ/WCC oscillator (FWO) acts as the transcriptional-translational feedback loop (TTFL) (Nsa *et al.*, 2015; Lakin-Thomas *et al.*, 2011; Lakin-Thomas and Brody 2004). As shown in the Figure 1.10, the core FRQ/WCC oscillator contains negative elements FREQUENCY (FRQ) and FRQ-interacting RNA helicase (FRH), the blue light photoreceptor WHITE COLLAR 1 (WC-1) and WHITE COLLAR 2 (WC-2) (Nsa *et al.*, 2015; Lakin-Thomas *et al.*, 2011; Lakin-Thomas and Brody 2004). WC-1 and WC-2 proteins bind together to form a heterodimeric transcription complex (the white-collar complex or WCC) that functions as the positive regulator in the oscillator loop to regulate transcription of the negative element gene *frq* (Nsa *et al.*, 2015; Lakin-Thomas *et al.*, 2011; Lakin-Thomas and Brody 2004; Liu and Bell-Pedersen 2006; Dunlap and Loros 2006). WC-1 and WC-2 are PAS domain-containing GATA –type zinc finger transcription factors primarily

located in the nucleus of cells (Ballario *et al.*, 1996; Lee *et al.*, 2000; Linden and Macino 1997; Schwerdtfeger and Linden 2000; Lakin-Thomas *et al.*, 2011). WCC rhythmically binds to the Clock box (C box) or light response element (LRE) on the promoter of the *frq* gene and directly activates transcription of the *frq* gene (Froehlich *et al.*, 2003). After synthesis and accumulation of FRQ protein, it dimerizes by self-associating via a coil-coil region near the N-terminus and interacts with FRH to form a complex (the FRQ/FRH complex or FFC) (Cheng *et al.*, 2001b; 2005; Zhou *et al.*, 2018). FRQ-FRH complex, which acts as a negative regulator, enters the nucleus, physically interacts with WCC and promotes phosphorylations to inhibit its activity by recruiting Casein kinase I (Schafmeier *et al.*, 2005; He *et al.*, 2006; Liu and Bell-Pedersen 2006; Zhou *et al.*, 2018). Inhibition of WCC activity by phosphorylation suppresses its binding to the *frq* gene promoter and this results in reduced *frq* transcription and FRQ protein levels (Nsa *et al.*, 2015; Brunner and Schafmeier 2006; Lakin-Thomas *et al.*, 2011; Dunlap and Loros 2006). When FRQ protein levels are sufficiently decreased, FRQ-FRH directed inhibition of the activity of WCC is released via dephosphorylation and the cycle reactivates (Nsa *et al.*, 2015; Liu and Bell-Pedersen 2006; Brunner and Schafmeier 2006; Lakin-Thomas *et al.*, 2011; Dunlap and Loros 2006). FRQ is an Intrinsically Disordered Protein (IDP) and it does not have an enzymatic function (Hurley *et al.*, 2016). But the FRQ protein acts as a scaffold to hold the components of the oscillator together (Hurley *et al.*, 2016). However, FRQ protein is also involved in increasing the levels of WC-1 and WC-2 at either transcriptional (wc-2) or translational (wc-1) steps via a positive feedback loop (Liu and Bell-Pedersen 2006; Brunner and Schafmeier 2006; Lakin-Thomas *et al.*, 2011; Cheng *et al.*, 2001; Lee *et al.* 2000). Therefore, generation of

endogenous circadian rhythmicity is due to the cyclic activation, repression and reactivation of *frq* expression (Jinhu and Yi 2010).

Even though phosphorylation and dephosphorylation of FRQ is important for clock function, recent findings suggest that degradation of hyperphosphorylated FRQ is not necessary for the timing mechanism (Jinhu and Yi 2010; Larrondo *et al.*, 2015; Montenegro-Montero *et al.*, 2015). Newly synthesized FRQ proteins get hyperphosphorylated and attached to FWD-1 (a component of ubiquitin ligase: F-box/WD-40 repeat containing protein), which signals for FRQ protein degradation by ubiquitin/proteasome pathway (He and Liu 2005; Lakin-Thomas *et al.*, 2011). Calcium/calmodulin dependent kinase (CAMK-1), casein kinase 1a (CK-1a), casein kinase II (CKII), PRD-4 (chk2) and protein kinase A (PKA) have been identified as FRQ phosphorylating kinases (Lakin-Thomas *et al.*, 2011; Jinhu and Yi 2010). CK-1a and CKII are the main kinases that phosphorylate FRQ (Jinhu and Yi 2010). PP1, PP2A and PP4 are the protein phosphatases that regulate the phosphorylation status of FRQ and WCC by dephosphorylating them (Lakin-Thomas *et al.*, 2011; Jinhu and Yi 2010).

Circadian rhythms were observed in the levels of FRQ RNA and protein (Brunner and Schafmeier 2006; Lakin-Thomas *et al.*, 2011; Aronson *et al.*, 1994; Froehlich *et al.*, 2003). The levels of WC transcripts do not show rhythms, but WC-1 protein levels cycle and WC-2 do not cycle (He *et al.*, 2002; Lee *et al.*, 2000; Lakin-Thomas *et al.*, 2011).

WC-1 also acts as a blue light photoreceptor as it contains a specialized PAS domain called a LOV (light, oxygen and voltage) domain, which allows the fungus to sense the environment to reset and entrain the endogenous biological clock based on light signals (Lakin-Thomas *et al.*, 2011; Froehlich *et al.*, 2002; He *et al.*, 2002, He and Liu 2005). Light induces rapid binding of

WCC to the light responsive element (LRE) on the promoters of *frq* and other light induced genes to activate their transcription (Jinhu and Yi 2010; He and Liu 2005). In light / dark cycles, another light inducible blue light receptor, VVD (VIVID), which is a small PAS/LOV domain containing protein, physically interacts with the WCC to act as a light response repressor by forming an additional negative feedback loop (Zhou *et al.*, 2018; Gyöngyösi *et al.*, 2017). VVD is not required for *Neurospora crassa* clock function (Zhou *et al.*, 2018; Proietto *et al.*, 2015). However, it controls WCC linked circadian clock properties such as the pathway of light input to the clock and phasing light resetting responses (Zhou *et al.*, 2018).

1.4.4.4: The FRQ-less oscillator

Several studies on circadian rhythmicity have provided evidence for the presence of multiple oscillators, which are independent of the canonical clock gene network in cells and/or tissues (Nsa *et al.*, 2015; Lakin-Thomas *et al.*, 2011; de Paula *et al.*, 2006; Hunt *et al.*, 2012; Brody *et al.*, 2010). Therefore, well-established TTFLs cannot be used to explain the circadian rhythms in many organisms and researchers are focusing on non-transcriptional oscillators (Adhvaryu *et al.*, 2016; van Ooijen and Millar 2012; Reddy and Rey 2014).

During investigations of clock mechanisms in *Neurospora crassa*, several studies have shown the existence of rhythms in the absence of functional FRQ-WCC TTFL under certain growth conditions and/or specific genetic backgrounds (Nsa *et al.*, 2015; Lakin-Thomas *et al.*, 2011; de Paula *et al.*, 2006; Hunt *et al.*, 2012; Brody *et al.*, 2010). These include both developmental (conidiation) as well as molecular/biochemical rhythms observed in strains with null mutations in *frq* and *wc* genes (Aronson *et al.*, 1994; Dragovic *et al.*, 2002; Granshaw *et al.*, 2003; Lakin-Thomas 2006c;

Lakin-Thomas and Brody 2000; Lombardi *et al.*, 2007; Loros and Feldman 1986; Merrow *et al.*, 1999; Yoshida *et al.*, 2008; Schneider *et al.*, 2009; Brody *et al.*, 2010; Christensen *et al.*, 2004; Correa *et al.*, 2003; Ramsdale and Lakin-Thomas 2000; de Paula *et al.*, 2006; Lakin-Thomas *et al.*, 2011; Hunt *et al.*, 2012; Li *et al.*, 2011; Nsa *et al.*, 2015). Rhythmic conidiation has been seen in clock gene null mutants (*frq* null) grown on extra-long race tubes (Loros and Feldman 1986). Furthermore, rhythms can be induced in strains lacking functional FRQ-WCC TTFL (*frq* null mutant), by adding farnesol or menadione to the medium, by using temperature (warm/cold) cycles and short heat pulses to entrain the rhythm where the entrained phase is dependent on the T cycle (Temperature cycle) or by introducing either the fatty-acid requiring *cel* mutation or the choline requiring *chol-1* mutation (Granshaw *et al.*, 2003; Brody *et al.*, 2010; Lakin-Thomas 2006c; Lakin-Thomas and Brody 1985; Lakin-Thomas 1998 ; Merrow *et al.*, 1999 ; Roenneberg *et al.*, 2005). Rhythmic molecular oscillations in nitrate reductase activity, levels of neutral lipid diacylglycerol (DAG), the expression of the *cgc-16* gene, and the levels of WC-1 protein have also been seen in FRQ-less strains (Ramsdale and Lakin-Thomas 2000; Christensen *et al.*, 2004; de Paula *et al.*, 2006). In addition, double mutant strains *ult: frq¹⁰*, *ult: wc-2 null*, *sod-1:frq¹⁰* and *vvd:frq¹⁰* produce rhythms in the absence of functional FRQ-WCC TTFL (Lakin-Thomas *et al.*, 2011; Schneider *et al.*, 2009; Yoshida *et al.*, 2008).

Moreover, rhythmic spore development has been observed under constant light, in the absence of functional FRQ/WCC oscillator (*frq* and WCC null mutant) in strains carrying the *cog-1* (cry-dependent oscillator gate-1) mutation (Nsa *et al.*, 2015). This novel oscillator does not require the functional FRQ/WCC oscillator, but it does require the blue light photoreceptor CRYPTOCHROME (CRY) (Nsa *et al.*, 2015).

Even though the stability of the negative element FRQ is assumed to be the period determining factor of the feedback loop, recent research has shown the persistence of rhythms when this negative feedback element FRQ is not degraded (Larrondo *et al.*, 2015). This observation also suggests the complex nature of the circadian system.

The existence of FRQ-less rhythms indicates the presence of one or more FRQ-less oscillators (FLO) that generate rhythms in the absence of a functional FRQ/WCC oscillator and these rhythms are called FRQ-less rhythms (Iwasaki and Dunlap 2000; Lakin-Thomas *et al.*, 2011; de Paula *et al.*, 2006; Li *et al.*, 2011; Lakin-Thomas 2006a). Various FRQ-less rhythms have been assayed under different conditions in diverse genetic backgrounds. The number of FLOs, exact nature and components of the FLOs, relationship between FLO and FRQ/WCC oscillator and molecular mechanisms of FLOs are not fully understood (Lakin-Thomas *et al.*, 2011; de Paula *et al.*, 2006; Nsa *et al.*, 2015; Adhvaryu *et al.*, 2016).

Free running rhythms in *chol-1* mutant strains in constant dark and constant temperature in choline-deficient cultures and entrainment using different cyclic heat pulses in constant darkness (which does not depend on the *chol-1* mutation) are the two assay systems we use in our laboratory to study FRQ-less rhythms in *Neurospora crassa* (Lakin-Thomas 2006c; Lakin-Thomas 1996; Lakin-Thomas 1998; Lakin-Thomas and Brody 2000; Adhvaryu *et al.*, 2016). Using both these assay systems, our laboratory has shown the existence of rhythmic behavior, which is an indicator of a functional oscillator in *Neurospora crassa frq* or *wc-1* null mutant strains (Adhvaryu *et al.*, 2016).

1.4.4.4.1: *Chol-1* mutant

One of the most abundant phospholipids in membranes of *Neurospora crassa* is phosphatidylcholine (PtdCho) (Lakin-Thomas 1996). Sequential methylation of phosphatidylethanolamine (PtdEtn) is the usual pathway of phosphatidylcholine (PtdCho) synthesis (Lakin-Thomas 1996). The *chol-1* (choline) mutant strains of *Neurospora crassa* are defective in methylation of phosphatidylethanolamine (PtdEtn) to phosphatidylmonomethylethanolamine (PtdEtn-Me), due to the lack of S-adenosyl methionine phosphatidylethanolamine N- methyl transferase and therefore impaired synthesis of phosphatidylcholine (PtdCho) (Lakin-Thomas 1996). These *chol-1* mutant strains require choline or the precursor monomethylethanolamine (Etn-Me) or dimethylethanolamine (Etn-diMe) for normal growth (Lakin-Thomas 1996). Choline requiring *chol-1* mutant strains shows altered membrane phospholipid composition when growing in choline depleted media (Lakin-Thomas 1996).

Chol-1 mutant strains growing on choline depleted media show reduced growth rate, lengthened period of the conidiation rhythms in inverse proportion to the choline concentration in the medium and impaired temperature compensation (stability) of conidiation rhythms (Lakin-Thomas 1996; Lakin-Thomas *et al.*, 2011). The growth rates as well as the period of the conidiation rhythms of *chol-1* mutant strains become normal and similar to wild type on high levels of choline (above 20 μ M) in the growth medium (Lakin-Thomas 1996).

As mentioned above, rhythmic conidiation can be seen in the absence of functional *frq*, *wc-1* and *wc-2* genes and it can be described as “conditionally rhythmic” since the null mutants can display rhythms under conditions such as introducing additional mutations, by adding chemicals to the growth medium or

by changing the geometry of the culture vessel (Lakin-Thomas *et al.*, 2011). In choline-deprived media, double mutants of the choline-requiring mutant *chol-1* with *frq*, *wc-1* or *wc-2* null mutants produce robust conidiation rhythms (Lakin-Thomas *et al.*, 2011). Therefore, rhythms generated by *chol-1* mutant strains carrying *frq* or *wc-1* or *wc-2* null alleles are important for elucidating the mechanism as well as the architecture of the FRQ-less oscillator (FLO) and the relationship between FRQ-WCC oscillator (FWO) and FLO.

Even though the *chol-1* mutation is important for rhythm generation in strains carrying *frq* or *wc-1* or *wc-2* null alleles, we still don't know the molecular mechanism underneath this conidiation rhythm generation process. Most of our laboratory strains carry the *chol-1* mutation and it is useful to assay the FRQ-less rhythm in choline- depleted cultures. As mentioned above, when *chol-1* mutant strains are growing in media supplemented with choline, the defect in the *chol-1* mutant strain gets repaired and the cultures are indistinguishable from *chol+* strains in our assay system. This phenomenon allows us to assay the other FRQ-less rhythms which do not depend on choline depletion, such as heat pulse entrainment behavior, in the same genotypes (Adhvaryu *et al.*, 2016).

1.4.4.5: Novel mutation affecting FRQ-less rhythms: UV90

Searching for mutations affecting rhythmicity of FRQ-less strains can be used to identify the components of the FLO (Li *et al.* 2011). By using two genetic strategies, our laboratory has found three different mutations; *prd-1*, *prd-2* and UV90 that affect FRQ-less rhythms.

As the first strategy, our lab has looked for FLO-affecting mutations among genes known to affect the FRQ/WCC oscillator (Lakin-Thomas *et al.*, 2011; Li and Lakin-Thomas 2010). The *prd* mutations (*prd-1* and *prd-2*) have

been identified as mutations that affect the period of conidiation rhythms in *frq*⁺ strains. To check the effects on FLO, the *prd* mutant strains were crossed into FRQ-less strains, which resulted in *frq* null and *prd* double mutant strains. The triple mutants of *chol-1*, *frq*¹⁰ and period mutant (*prd-1* or *prd-2*) have been shown to disrupt the rhythmicity in choline-depleted cultures (Lakin-Thomas *et al.*, 2011; Li and Lakin-Thomas 2010). These strains were also grown on high choline to assay the effect of the *prd* mutations on the heat-entrainable oscillator in *frq*¹⁰ and large effects on the timing of the heat-entrainable peaks were observed (Lakin-Thomas *et al.*, 2011; Li and Lakin-Thomas 2010).

As the second strategy, a novel mutation was introduced into the *chol-1: frq*¹⁰ strain and it was shown to disrupt the rhythm without choline in the media (Li *et al.*, 2011). A mutagenesis screen was used to identify this novel FLO affecting mutation (Li *et al.*, 2011). Uninucleate microconidial spores of strains carrying both *frq*¹⁰ knockout allele and *chol-1* mutation were subjected to UV light treatment (Li *et al.*, 2011). Colonies derived from mutagenized spores were arbitrarily numbered and they were individually analyzed on race tubes under high choline and choline deficient conditions (Li *et al.*, 2011). The 90th spore analyzed after UV mutagenesis produced an arrhythmic phenotype in the typical rhythmic *chol-1: frq*¹⁰ strain on choline deficient conditions and this new mutation was named UV90 (Figure 1.11) (Li *et al.*, 2011; Lakin-Thomas *et al.*, 2011).

The heat-entrainable conidiation rhythm in choline-rich cultures was severely affected by the UV90 mutation (Lakin-Thomas *et al.*, 2011). UV90 mutation damps the amplitude of the conidiation rhythms (Figure 1.11) and FRQ protein rhythms when FRQ/WCC oscillator is functional (Li *et al.*, 2011; Lakin-Thomas *et al.*, 2011). Furthermore, UV90 mutant background increases the response of strains carrying a functional FRQ/WCC oscillator to phase

resetting by pulses of either light or high temperature (Li *et al.*, 2011; Lakin-Thomas *et al.*, 2011). Therefore, UV90 gene product is an important component of the FLO and it also interacts with FRQ/WCC oscillator to maintain the amplitude of rhythms, indicating the influence of FLO on FRQ/WCC oscillator (Li *et al.*, 2011; Lakin-Thomas *et al.*, 2011).

1.5: TOR signaling pathway

TOR is an acronym for “target of Rapamycin”. *Saccharomyces cerevisiae* has contributed greatly to discovering TOR and elucidating its functions (Loewith and Hall 2011). In yeast, the proteins encoded by TOR1 and TOR2 are large (2470 and 2474 amino acids respectively), highly similar (67% identical), serine/threonine kinases and structurally related to phosphatidylinositol 3-kinase (Loewith and Hall 2011). TOR is the founding member of the phosphatidylinositol kinase-related protein kinase (PIKK) family (Loewith and Hall 2011).

After the discovery of TOR in *Saccharomyces cerevisiae*, a mammalian equivalent mTOR (also known as FRAP, RAFT or RAPT) was identified (Brown *et al.*, 1994; Chiu *et al.*, 1994, Sabatini *et al.*, 1994, Sabers *et al.*, 1995). Up to the present time, TOR has been identified in all other eukaryotes including fungi, mammals, flies, worms and plants. For example: *S. pombe* (SpTOR; Kawai *et al.*, 2001; Weisman and Choder 2001), *Cryptococcus neoformans* (CnTOR; Cruz *et al.*, 1999), *Candida albicans* (CaTOR; Cruz *et al.*, 2001), *Drosophila melanogaster* (dTOR; Oldham *et al.*, 2000; Zhang *et al.*, 2000), *Caenorhabditis elegans* (CeTOR; Long *et al.*, 2002), unicellular green alga *Chlamydomonas reinhardtii* (CrTOR ; Pérez-Pérez *et al.*, 2017; Pérez-Pérez and Crespo, 2010) and *Arabidopsis thaliana* (AtTOR; Menand *et al.*, 2002). These findings suggest that TOR proteins are highly conserved in all

eukaryotic life forms. Lower eukaryotes like yeast and other fungi (*Saccharomyces cerevisiae*, *S. pombe* and *Cryptococcus neoformans*) contain two TOR genes and higher eukaryotes possess only one TOR gene.

All TOR protein orthologs have a similar domain structure and the presence of many protein-protein interaction domains in TOR1 and TOR2 suggested that the TORs form large complexes. TOR1 and TOR2 proteins in yeast function in two distinct multi-protein complexes named TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Loewith and Hall 2011). TOR complexes have been identified in other eukaryotes, including plants, worms, flies and mammals and each complex appears to be conserved like TOR itself from yeast to man (Loewith and Hall 2011). Furthermore, only TORC1 is sensitive to rapamycin (Loewith and Hall 2011).

In yeast, TOR complex 1 is concentrated on the limiting membrane of the vacuole and in other higher eukaryotes on the limiting membrane of the lysosome, which is the counterpart of yeast vacuoles (Loewith and Hall 2011). Loewith and Hall 2011 suggested TORC2 appears to be at multiple locations, the plasma membrane and one or possibly more other sites. Mammalian TORC2 has commonly been identified in proximity to mitochondria, the endoplasmic reticulum and mitochondria associated membranes (MAM) (Betz and Hall 2013). mTOR2 also appears to be present in the nucleus and at the plasma membrane (Betz and Hall 2013).

The two TOR complexes regulate many characteristics of eukaryote physiology, which are mainly determining the mass of the cell. Therefore, TOR is the central controller of cell growth. Like TOR itself, its function is conserved from yeast to man. TOR proteins perform overlapping functions via the rapamycin sensitive TORC1, and TOR2 performs additional unique functions through the rapamycin resistant TORC2 (Loewith and Hall 2011).

The essential functions mediated by TOR2 have been referred to as the “TOR-shared” function, which is redundant with TOR1, and “TOR2-unique” function that is unique to TOR2. Rapamycin sensitive TORC1, which consists of either TOR1 or TOR2, mediates the TOR-shared pathway and rapamycin insensitive TORC2, which specifically contains TOR2, mediates the TOR2-unique pathway. The temporal control of cell growth is mediated by the TOR-shared function and TOR2-unique function mediates spatial control of cell growth, each via separate signaling pathways which occupy structurally and functionally distinct TOR complexes (Loewith and Hall 2011). TOR-shared signaling pathway controls protein synthesis and degradation, mRNA synthesis and degradation, ribosome biogenesis, nutrient transport, cell cycle progression and autophagy to determine the mass of the cell (Loewith and Hall 2011). TOR2-unique pathway controls cell cycle-dependent polarized organization of the actin cytoskeleton, endocytosis and sphingolipid synthesis (Loewith and Hall 2011).

The availability of nutrients dictates basic cellular functions and determines whether or not cells can grow and develop. Target of rapamycin (TOR) signaling pathway, the major regulator of growth in eukaryotes, integrates both intracellular signals that depend on nutrient availability and extracellular signals to balance protein synthesis and protein degradation in response to nutrient quality and quantity. TOR signalling is active in the presence of sufficient nutrients thereby promoting translation, ribosome biosynthesis, and cell cycle progression. At the same time, it inhibits starvation-induced gene transcription and prevents autophagy.

Yeast TORC1 responds to nutrients such as nitrogen and carbon availability. EGO-GTPase complex, which resides on the vacuolar membrane and its orthologs, was shown to convey amino acid signals for TORC1 activity

in yeast, insects and mammals (Binda *et al.*, 2009; Kim *et al.*, 2008; Gao *et al.*, 2006; Sancak *et al.*, 2008). “Ragulator” complex that resides on the lysosomal membrane is the vertebrate functional homolog of yeast EGO-GTPase complex. Ragulator complex together with Rags convey amino acid sufficiency signals to mTORC1 (Kim and Guan 2011). Both in metazoans and yeast, TORC1 activity responds to glutamine and branched-chain amino acid leucine (Crespo *et al.*, 2002; Binda *et al.*, 2009).

EGO complex responds to intracellular leucine levels and intravacuolar amino acid levels via Gtr1 guanine nucleotide exchange factor Vam6 (Binda *et al.*, 2009). In the Gtr1^{GTP} and Gtr2^{GDP} configuration, the EGO complex activates TORC1 (Loewith and Hall 2011). mTORC1 is regulated by a variety of upstream signals including growth factors and nutrients (Kim *et al.*, 2013). In animals the best-characterized mTORC1 activation route is the insulin-signaling pathway (van Dam *et al.*, 2011). Growth factors bind to a specific receptor tyrosine kinase and it activates the PI3K-Akt and/or Ras-MAPK pathway (Mendoza *et al.*, 2011). These pathways activate mTORC1 primarily by phosphorylating and inhibiting the tuberous sclerosis complex 2 (TSC2), which is in a complex with TSC1 and functions as a GTPase activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain) (Kim *et al.*, 2013). Rheb is active in its GTP-bound form and it activates mTORC1 in response to all stimuli (Kim *et al.*, 2013).

In yeast, the AGC kinase Sch9, PP2A-like phosphatase Sit4 and Tap42-PP2A act as immediate effectors of TORC1 (Rohde *et al.*, 2004; Urban *et al.*, 2007). Ribosomal S6 kinase (S6K) is an immediate effector of mTORC1 and it is analogous to yeast AGS kinase Sch9 (Loewith and Hall 2011). Eukaryotic translational initiation factor, 4E-binding protein (4E-BP) also acts as a substrate of mTORC1 (Laplane and Sabatini 2012; Ma and Blenis 2009).

Protein kinase Ypk acts as an effector of yeast TORC2. Ypk1 and Ypk2 is an essential pair of homologous kinases, which belongs to the AGC kinase family (Loewith and Hall 2011).

The TOR signaling pathway has been extensively studied in yeast and mammals. However, very little is known about the TOR signaling pathway in *Neurospora crassa* and other filamentous fungi that includes *Aspergillus fumigatus*, *Aspergillus nidulans*, *Fusarium oxysporum*, *Podospora anserina*, *Fusarium fujikurio*, *Candida albicans* and *Fusarium graminearum* (Yu *et al.*, 2014; Flanagan *et al.*, 2017; Liu *et al.*, 2017 ; Teichert *et al.*, 2006; Pinan-Lucarré *et al.*, 2006; López-Berges *et al.*, 2010 ; Fitzgibbon *et al.*, 2005; Falloon *et al.*, 2015). Rapamycin or FK506 binding proteins (FKBPs that include FKR-2, FKR-3, FKR5 & FKR-5) have been identified in *Neurospora crassa* (Solscheid and Tropschug, 2000; Pinto *et al.*, 2008).

TOR proteins are conserved amongst eukaryotes and the best studied fungal examples are TOR1 and TOR2 from *S. cerevisiae* and *S. pombe*. A screen of Ser/Thr kinase gene deletions in *N. crassa* identified a homolog of yeast TOR1/TOR2 by sequence similarity (Park *et al.*, 2011) and *N. crassa* possesses only a single copy of TOR, with higher homology to TOR2 (45% amino acid sequence homology) than to TOR1 (19% amino acid sequence homology) in *S. cerevisiae*. *Neurospora crassa* TOR deletion strain is not available as a homokaryon from the Fungal Genetics Stock Center and it suggests that the deletion may be lethal, although this has not been rigorously tested. Park *et al.*, 2011 also report STK-10 as a homolog of the yeast Sch9 gene (homologous to the TOR kinase substrate S6K in mammals) in *Neurospora crassa* and found that the deletion strain had impaired growth and impaired osmoregulation. Apart from those, some other components of the basic TOR signaling cascade, including orthologs of the SIT4 (serine/threonine-

protein phosphatase PPH-1), KOG1, GTR1, GTR2, Tap42- protein catalytic subunit (TAP42) are present in *Neurospora crassa*. Genes encoding components of the TOR pathway have been identified throughout the fungal kingdom and are (with a few exceptions) highly conserved (Shertz *et al.*, 2010).

In filamentous fungus *Aspergillus nidulans* TOR signaling pathway plays only a minor role in regulating nitrogen metabolism (Fitzgibbon *et al.*, 2005) and in the fungal plant pathogen *Fusarium graminearum*, this pathway is involved in virulence and vegetative development (Yu *et al.*, 2014). Nitrogen responsive TOR signaling pathway in plant pathogenic fungi *Fusarium oxysporum* also controls its virulence function (López-Berges *et al.*, 2010). Furthermore, in *Fusarium fujikurio* TOR signaling pathway is involved in nitrogen regulation of secondary metabolism (Teichert *et al.*, 2006) and in the human fungal pathogen *Candida albicans*, TOR participates in regulating morphogenesis, nitrogen acquisition, responses to host cells, phosphate sensing as well as formation of biofilms (Liu *et al.*, 2017; Flanagan *et al.*, 2017).

1.6: Hypotheses and Objectives

Our hypotheses are that the *Neurospora* circadian system is comprised of mutually interacting multiple components such as the FRQ-less oscillator (FLO) and the FRQ/WCC oscillator; and the UV90 gene product is a component of the FLO (Figure 1.12).

My long-term objectives are to define the architecture of the *Neurospora* circadian system at the biochemical/molecular level by describing the interactions between FLO and the FRQ/WCC oscillator at the molecular and phenotypic level.

My short-term objectives are to confirm the identity of the UV90 gene, identify the FLO-affecting UV90 mutation and identify the functions of the UV90 gene product.

During the course of this research I found that the UV90 gene codes for a protein in the TOR (Target of Rapamycin) nutrient sensing pathway. The TOR signaling pathway is a highly conserved pathway that controls cellular growth as well as stress responses according to the nutritional state. Therefore, I would also like to investigate the contribution of the TOR signaling pathway to clock function.

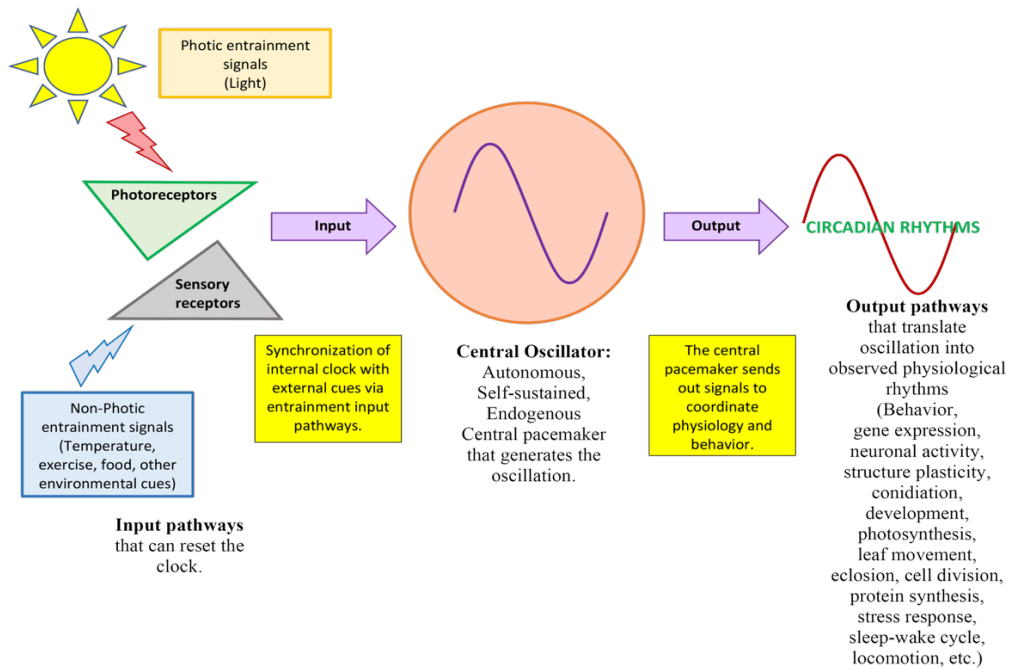


Figure1.1: The model of circadian systems.

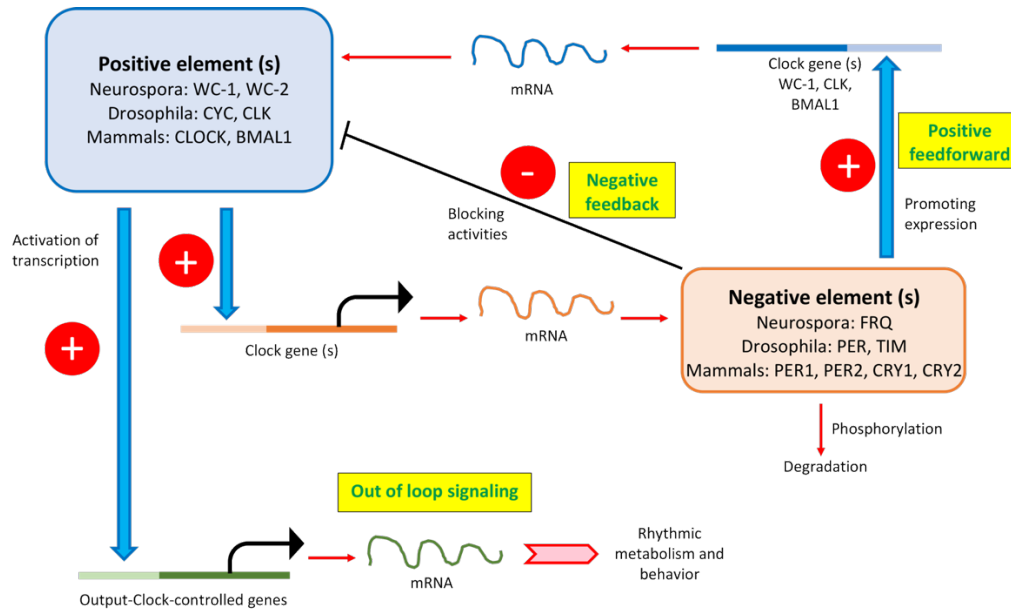


Figure 1.2: Transcription – translation feedback loop (TTFL) model for the circadian mechanism. A set of canonical clock genes gets transcribed, translated and their protein products inhibit the transcription and translation of those same genes, after some delay. Those genes and their products also regulate expression of some other genes that the cell uses in its daily functions.

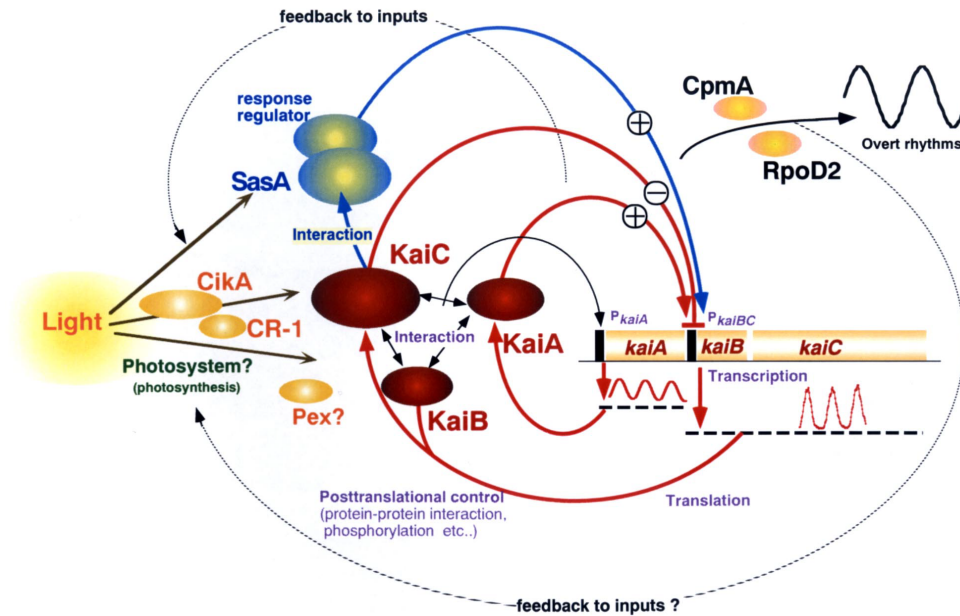


Figure 1.3: A molecular feedback model for the cyanobacterial circadian clock. As shown in the red colored basic timing loop, KaiC negatively regulates its own (*kaiBC*) expression, which generates a molecular feedback loop. However, KaiA activates *kaiBC* expression as a positive element to make the loop oscillate. To regulate their functions, posttranslational events such as protein-protein interactions and phosphorylation may be required. SasA is a KaiC-interacting sensory histidine kinase. SasA enhances expression of *kaiBC* genes via its cognate response regulator. SasA is not an essential component of a minimum feedback loop and SasA forms a secondary loop (blue) coupling with the minimal loop which act as a circadian amplifier. SasA also likely adjusts sensitivity of robustness of the oscillator to the influence of light. CikA is a phytochrome-related histidine kinase that is a circadian photoreceptor. The CR-1 and PEX proteins may also associate with some circadian photoreceptive pathways, while CpmA and RpoD2 are components of the output pathways. The photosynthesis machinery would be both inputs and outputs of the

circadian system. All the photoreception genes are expected to express under the control of the clock, involving feedback from the clock and its outputs to the input pathways (dotted lines) (Adapted from Iwasaki and Kondo 2000).

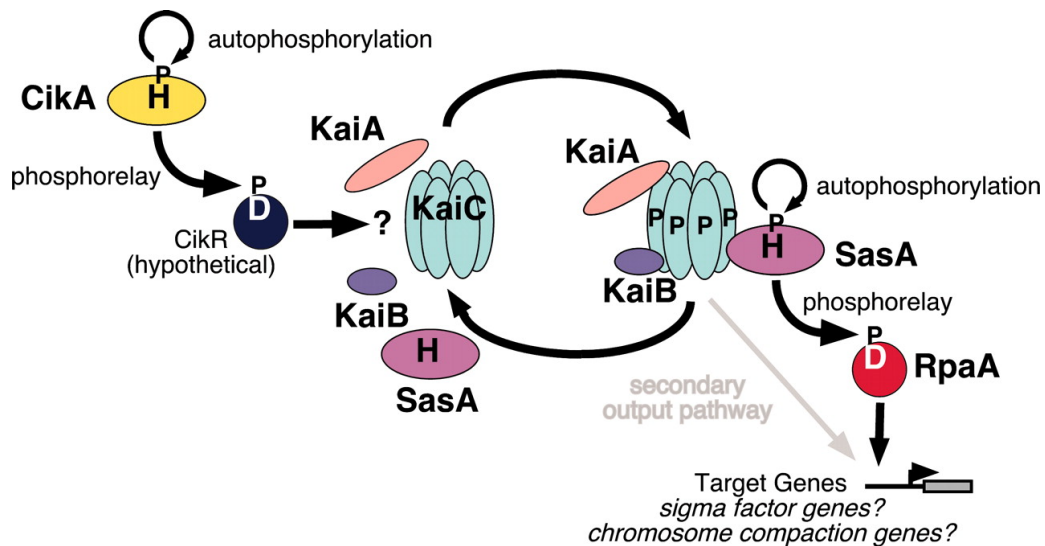


Figure 1.4: Transmission of environmental cues to internal regulators in *Cyanobacteria*. Environmental inputs are transmitted to the periodosome via the histidine kinase, CikA, and cognate response regulator, CikR. KaiA consist with a response regulator like domain at the N terminus and it also directly involved in transmitting environmental signals to stimulate the autophosphorylation of KaiC. SasA autophosphorylation is stimulated by phosphorylated KaiC, initiating the phosphorelay to its cognate response regulator, RpaA. (Adapted from McClung 2006b).

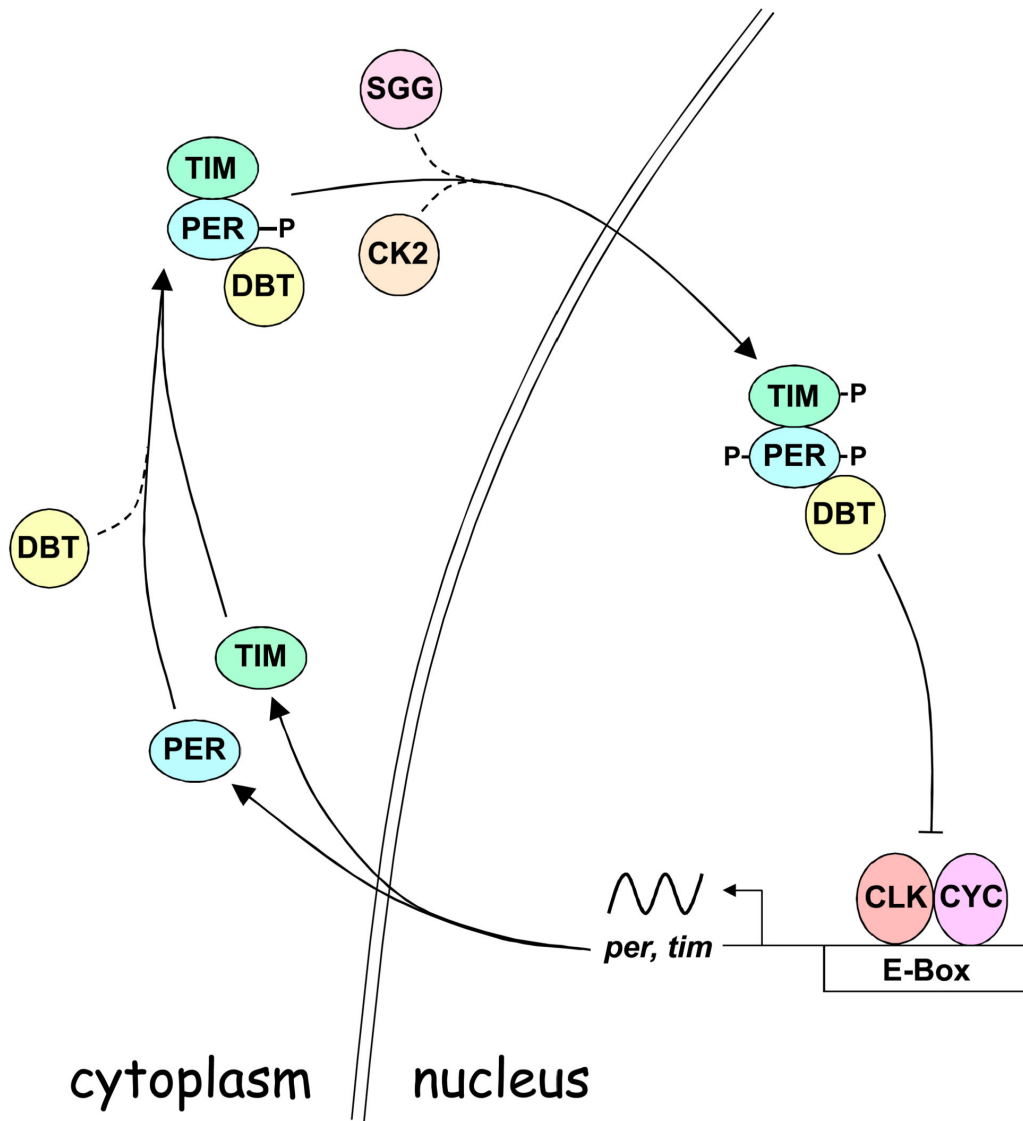


Figure 1.5: The core feedback loop in *Drosophila*. In the core feedback loop in *Drosophila*, *per* and *tim* transcription is activated by CLK-CYC heterodimer. The period (PER) and timeless (TIM) proteins accumulate in the cytoplasm and form PER-TIM heterodimers and translocate into the nucleus to inhibit CLK-CYC, which stops transcription of *per* and *tim* genes. (Adapted from Hardin 2011)

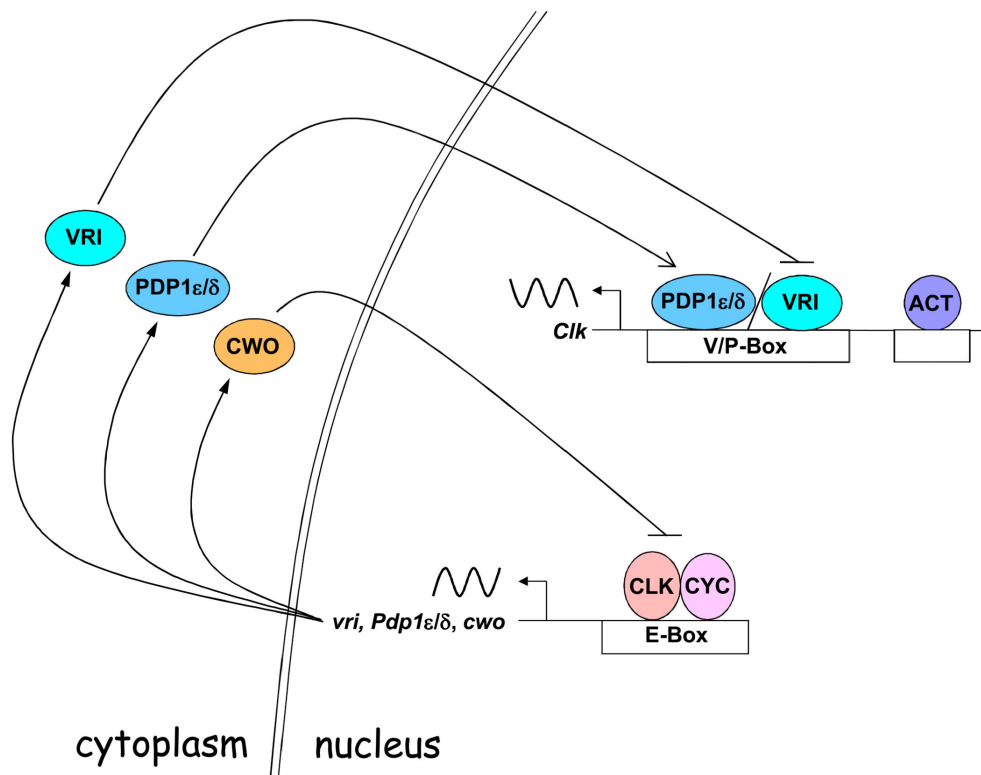


Figure 1.6: The *Clk* feedback loop in *Drosophila*. In the *Clk* feedback loop, *Clk* transcription is repressed by Vrille (VIR) and activated by PDP1 ϵ/δ . (Adapted from Hardin 2011)

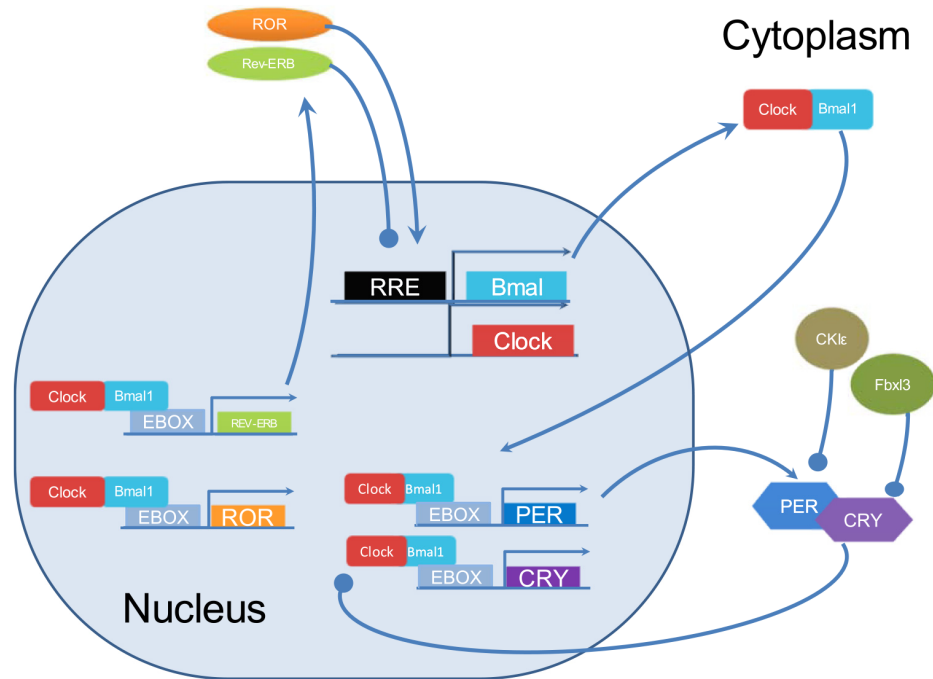


Figure 1.7: Mammalian core circadian clock. Schematic representation of the core circadian clock. BMAL and CLOCK transcription products translocate to the cytoplasm and dimerize. They then return to the nucleus, binding to E-box regions and promoting Period (PER), Cryptochrome (CRY), REV-ERB and ROR transcription. PER and CRY proteins are synthesized in the cytoplasm, dimerize and return to the nucleus inhibiting the binding of the CLOCK/BMAL complex. PER and CRY are subsequently degraded following binding to casein kinase I ϵ (CKI ϵ) and Fbxl3 respectively. REV-ERB inhibits transcription of Bmal1 whilst ROR promotes Bmal1 expression. (Adapted from Robinson and Reddy 2014)

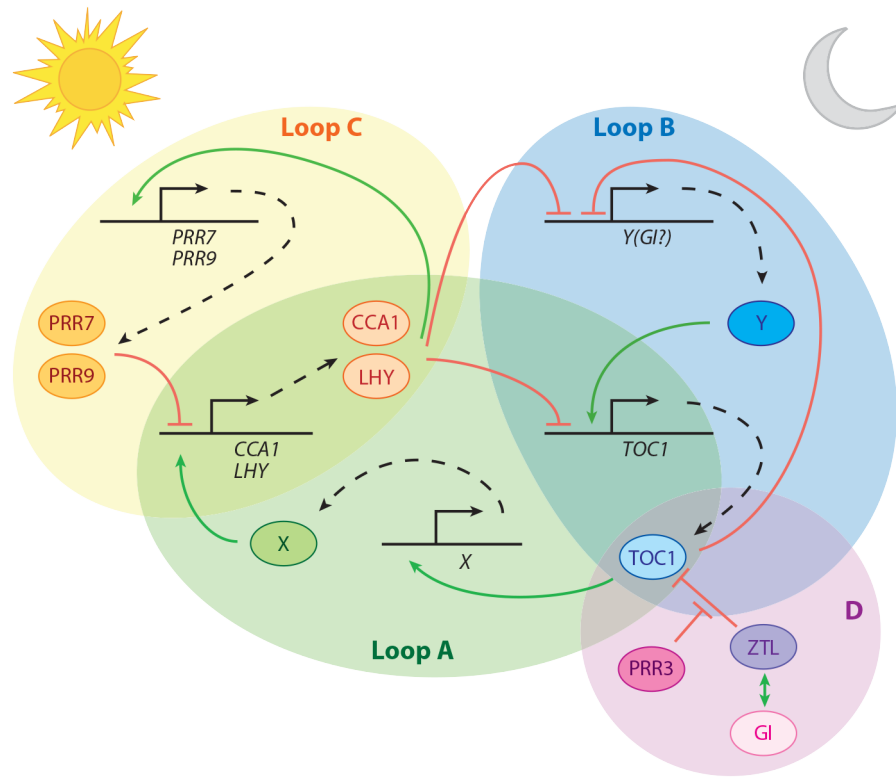


Figure 1.8: Molecular model for plant circadian network. Model of the plant clock. The first-identified transcriptional feedback loop (*loop A*) consists of the dawn-phased Myb-like factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which negatively regulate expression of *TIMING OF CAB EXPRESSION 1* (*TOC1*). *TOC1* is postulated to directly or indirectly activate component X, an as-yet-unidentified factor that induces expression of *CCA1* and *LHY*. The second loop (*loop B*) is thought to be composed of two or more evening-phased genes, an unknown factor designated Y, and *TOC1* [note GIGANTEA (GI) may provide a portion of Y activity]. The third loop (*loop C*), consists of the morning-phased genes *PSEUDO-RESPONSE REGULATOR 7* (PRR7), *PRR9*, *CCA1*, and *LHY*.

Posttranscriptional modifications are also very important for clock function (*D*). ZTL negatively regulates TOC1 protein abundance; its activity is regulated by GI and PRR3. Other genes implicated in clock function have been omitted for clarity. (Adapted from Harmer 2009)

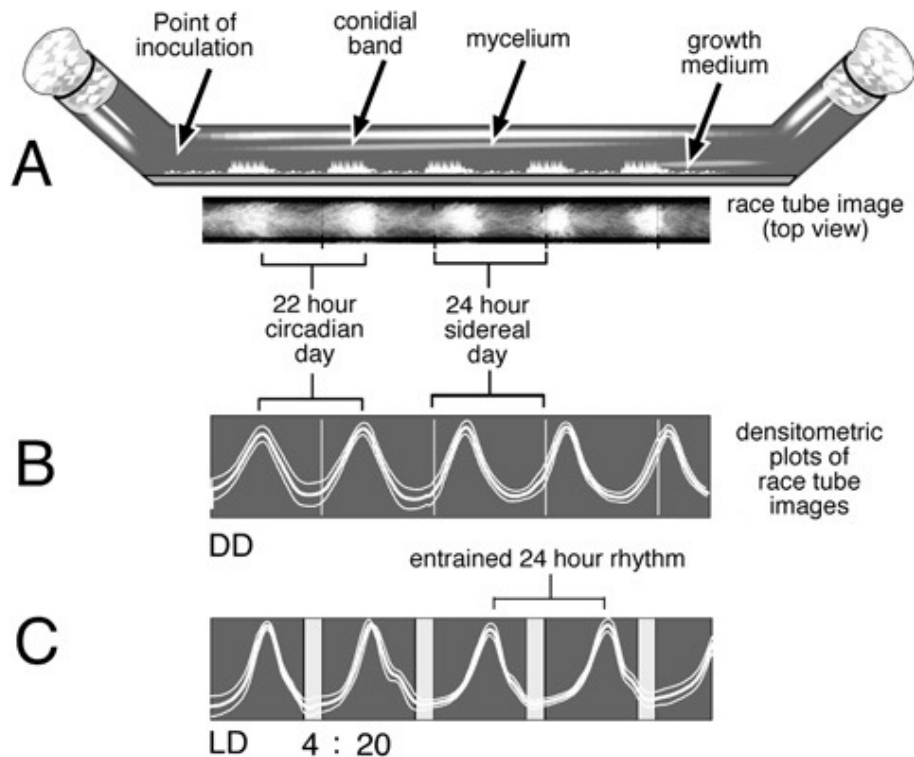


Figure 1.9: The race-tube assay. (A) Schematic representation of a race tube in side view (top) and actual photograph of a race tube in top view (below). Grown on suitable medium, *Neurospora* switches from hyphal growth to asexual spore formation approximately once every 22 h when grown in constant darkness (DD) and temperature (25°C). (B) Densitometric analysis of race tubes held in constant darkness. Notice the free-running 22 h rhythm. (C) Densitometric analysis of *Neurospora* cultures that were entrained to a 4 h light/20 h dark cycle (LD, 4:20). Light-shading symbolizes light, dark-shading represents darkness. Notice the entrained 24 h rhythm. (Adapted from Price-Lloyd *et al.*, 2005)

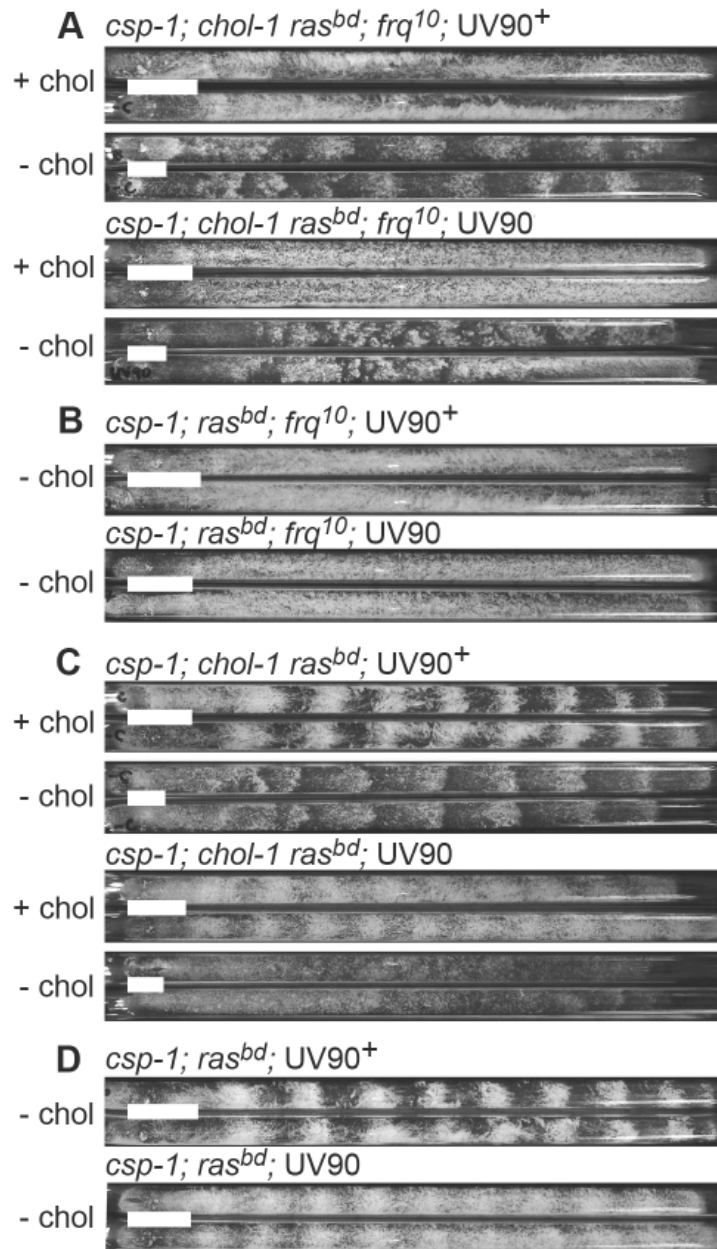


Figure 1.11: Phenotypes of *UV90* mutants. Cultures were grown at 22°C in DD in race tubes on MA medium with (+ chol) or without (- chol) 100 mM choline. Growth was from left to right. White bars represent an average distance

for 24 h of growth. Two sibling strains from a cross are shown in pairs. All strains carry the *csp-1* mutation. A– D: Progeny are from the backcross of *csp-1; chol-1 ras^{bd}; frq¹⁰; UV90* to *ras^{bd}*. A. All strains carry the *ras^{bd}*, *frq¹⁰* and *chol-1* mutations and were grown with or without choline. B. All strains carry the *ras^{bd}* and *frq¹⁰* mutations and were grown without choline. C. All strains carry the *ras^{bd}* and *chol-1* mutations and were grown with or without choline. D. All strains carry the *ras^{bd}* mutation and were grown without choline.
(Adapted from Li *et al.*, 2011)

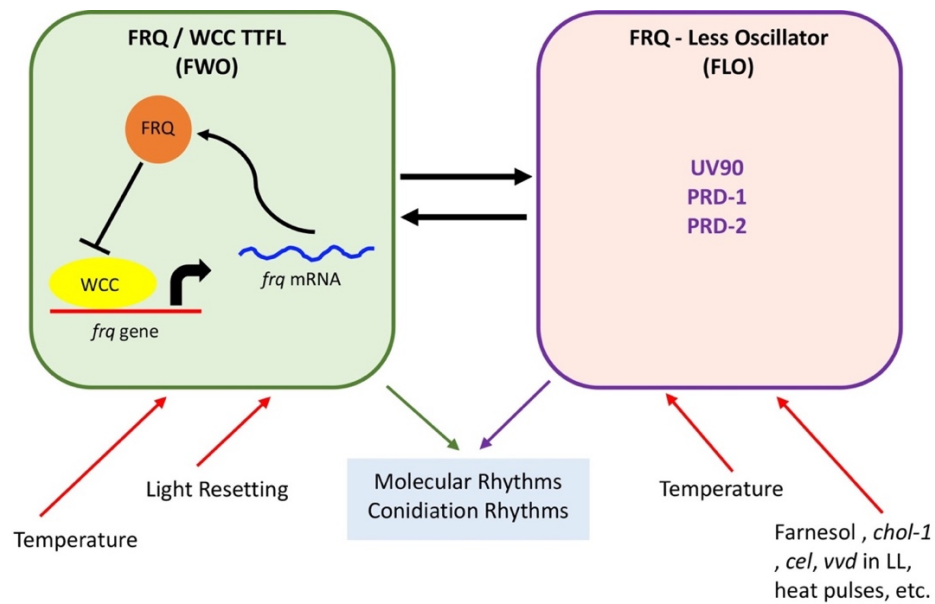


Figure 1.12: The proposed model of the circadian system in *Neurospora crassa*. This model is based on a single FRQ-less oscillator (FLO) that interacts with FRQ/WCC oscillator (FWO). According to this proposed circadian network model: When FRQ is present and functional, all rhythmic processes are integrated into a single orchestrated system that maintains a stable period and is responsive to light. When FRQ is absent or non-functional, rhythmicity continues, but is poorly regulated and not buffered against changes in temperature or metabolic state. Therefore, both oscillators are required in the intact system to maintain the period, temperature compensation, amplitude and stability of the output.

CHAPTER 02: General Methods

2.1: *Neurospora crassa* strains

Neurospora crassa strains used in this study are listed in Appendix I along with their genotypes. These strains were obtained either from Fungal Genetics Stock Center (FGSC – Kansas State University, Manhattan, KS) or constructed in our laboratory by standard *Neurospora crassa* genetic crossing techniques. All the strains were derivatives of Oak Ridge standard (OR-ST) genetic background. All the mutations considered in this study are listed in Table 2.1. Most of the strains used in this study have the *chol-1*, *ras-1^{bd}* and *csp-1* mutations. Strains carrying molecularly validated gene deletions / knockouts produced by the *Neurospora* Genome project (Colot *et al.*, 2006) were obtained from FGSC.

The *csp-1* mutation, that is included in the genotypes of most of the strains used in this study, was identified as a morphological mutation and it prevents the separation of conidiospores from aerial hypha. Therefore, it has been named “conidial separation” (*csp*). This mutation was introduced into strains developed for circadian rhythm assays, as it greatly reduces contamination of cultures with free-flying spores that happen with *csp-1* wild type strains (Lakin-Thomas *et al.*, 1990). The CSP-1 gene product is regulated by light and it has been identified as a transcriptional repressor that affects transcription of a number of genes in response to light (Sancar *et al.*, 2011). My experiments were conducted in constant darkness, removing the complications of light regulation. CSP-1 also dampens the amplitude and shifts the phase of a number of rhythmically transcribed genes (Sancar *et al.*, 2015). The NCU05950 gene that I worked with is not controlled by *csp-1* (Sancar *et al.*, 2011). The *csp-1* mutation that we have used is likely to be a null mutation and

this mutation causes a small effect on the period of the conidiation rhythm relative to wild type (Lambreghts *et al.*, 2009; Sancar *et al.*, 2012; Schneider *et al.*, 2009).

2.2: Maintenance of *Neurospora crassa* strains

Stocks of *Neurospora crassa* were maintained in glass tubes containing slants of 1xVogel's salts (Vogel, 1956) with 2% glucose along with any other required nutritional supplements such as choline (100µM), histidine (500 µg / mL), inositol (250 µM) at -20°C. In order to avoid accumulation of background mutations, no further sub-cultures were made and silica stocks were always used to initiate cultures.

Anhydrous silica gel was used to preserve the strains as described in Wilson, 1986 and given the silica gel stock number (SG# __) to each strain depending on the order in which they were made.

2.3: *Neurospora crassa* culture methods

2.3.1: Solid agar culture methods

2.3.1.1: Race tube experiments

To assay rhythmic development of conidia, period and linear growth rate measurements were taken from cultures grown on solid agar medium in 30 cm or 40 cm long glass tubes called “race tubes”. These are cylindrical hollow tubes bent at both ends to hold the partially filled solid growth medium. Maltose-arginine (MA) growth medium (Appendix II) containing 1x Vogel's salts, 0.5% maltose, 0.01% L-arginine and 2% agar was used and 100µM choline was added as indicated.

In order to make starter cultures, 10 cm petri plates containing solid MA medium with or without choline were inoculated with conidia from stock tube cultures at the edge of the plate and incubated at either 30 °C or 22 °C in constant light (LL) for 2 days. Inoculation of race tubes was performed either by transferring a small amount of conidia from stock tube cultures using sterile inoculating loops or small plugs cut from the growing front of the starter culture plates using the wide end of sterile glass pasture pipettes.

The race tubes were incubated at 30 °C in LL for a day to germinate and synchronize the individual clocks and then transferred to constant dark (DD) at 22 °C until the mycelial front reach the end of the tube. The growth front was marked everyday under red safe light, noting the date and time. The linear growth rate and period (for the strains carrying *ras-1^{bd}* mutation) were calculated as previously described (Lakin-Thomas, 1998) using the scanned race tube pictures with daily growth marks and Microsoft Excel program “MacTau” developed by Dr. Patricia Lakin-Thomas.

2.3.1.2: Heat pulse experiments

To entrain the conidiation rhythm to various T-cycles (T=total number of hours in the cycle) such as 16-hr, 20-hr and 24-hr, 2-hour heat pulses of 32°C were used on cultures growing at 22°C in race tubes containing maltose-arginine (MA) medium with 100 µM choline in DD. Density traces were collected and analyzed as described in Lakin-Thomas, 2006 & Li and Lakin-Thomas, 2010. Average density traces were calculated from the last two (or four) complete cycles in each experiment and five or six tubes per set i.e. N=10/12. Standard errors were calculated for each average pixel value. Average density profiles were normalized by setting the minimum and maximum pixel values to 0 and 1, and confidence intervals were plotted as $\pm$

one S.E.M. (Li *et al.*, 2011). Changes in peak timing and peak shape in different T-cycles were monitored.

2.3.1.3: Time course experiments

To analyze molecular rhythms at the protein level, cultures were grown on MA medium with 100 μ M choline in large 15 cm petri plates on top of a layer of cellophane. Dry cellophane circles were cut using the 15 cm petri plate lid as a template. Cellophane circles were soaked and washed in distilled water prior to sterilization by autoclaving at 121°C for 15 minutes. Sterile cellophane circles were laid on solidified MA medium with 100 μ M choline in 15 cm petri plates. Plates were inoculated by placing small mycelial plugs cut from the growing front of starter culture plates at the edge of the petri plate. All the plates were inoculated at the same time and initially incubated at 30 °C in LL for various lengths of time. Plates were then transferred from 30 °C in LL to 22°C in DD at 4-hour intervals and all plates were harvested after 72 hours total growth. Hours in darkness were varied by varying the time at which cultures were transferred from light to dark. Samples were collected every 4 hours under red safe light to collect samples from 0 – 48hr in DD. To harvest samples, mycelium from the entire growth front of a culture was scraped off of the cellophane using a plastic spatula that removed a 1 cm wide strip. Samples were immediately frozen in liquid nitrogen and stored in -80°C (Li *et al.*, 2011 , Schneider *et al.*, 2009).

2.3.2: Liquid culture method

2.3.2.1: Time course experiments

Samples were harvested at different clock times from liquid cultures to assay the molecular rhythms at protein level as well as RNA level and this method is modified from Edgar *et al.*, 2012. Cultures were initiated by inoculating conidiospores in 1 ml of Vogel's medium containing 2% glucose, 0.5% arginine, 10 ng ml⁻¹ biotin, 0.2% Tween 80 and 100µM choline (high-glucose medium) in 24-well microtiter plates. Plates were incubated under LL for 2 days at 25°C. Three of the resulting hyphal mats were placed in each of a series of 125 ml flasks containing 50 ml of Vogel's medium with 0.03% glucose, 0.05% arginine, 10 ng ml⁻¹ biotin and 100µM choline (low-glucose medium) and shaken at 150 r.p.m on an orbital shaker. These were incubated at 25°C under LL for at least 2 hrs before staggered transfers to DD at 25°C with shaking at 150 r.p.m. and harvested after 48hrs of total growth. Cultures were transferred to DD at various times to set the circadian clock at different phases, from 0 to 48 hours in DD. In some experiments, to reset the circadian phase (hours in dark), 2 hrs of heat pulse at 35°C were given under LL and then transferred from LL to DD. Mycelia were harvested by vacuum filtration, at 4hrs intervals and samples were collected from 0 hr to 48 hrs of growth in flasks under DD condition. After harvesting mycelia were washed with distilled water, dried on filter paper, frozen in liquid N₂, and stored at -80°C. All manipulations in the dark were performed under safe red light.

2.4: *Neurospora crassa* crosses

2.4.1: Mating type determination

The highly fertile aconidiate mutant *fluffy* (*fl*) strains were used in mating type determination. The *fl* strains are superior female parents compared

to wild type as these *fl* strains form abundant perithecia more quickly than other fertile strains and also the perithecia are more readily seen. The *mat A* and *mat a fluffy* strains were used as protoperithecial parental strains and allowed to grow on synthetic crossing medium (SCM) (Appendix II) with 100µM choline on 10 cm petri plates individually at 25°C until the plate was covered with lawns of the *fl* strain. Conidia from strains to be tested were inoculated on the surface of the *fl* strain lawn at marked positions. Parallel tests were performed on both *fl mat A* and *fl mat a* to provide assurance against false-negative tests, infertility and spotting errors. Strains of known mating type were used as controls. This mating type determination test was performed according to the protocol obtained from FGSC.

2.4.2: Setting up and analyzing *Neurospora crassa* sexual crosses

Neurospora crassa standard genetic crosses were conducted according to the protocols derived from *Neurospora* Methods Manual in FGSC. Strains with opposite mating types were selected as male (fertilizing) and female (protoperithecial) parents for the cross. Usually a *csp+* strain was used as the female parent and grown on slants of synthetic crossing medium (SCM) (Appendix II) with or without 100 µM choline in glass tubes at room temperature for 7-10 days or until protoperithecia were visible. The male parent was grown on slants of 1xVogel's salts (Vogel, 1956) with 2% glucose along with any other required nutritional supplements for 7-10 days at room temperature. Fertilization was done by inoculating 7-10 days old spores of the male parent on to the female parent using a sterile inoculating loop. Fertilized strains were incubated at room temperature in laboratory LD conditions for 4 weeks. Ejected ascospores were seen on the opposite wall of the tube by the end of the 4th week.

Individual ascospores were picked into slants containing 1xVogel's salts (Vogel, 1956) with 2% glucose along with any other required nutritional supplements. To activate the ascospores slants were heat shocked for 30 minutes in a 60°C water bath and slants were incubated at room temperature in laboratory LD or 30°C LL conditions until germination.

Genotypes and phenotypes of the germinated progeny from the cross were identified by various criteria; *csp-1* (Conidial separation-1) by tapping the tubes (Tap test) to look for failure of spore separation, gene deletion by hygromycin resistance (produced by cassette used for gene replacement in *Neurospora crassa* gene knockout project by Colot *et al.*, 2006) or polymerase chain reaction (PCR) with gene specific primers to confirm gene replacement, *uvr90* mutation by failure to produce PCR product from NCU05950 – specific primers. Race tube experiments were carried out to identify *ras^{bd}* by slower growth and banding phenotype, *chol-1* by slow growth without choline supplementation, *frq^{null}* by arrhythmic conidiation on high choline media and presence of banding rhythms in *chol-1* strains in no choline culture media, and *uvr90* mutation by damped or arrhythmic conidiation phenotype depending on the genetic background. In order to confirm the genotypes and segregation of the mutations, strains with multiple mutations were backcrossed, when needed.

2.5: *Neurospora crassa* genomic DNA extraction

Genomic DNA extraction was performed as described in Jin *et al.*, 2007. Strains were grown in 24-well microtiter plates containing 1 mL of liquid minimal media containing 1xVogel's salts (Vogel, 1956), 2% glucose along with any other required nutritional supplements at room temperature for 3 days or at 30°C for 2 days under LL conditions.

Mycelia were harvested by transferring mycelial pads into vacuum filtration system layered with Whatman P8, 3.5 cm filter papers. Mycelial pads were rinsed 3 times with distilled water and vacuum filtered until just damp. Samples were then transferred to 1.5 mL micro-centrifuge tubes and immediately frozen in liquid nitrogen. Frozen samples were stored at -80°C until DNA extraction was performed.

Frozen samples were ground to a fine powder using mortar and pestle, which were pre-cooled 3 times with liquid nitrogen. Powdered fungus was transferred to 1.5 mL micro-centrifuge tubes containing 600 µL of DNA extraction buffer (100 mM Tris- HCl pH 8.0, 50 mM EDTA, 1% SDS) and 3 µL Proteinase K (20 mg/mL in 20 mM Tris-HCl, 1 mM CaCl₂, 50% glycerol, pH 7.5). Samples were mixed vigorously and incubated at 65°C for 1 hour. After the 1 hour incubation, samples were again mixed thoroughly by vortexing and 200 µL of 7.5 M ammonium acetate was added. Sample tubes were then mixed vigorously and incubated on ice for 5 min. Samples were centrifuged for 3 minutes at 16,000 x g, 4°C and supernatants were transferred into fresh, labeled 1.5 mL screw-capped tubes. 6 µL of RNase A (5 mg/ml in Tris-HCl, pH 8.0, 50% glycerol) was added and samples were vortexed prior to incubation at 37 °C for 1 hour.

Each sample was extracted with 500 µL of chloroform followed by centrifugation for 5 minutes at 14000 x g, 4°C. The aqueous phase containing supernatants was carefully transferred into fresh 1.5 mL micro-centrifuge tubes and genomic DNA was precipitated with 650 µL of isopropanol followed by centrifugation for 15 minutes at 14000 x g, 4°C. DNA pellets were washed once with 1 mL of 70% ethanol and centrifuged for 5 minutes at 7500 x g, 4°C. DNA pellets were air dried for 1 – 2 hours or until ethanol evaporated and pellets were dissolved in 100 µL of TE buffer (10 mM Tris , 1 mM EDTA, pH 8.0).

2.6: Transformation of *Neurospora crassa*

2.6.1: Growing conidia

Recipient strains (SG#274: *csp-1 his-3; chol-1 ras^{bd} mat a* & SG# 359: *csp-1 his-3; chol-1 ras^{bd}; NCU05950^{KO} mat a*) were allowed to grow on 3 mL of minimum media slants containing 1xVogel's salts (Vogel, 1956), 2% glucose, 100 µM choline, 500 µg/mL histidine and 2 % agar for 3-7 days at 30°C under LL condition. For each strain 5 starter cultures were prepared. 1L flasks containing 100 mL of minimum media containing 1xVogel's salts (Vogel, 1956), 2% glucose, 100 µM choline, 500 µg/mL histidine and 2% agar were inoculated evenly with the contents of slants using a sterile inoculating loop and incubated at 30°C under DD condition for 3 days and then transferred to laboratory LL conditions at room temperature for 5-8 days.

2.6.2: Harvesting and preparation of macroconidia

50 mL of sterile distilled water was added to each flask with conidia and conidia were scraped off using a sterile loop. The contents of one flask was poured into a 50 mL sterile conical tube and washing was repeated with another 50 mL of sterile distilled water into a second sterile conical tube. Both the tubes were vortexed vigorously and the contents were poured through 4 layers of sterile muslin cloth in a funnel into fresh 50 mL sterile conical tube. For maximum spore recovery, the muslin cloth was squeezed. This was repeated for all the flasks and tubes were centrifuged at 5000 rpm for 5 minutes at 25°C. Supernatants were discarded into bleach and all pellets from five flasks were pooled into one 50 mL conical tube. The final pellet was washed 3 times with 40 mL of sterile distilled water and centrifuged at 5000 rpm for 5 minutes at 25°C. After the final wash and centrifugation, the supernatant was poured off

and the conidia pellet was re-suspended in 2 mL of 1M sorbitol. 200 µL of spore aliquots were transferred to 1.5 mL screw-cap tubes and stored at -80°C.

To prepare the spores for transformation, 200 µL of recipient spores in 1.5 mL tubes were centrifuged at 5000 rpm for 5 minutes at 4°C. Supernatant was discarded and 1 mL 1M sorbitol was added and mixed by vortexing. Samples were centrifuged again at 5000 rpm for 5 min at 4°C. Supernatant was removed and 40 µL of 1M sorbitol was added. Samples were mixed gently by flicking the tube and placed on ice.

2.6.3: Electroporation and plating

Electroporation cuvettes with 2 mm gap were pre-cooled on ice. 10-15 µg of linearized DNA in 15 µL of sterile water was added to prepared spores and gently mixed by flicking the tube. If the plasmid DNA sample was not concentrated enough, samples were precipitated as described in section 2.14.1 to concentrate. The sample tube was kept on ice for 5 min. The viscous conidia-DNA mixture was transferred to the pre-cooled electroporation cuvette, which was placed into the BioRAD Gene Pulser that was adjusted to deliver 1.5 kV (7.5 kV / cm) voltage, 25 µF capacitance and 600 ohms resistance. 1 mL of recovery medium (Appendix II) was immediately added to electroporated sample cuvette using sterile Pasteur pipette and gently mixed by pipetting up and down. The mixture was transferred to 1.5 mL screw capped tube and incubated at room temperature for one hour with gentle shaking by placing horizontally on a shaker platform at about 9 rpm.

After the recovery step, spores were diluted to make 1 mL of a 1/10 dilution (100 µL of spores + 900 µL of recovery medium) and 1 mL of a 1/100 dilution (100 µL of 1/10 dilution + 900 µL of recovery medium) for each sample.

For each dilution 60 mL of plating media containing top agar and FIGS (Appendix II) was prepared. Undiluted transformed spores and two diluted spore samples were transferred to 100 mL flasks containing 60 mL of plating media at about 40 °C. Flasks were swirled to mix well and immediately poured into three, labeled 10 cm Petri plates (about 20 mL per plate). Plates were allowed to solidify and sealed with micropore tape. Plates were incubated face up for 3-7 days at 30°C under LL condition for colonies to develop.

2.6.4: Growing transformants and analysis of transformants

Individual colonies were punched out using the narrow tip of sterile Pasteur pipettes and transferred to slant tubes with 3 mL of minimum media containing 1xVogel's salts (Vogel, 1956), 2% glucose, 2% agar and other supplements but without histidine to select *his-3*⁺ transformants. Tubes were incubated for 3-7 days at 30°C under LL.

Positive transformants were able to grow on media without histidine supplement and successful integration of the transgene at the *his-3* locus was confirmed by PCR using combination of *his-3* locus specific and transgene specific primers.

2.7: Microconidia preparation

Conidiospores used for the transformation are usually multinucleated. Therefore, transformants are likely to be heterokaryotic with transformed and untransformed nuclei. Microconidia are predominantly uninucleate (Horowitz and Macleod 1960; Baylis and DeBusk 1967) and iodoacetate (IAA) supplemented media can be used to induce microconidia (Pandit and

Maheshwari 1993). A method adapted from Ebbole and Sachs 1990 and Pandit and Maheshwari 1993 was used to produce microconidia.

Dry cellophane circles were cut using the 10 cm petri plate lid as a template. Cellophane circles were soaked and washed in distilled water prior to sterilization by autoclaving at 121°C for 15 minutes. Sterile cellophane circles were laid on solidified microconidia inducing media (0.1 X SCM, 0.5% sucrose, 2% agar, 1 mM Na-iodoacetate [Appendix II]) with 100 µM choline in 10 cm petri plates.

The center of the plate was marked on the underside with a marking pen and a hole was pierced over the mark in the center of cellophane using a sterile needle. This method facilitates the fungus to grow between the cellophane and solid medium. A few spores were inoculated near the hole and plates were sealed with micropore tape. Plates were turned upside down and incubated for 7-12 day, at room temperature inside a sealed box with a dish of water that created a humid atmosphere.

The cellophane was peeled off after 12 days and petri plate lids were replaced with new sterile lids to avoid carrying over macroconidial contaminations from old lids. Plates were left at room temperature for another day and microconidia were harvested 24hrs after the removal of the cellophane layer. 2 mL of sterile water was added to the plate and solid media surface was washed by tilting the plate few times. Microconidia suspension was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 14000 rpm for 1 minute at room temperature. Most of the supernatant was removed and microconidia were resuspended in residual water by pipetting up and down and also by vortexing.

Microspores were counted using a hemacytometer. The volume needed to get 100 spores was calculated and transferred to a sterile 100 mL flask containing 60 mL of sorbose plating medium (0.1% fructose, 0.1% glucose, 2%

sorbose, 1X Vogel's salts, 2% agar [Appendix II]) at about 40 °C. Flasks were swirled to mix well and immediately poured into three, labeled 10 cm Petri plates (about 20 mL per plate). Plates were allowed to solidify and sealed with micropore tape. Plates were incubated face up for 3-7 days at 30°C under LL condition for colonies to develop.

2.7.1: Growth and analysis of microspore derived strains

Individual colonies were punched out using the narrow tip of a sterile Pasteur pipette and transferred to slant tubes with 3 mL of minimum media containing 1xVogel's salts (Vogel, 1956), 2% glucose, 2 % agar and other supplements but without histidine to select *his-3*⁺ microspores. Tubes were incubated for 3-7 days at 30°C under LL condition.

Microconidia originating from positive transformants were able to grow on media without histidine supplement and successful integration of transgene at the *his-3* locus as well as the purification of transformants to homokaryons were confirmed by PCR using *his-3* locus specific primers (HIS3-F1: CTCATGTTCAACCCTTTGGATGG and HIS3-R: GTCATGCAGTTGGCTAAGGTTGAG).

2.8: *E.coli* strains

The bacterial strain used in this study was *E.coli* DH10β with the genotype $\Delta(ara-leu)$ 7697 *araD139 fhuA* $\Delta lacX74$ *galK16 galE15 e14- ϕ 80dlacZAM15 recA1 relA1 endA1 nupG rpsL (Str^R) rph spoT1 $\Delta(mrr-hsdRMS-mcrBC)$* and was obtained from NEB as high efficient competent cells.

2.8.1: Growth and storage of *E.coli*

The *E.coli* strains were grown either in liquid Luria – Bertani (LB) medium (Appendix II) with shaking at 250 rpm or solid LB medium (Appendix II) at 37°C for about 16 hours. Working stock cultures were kept on LB plates at 4 °C. For selection of strains bearing plasmids with antibiotic resistance genes, ampicillin was added to growth media to final concentration of 100 µg/mL.

For long-term storage, a single colony was allowed to grow overnight in liquid LB medium and glycerol stocks were prepared by mixing 500 µL of fresh bacteria culture with 500 µL of 60% sterile glycerol (Appendix II) in a 1.5 mL screw capped tube. Tubes were mixed well, frozen in liquid nitrogen and stored at -80°C. When recovering bacteria from glycerol stocks, an aliquot was streaked on a selective solid LB plate to check the selective markers as well as to get a single colony.

2.9: Plasmids, primers and synthetic gene fragments

2.9.1: Plasmids

All the plasmids constructed during this study are listed on Appendix I.

2.9.1.1: pBM61

Neurospora crassa his-3 locus targeting plasmid pBM61 (Margolin *et al.*, 1997) was used clone the NCU05950 gene and thereby to rescue the *uvr90* mutant phenotype. This plasmid is 6836 bp and consists of beta-lactamase encoding bla gene that confers resistance to the antibiotic ampicillin (Amp^R), 5'-truncated *his-3* gene fragment (5'-truncated *his-3* exon 1:: *his-3* intron :: *his-*

3' exon 2 -3'), the multiple cloning site from pBluescript KS(+) and 3' *his-3* gene flanking region (Figure 2.1). The plasmid was obtained from FGSC.

2.9.1.2: pCCG::C-Gly::GFP

Neurospora crassa expression vector pCCG::C-Gly::GFP (Honda and Selker, 2009) was used to clone NCU05950 as well as NCU000376 genes and thereby to detect the subcellular localization of the protein. This vector is 8509 bp in size and contains beta-lactamase encoding *bla* gene that confers resistance to the antibiotic ampicillin (Amp^R), 3' flank region of *Neurospora crassa his-3* gene, high-level gene expression *Neurospora* promoter *cgc-1* (*Pccg-1*), multiple cloning site (MCS) and flexible poly-glycine linker (10x Gly) from pRS416-10xGly plasmid, green fluorescent protein (GFP) and 5' truncated *his-3* gene fragment (Figure 2.2 & Figure 2.4). The GFP tag was added to the C – terminal of the protein of interest by using this plasmid. The gene of interest was added between the XbaI and PacI sites in the MCS to express under *cgc-1* promoter and the gene of interest with its endogenous promoter was cloned between NotI and PacI to replace the *Pccg-1*. The plasmid was obtained from FGSC.

2.9.1.3: pCCG::C-Gly::3xFLAG

Neurospora crassa expression vector pCCG::C-Gly::3xFLAG (Honda and Selker, 2009) was used to clone NCU05950 gene to detect and isolate proteins and protein complexes. This vector is 8019 bp in size and contains beta-lactamase encoding *bla* gene that confers resistance to the antibiotic ampicillin (Amp^R), 3' flank region of *Neurospora crassa his-3* gene, high-level gene expression *Neurospora* promoter *cgc-1* (*Pccg-1*), multiple cloning site

(MCS) and flexible poly-glycine linker (10x Gly) from pRS416-10xGly plasmid, the epitope tag 3xFLAG and 5' truncated *his-3* gene fragment. The 3xFLAG tag was added to the C –terminal of the protein of interest by using this plasmid (Figure 2.3 & Figure 2.4). The gene of interest was added between the XbaI and PacI sites in the MCS to express under *ccg-1* promoter and the gene of interest with its endogenous promoter was cloned between NotI and PacI to replace the *Pccg-1*. The plasmid was obtained from FGSC.

2.9.1.4: pRFP-vam-3 (*N. crassa* vam-3 tagged with tdimer2 in pMF334)

10.1 kb long pRFP-vam-3 (Bowman *et al.*, 2009) plasmid consists of beta-lactamase encoding *bla* gene that confers resistance to the antibiotic ampicillin (Amp^R), 3' flank region of *Neurospora crassa his-3* gene, high-level gene expression *Neurospora* promoter *ccg-1* (*Pccg-1*), red fluorescence protein (RFP) tag from *tdimer2(12)* gene, NCU06777 (*vam-3*) gene and 5' truncated *his-3* gene fragment. The plasmid was obtained from FGSC. pMF334 (9177 bp) was used as the host vector and *vam-3* gene was inserted between MluI and BglII restriction sites (Figure 2.5) (Bowman *et al.*, 2009).

2.9.2: Primers

Primers were received from Invitrogen or Integrated DNA Technologies (IDT). Both 1 mM primer stocks and 10 µM working primer tubes were stored at –20°C. Details of primers used to analyze the *uv90* mutant are given in Appendix III. Details of primers used to clone NCU05950 gene and NCU00376 gene are given in Appendix IV. Details of primers used to identify gene knockout strains are given in Appendix V. Details of primers used to distinguish different genotypes are given in Appendix VI.

2.9.3: Synthetic gene fragments

Integrated DNA technologies (IDT- Integrated DNA Technologies, Inc., Coralville, IA, USA) MiniGene constructs were used to generate strains expressing UV90 defective protein under its native promoter. Commercially synthesized gene fragments of Nat-Del1-UV90 (Δ 1-NCU05950) = 611bp, Nat-Del7-UV90 (Δ 7-NCU05950) = 593bp and Nat-Del10-UV901(Δ 10-NCU05950) = 584bp (Appendix VII) with *Sal*I and *Bgl*II restriction sites on either sides were received on ampicillin resistant pUCIDT (2752 bp) vector.

2.10: Plasmid DNA extraction

Plasmid mini-preparations were used to obtain small amounts of plasmid DNA. Plasmids were extracted from overnight grown cultures using QIAprep Spin Miniprep kit from QIAGEN or High-Speed Plasmid Mini kit from Geneaid according to the instructions provided by the manufacture.

Plasmid Midi-preparations were performed to obtain large amount of plasmid DNA. Plasmids were extracted from overnight grown cultures using plasmid midi kit from QIAGEN or Midiprep Plasmid DNA kit from Geneaid according to the instructions provided by the manufacture.

2.11: Transformation of *E.coli*

NEB 10-beta chemically competent *E.coli* cells were purchased from New England BioLabs (NEB) inc. A tube containing 50 μ L aliquot of NEB 10-beta chemically competent *E.coli* cells was thawed on ice for 10 minutes. 1-5 μ L of plasmid DNA with 1 pg – 100 ng was added to the cell mixture and the tube was carefully flicked 4-5 times to mix cells and DNA. The DNA-cell mixture was incubated on ice for 30 minutes and it was subjected to heat shock

at 42°C for exactly 30 seconds. The tube was immediately placed on ice for 5 minutes. 950 µL of NEB 10-beta Outgrowth Medium (supplied with the NEB 10-beta chemically competent *E.coli* cells) at room temperature was added to the heat shocked DNA-cell mixture and it was incubated at 37°C with vigorous shaking (250 rpm) for 60 minutes. LB-agar plates with appropriate antibiotic were warmed to 37°C. Transformed cells were mixed thoroughly by flicking and inverting. Several 10-fold serial dilutions were made using NEB 10-beta Outgrowth Medium and 50-100 µL from each dilution were spread on selection plates. Plates were allowed to dry for 10-15 minutes and incubated overnight (approximately 16 hours) at 37°C.

2.11.1: Screening of positive *E.coli* transformants

Colonies grown after 16 hours of incubation were streaked on a LB-agar plate with appropriate antibiotic and incubated overnight (approximately 16 hours) at 37°C. Colony PCR was performed to select positive transformants and plasmid DNA was extracted from positive transformants. To confirm the plasmid, restriction enzyme digestion of the plasmid DNA was performed with appropriate restriction enzymes.

2.12: Polymerase Chain reaction (PCR)

For cloning and sequencing applications, Q5 high fidelity DNA polymerase from New England BioLabs inc. (NEB) was used in accordance to manufacturer's instructions.

Annealing temperature was calculated using the NEB Tm calculator application and temperature gradient PCR reactions were performed for each primer pair with $T_m \pm 5^\circ\text{C}$ to optimize the primer annealing temperature.

2.12.1: Colony PCR

Colony PCR was used to screen *E.coli* colonies developed after transformation by determining the presence or absence of the desired plasmid directly from bacterial colonies without culturing bacteria or plasmid purification. PCR reaction mixtures were prepared and as the template DNA, a minute amount of bacterial colony was added directly to the PCR reaction mixture using sterile micropipette tip or inoculating needle. Q5 high fidelity DNA polymerase was used for colony PCR reactions as it can amplify long, difficult amplicons. During the initial denaturation / heating step bacterial cells will be lysed and plasmid DNA will be released from cells to serve as template for the amplification process. Inserted DNA fragment specific primers or vector plasmid DNA specific primers flanking the insert were used to determine presence of the insert as well as to determine whether the insert has the correct molecular size. Insert specific primers were used to acquire information on both the specificity and size of the DNA insert. Vector specific primers were used to screen multiple constructs simultaneously. To determine the insert orientation, insert specific primers along with vector specific primers were used. Extension and final extension steps of the colony PCR were performed at 68°C.

2.12.2: Spore PCR

Spore PCR was carried out to save time when analyzing large numbers of fungal strains. This method eliminates fungal culture preparation, DNA extraction and purification. 50 µL PCR reaction mixtures were prepared using Terra™ PCR Direct Polymerase Mix (Takara Bio Inc.) and as the template DNA, a minute amount of fungal spores were added directly to the PCR reaction mixture using sterile micropipette tip or inoculating needle. Depending

on the objective of the experiment, different primer pairs were used for spore PCR analysis.

2.12.3: Long-range PCR

To amplify extremely long DNA fragments (up to 40 kb DNA) long-range PCR was performed using QIAGEN LongRange PCR Kit according to manufacturer's guidelines.

2.12.4: PCR product cleanup

PCR products were cleaned to using one of the following commercial PCR purification kits according to manufacturer's recommendations: QIAquick PCR Purification Kit or Geneaid Gel/PCR DNA fragments kit or Monarch PCR & DNA Cleanup Kit. DNA was eluted in pre-warmed elution buffer from the kit or sterile dH₂O.

2.13: DNA methods

2.13.1: Quantification of DNA

The concentration and purity of double stranded DNA was determined using the Thermo-Fisher Scientific Nanodrop2000 UV/Visible spectrophotometer. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and ratio of ~1.8 is generally accepted as "pure" for DNA.

2.13.2: Restriction Enzyme digestion

All restriction digestions were carried out with enzymes obtained from New England Biolabs (NEB) and ThermoFisher Scientific. The enzymes were used according to the manufacturer's guidelines. New England Biolabs website (<https://www.neb.com>) was used to determine if the enzyme needs Bovine Serum Albumin (BSA), optimum incubation temperature and buffer that gives highest activity for the enzyme.

2.13.3: Precipitation of DNA

0.1 volume 3 M sodium acetate (pH 5.2) was added to the DNA solution in an Eppendorf tube. Two volumes of chilled (- 20 °C) absolute alcohol were added and DNA allowed to precipitate overnight at - 80 °C. The mixture was centrifuged at 13000 rpm and 4 °C for 15 minutes and the supernatant was removed. The DNA pellet was washed with 1 volume of 75 % ethanol and centrifuged for 5 min at 13000 rpm at 4 °C. After discarding the supernatant the pellet was dried for 30-60 minutes at room temperature and re-suspended in desired volume of Tris-EDTA buffer (TE buffer) or ddH₂O.

2.13.4: DNA agarose gel-electrophoresis

DNA agarose gel-electrophoresis was carried out according to standard procedures (Green and Sambrook 2012). The agarose gel was prepared with agarose concentration appropriate for the size of DNA fragments to be separated. Desired amount of agarose was added and dissolved in 1X TAE buffer by heating. This solution was allowed to cool and 0.005% RedSafe™ nucleic acid staining solution (iNtRON Biotechnology) was added. Gel electrophoresis was carried out in a horizontal tank containing 1x TAE buffer.

Either 100 bp ladder or 1 kb ladder was used depending on size of DNA fragment. Gels were imaged using the FluoroChem FC2 Imaging System (Alpha Innotech).

2.13.5: Purification of DNA from gel

DNA bands were excised from agarose gels and purified with one of the following gel extraction kits according to the manufacturer's recommendations: QiaQuick Gel Extraction Kit or Geneaid Gel/PCR DNA fragments kit or Monarch® DNA Gel Extraction Kit. DNA was eluted in pre-warmed elution buffer from the kit or sterile dH₂O.

2.13.6: DNA ligation

DNA fragments for ligation were prepared by restriction enzyme digestion, separation by agarose gel electrophoresis and gel extraction, as described above. DNA was ligated using T4 DNA ligase (from New England Biolabs) according to the manufacturer's instructions.

In order to calculate the insert mass (X ng) required, the following equation was used;

$$\text{Mass of insert (X ng)} = (\text{Molar ratio of insert : vector}) \times (\text{Insert length in bp} / \text{Vector length in bp}) \times (\text{Vector mass in ng})$$

Appendix VIII contains an overview of ligation reactions performed in this study.

2.13.7: DNA sequencing

Sanger DNA Sequencing facility at Bio Basic (Markham, Ontario, Canada) was used and samples were prepared according to sample submission

guidelines. Sequence analysis was performed using ApE software (A plasmid Editor by M. Wayne Davis, University of Utah, Salt Lake City, Utah, USA, <http://biologylabs.utah.edu/jorgensen/wayned/apex/>).

2.13.8: Southern Blotting

Southern analysis was carried out according to standard procedures (Green and Sambrook 2012). 5 µg of genomic DNA was digested over night with restriction enzymes and digested samples were separated by electrophoresis on a 0.8% agarose gel. Standard 1 kb ladder or Digoxigenin labeled DNA Molecular Weight Marker VII (Roche) was used to estimate the size of the fragments. Separated DNA fragments were denatured by treating the gel with 0.25 M HCl at room temperature for 10 to 20 minutes or until the tracking dye turned completely yellow followed by treating the gel with 0.4 M NaOH at room temperature for 10 to 20 minutes or until the tracking dye turned back to blue. Denatured DNA was then transferred from the gel to a negatively charged nylon membrane (Hybond-XL) in an alkaline transfer buffer (i.e in 0.4 M NaOH). After overnight transfer DNA was cross-linked to the membrane by exposing to ultraviolet light using the “optimal crosslink” setting on the cross linker.

Digoxigenin-labeled probe was prepared by using a digoxigenin labeling kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche) according to the manufacturer’s instructions. DNA probe fragment was amplified by PCR and 1 µg of DNA probe was labelled.

All the washes, hybridization and immunodetection were done in a hybridization oven with ~ 10 rpm rotor speed. DNA cross-linked membrane was washed four times (15 minutes each) in 20x SSC buffer (Appendix II) at 50°C and it was pre-hybridized in 15 mL of DIG Easy-Hyb buffer (DIG High

Prime DNA Labeling and Detection Starter Kit II, Roche) for 30 minutes at 42°C. Labeled DNA probe was denatured by boiling 5 minutes at 95°C and snap-freezing for 5 minutes. Denatured probe was added to the DIG Easy-Hyb buffer in the hybridization bottle with the membrane and incubated overnight at 42°C to facilitate hybridization of DIG labeled probe to target DNA fragments on the membrane. When using previously made hybridization solution, the probe was denatured by incubating the hybridization solution at 68°C for 10 minutes.

After overnight hybridization, two stringency washes (5 minutes each) were performed with 20 mL of Stringency Wash buffer 1 (SW#1-Appendix II) at 30°C and another two stringency washes (15 minutes each) were performed with 20 mL of Stringency Wash buffer 2 (SW#2- Appendix II) at 65°C. Immunochemical technique was used to detect the hybridization signal on the Southern blot and all the steps were done at 30°C. The membrane was rinsed for 5 minutes in 20 mL Washing buffer (Appendix II) and incubated for 30 minutes in 20 mL of Blocking solution (Appendix II) followed by incubation for 30 minutes in 20 mL of Antibody solution (Appendix II). Then the membrane was washed twice (15 minutes each) in 20 mL of Washing buffer to remove any un bound antibodies and equilibrated in 20 mL of Detection buffer (Appendix II) for 10 minutes to adjust the pH to 9.5.

The membrane was placed in a plastic folder (facing the DNA side up) and 1 mL of CPSD (chemiluminescent substrate for alkaline phosphatase – Roche) was applied. The plastic folder was closed and opened several times to facilitate even distribution of CPSD on the membrane and incubated at room temperature for 10 minutes. Excess CPSD was removed from the membrane by pat drying with paper towels and the light produced by chemiluminescent substrate was recorded with X-ray film or CCD camera on MicroChemi imager.

2.14: Protein methods

2.14.1: Protein extraction

Strains were grown in 24-well microtiter plates containing 1 mL of liquid minimum media containing 1xVogel's salts (Vogel, 1956), 2% glucose along with any other required nutritional supplements at room temperature for 3 days or at 30°C for 2 days under LL or DD conditions.

Mycelia were harvested by transferring mycelial pads onto a vacuum filtration system with Whatman P8, 3.5 cm filter papers. Mycelial pads were rinsed 3 times with distilled water and vacuum filtered until just damp. Samples were then transferred to 1.5 mL micro-centrifuge tubes and immediately frozen in liquid nitrogen. Frozen samples were stored at -80°C until extraction was performed.

Total protein was extracted by grinding the frozen samples in liquid nitrogen with a pre-cooled mortar and pestle. The frozen powder was suspended in ice-cold SDS extraction buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA- Na_2 and 1mM PMSF) at a volume ratio of 1:1 (buffer: mycelia powder). Vortexed samples were immediately heated to 100°C for 5 min and then cooled on ice. Samples were then centrifuged at 25°C at 12000xg for 5 minutes and supernatants were transferred to a fresh tube. To make dilutions for protein assay 5 μl of supernatant was mixed with 45 μl of water to get 1/10 dilution and 5 μl of 1/10 dilution was mixed with 20 μl of water to get 1/50 dilution.

2.14.2: Protein quantification

To prepare the standard curve 0.5 mg/mL BSA was used. 0, 2.5, 5, 10, 15, 20 μ l of BSA was added to 1.5 mL tubes with 25, 22.5, 20, 15, 10, 5 μ l of water respectively.

2.14.2.1: Bradford assay

Protein concentration was measured using the Bradford assay method (Bradford, 1976). Bradford reagent was purchased from BioShop /BioBasic. An aliquot of the sample was diluted (1:10 and 1:50) and 25 μ l of the dilution was added to 990 μ l of Bradford reagent. The samples were incubated for 5 min at room temperature and then the OD595 was measured with a spectrophotometer. The protein concentration was calculated in mg/ml.

2.14.2.2: BioRad DC protein assay

BioRad DC protein assay was performed according to the manufacturer's instructions. 3 x 300 μ l of each sample were read on a plate reader spectrophotometer and the closest two replicates were averaged. The protein concentration was calculated in mg/ml.

Note: When using BioRad protein assay for protein quantification, DTT was not added to the protein extraction buffer.

2.14.3: Protein electrophoresis by SDS-PAGE

Discontinuous SDS-PAGE was performed to separate proteins. For NCU05950 /UV90 protein 15 % acrylamide gels (Appendix II) and for FRQ protein 7.5% acrylamide gels (Appendix II) were used. Protein concentration was assayed and for NCU05950 /UV90 protein 20 μ g of total protein or for

FRQ protein 50 µg of total protein was used. 0.1 M DTT and 0.1% bromophenol blue were added to the protein samples and the total protein sample volumes were adjusted to the same volume with SDS extraction buffer. The samples were heated at 95°C for 5 min and loaded on the gel. Tris-glycine-SDS buffer (Appendix II) was used as the running buffer and separation of the proteins was performed at 50 mA current per gel for about 30-45 minutes.

2.14.4: Western Blotting

After electrophoresis, proteins were transferred to the PVDF microporous membrane (Millipore - Immobilon-P transfer membrane) using the semi-dry blotter and three buffer system (Anode buffer I, Anode buffer II and Cathode buffer- Appendix II) as instructed by Millipore. The area of the gel was calculated and the transfer was done at 2 mA/cm² constant current for 45 minutes for NCU05950 / UV90 protein or 1.5 hours for FRQ protein.

2.14.5: Immunodetection

For immunodetection, the blot was wetted in 100% methanol for 15 seconds or until it changed from opaque to semitransparent. It was rinsed with water and incubated in 1X blocking buffer (10X blocking buffer: 5% BSA + 5% skimmed milk in 1x TBST, 1X TBST: 150mM NaCl + 50mM Tris, pH 7.5 + 0.05% Tween-20) for one hour with gentle agitation at room temperature. After blocking the blot was probed sequentially with primary antibody (monoclonal Anti-FLAG M2 from Sigma-Aldrich, diluted 1:10000 in freshly diluted 1X blocking buffer or Anti-GFP or Anti-FRQ monoclonal primary antibody, diluted 1:40 in freshly diluted 1X blocking buffer) and secondary antibody (goat anti-mouse IgG-horseradish peroxidase conjugate from Origene,

diluted 1:50000 for UV90 protein and 1:1500 for FRQ protein in freshly diluted 1xblocking buffer). The FRQ antibody was generously supplied by M. Merrow and M. Brunner (Gorl *et al.*, 2001). After washing the membrane with TBST, chemiluminescent detection reagent (Immobilon ECL reagents from Millipore) was added and incubated for 5 minutes. The blot was exposed to X-ray films or a CCD camera on the MicroChemi imager to detect the light produced by chemiluminescent substrate. ImageJ software was used to quantitate the signals by measuring the mean grey value of the band of interest within a box of constant dimensions. The brightness of each band was determined by subtracting the brightness of the background. NCU05950 / UV90 protein was normalized against total protein by staining the membrane with Coomassie Blue after immunodetection. The ratio between the measurement of chemiluminescent signal and measurement of Coomassie Blue staining was considered as the relative expression level of the protein. FRQ phosphorylation state was quantitated by calculating the ratio between the density of the upper band of FRQ protein (highly phosphorylated) to the density of the lower FRQ band (relatively dephosphorylated).

2.14.6: Staining of PVDF membrane with Coomassie Blue

The membrane was washed with water for 10 minutes and rinsed in 100% methanol for 1 minute. The membrane was then incubated in Coomassie Blue solution (0.1% Coomassie R-250, 50% Methanol, 10% Acetic acid) with gentle agitation for few minutes or until the bands were visible. The stained membrane was then washed with de-staining buffer (50% Methanol, 10% Acetic acid) for at least three solvent changes or until the background was nearly clear.

2.14.7: Colloidal Coomassie G-250 staining for proteins in polyacrylamide gels

The method described in Dyballa and Metzger 2009 was used to stain the gels. The polyacrylamide gel was washed with water for 10 minutes and incubated overnight with Colloidal Coomassie G-250 staining solution (Appendix II) on a rotating platform at 20 rpm. The stained gel was de-stained with ddH₂O until clear bands appear.

After staining and de-staining, the gel was immersed in gel drying solution (Appendix II) for 30 minutes or longer. A sheet of plastic wrap (a thin page protector) was soaked. The gel was placed on top of the plastic sheet and a second sheet of plastic wrap was placed on the gel. The gel was scanned by the scanner and allowed to dry for 1 or 2 days.

2.15: RNA methods

2.15.1: RNA extraction

Total RNA was extracted from mycelial biomass grown as described in section 2.3.2. The frozen mycelial samples were grounded in liquid nitrogen with a pre-cooled mortar and pestle. The mycelial powder was immediately transferred to 1.5 mL screw-cap tubes containing 1 mL of ice-cold TRIZOL reagent (Molecular Research Center Inc.) and vortexed. Tubes were incubated at room temperature for 5 minutes and 0.2 mL of chloroform was added to each tube and mixed by shaking vigorously for 15 seconds. The mixtures were incubated at room temperature for 15 minutes and centrifuged at 12000g for 15 minutes at 4°C. The supernatant was transferred to a fresh tube with 0.5 mL of isopropanol and tubes were incubated at room temperature for 5-10 minutes to facilitate RNA precipitation. Tubes were centrifuged at 12000g for 8 minutes at

4°C and the RNA pellets were washed with 1mL of 75% ethanol. The RNA was pelleted by centrifuging at 7500g for 5 minutes at 4°C. Ethanol was removed and the RNA pellet was air-dried. RNA pellets were dissolved in 100 µL of Di-Ethyl-Pyro-Carbonate (DEPC) treated water by incubating in a water bath for 10-15 minutes at 55-60°C and stored at -80°C.

2.15.2: Spectrophotometric quantitation of RNA

The concentration of RNA was determined in a spectrophotometer. RNA was diluted by mixing 2.5 µL of RNA with 97.5 µL of DEPC treated water in 100 µL cuvette. Absorbance at 260 and 280 nm were measured. OD260 measurement was used for the amount of RNA and the ratio of OD260:OD280 was used to determine the purity of the samples (OD260:OD280 of 1.6-1.8 was considered as a good sample). Calculations assumed that at 260 nm, an absorbance of 1.0 contains 40 µg/mL.

2.15.3: RNA gel electrophoresis and Northern blotting

Formaldehyde-agarose gels (Appendix II) with 1x RedSafe (iNtRON Biotechnology) were used to separate RNA. The required amount of RNA (20 or 40 µg) was mixed with 1/10th volume of 3M Sodium acetate and 2 volumes of 95% ethanol and incubated overnight at -80°C for complete precipitation. Centrifuging at 12000g at 4°C pelleted the RNA and the pellet was washed with 100 µL of 70% ethanol. Ethanol was removed and the RNA pellet was air-dried. The air-dried RNA pellet was dissolved by incubating at 75°C for 5 minutes in 5 µL of water and 20 µL of denaturation cocktail (Appendix II). Samples were allowed to cool on ice and used for RNA gel electrophoresis. 1x MOPS was used as the running buffer and the gel was run at about 150 V for 3

hours at about 90 mA or until dark blue dye was at the end and light blue dye was about 1/3 from start.

The RNA gel was photographed under UV light using FluoroChem FC2 Imaging System (Alpha Innotech) and transferred to positively charged BrightStar-Plus® nylon membrane (Ambion) as instructed by the manufacture. Membrane was washed in 2X SSC for a few minutes and exposed to UV cross-linker, 2 times (12000 joules/cm²), to fix RNA onto the membrane.

Digoxigenin-labeled DNA probe was prepared by PCR using 10x concentrated DIG DNA Labeling Mix (Roche) and Q⁵ High-Fidelity DNA polymerase (NEB). pBM61-NCU05950 plasmid was used as the template to generate a 251 bp long DIG labeled NCU05950 specific probe. UV90Ex3F (3' ATCTATGATATGGTCCCGCATG 5') and UV90Ex3R (3' GCAGCGGCA TCGGTAAAGTT 5') primers were used and PCR reaction was run as indicated in Table 2.2 and Table 2.3. Genomic DNA from wild type strain SG#26 was used as the template to generate a 484 bp long DIG labeled FRQ specific probe. FrqF and FrqR primers were used and PCR reaction was run as indicated in Table 2.4 and Table 2.5. The purity and size of the DIG-labeled probe was tested by agarose gel electrophoresis and the concentration of the probe was measured using the Thermo-Fisher Scientific Nanodrop2000 UV/Visible spectrophotometer.

All the washes, hybridization and immunodetection were done in a hybridization oven with ~ 10 rpm rotor speed and membrane was placed in a hybridization bottle. RNA cross-linked membrane was pre-hybridized in 10-15 mL of pre- heated (to 68°C) ULTRAhyb® Ultrasensitive hybridization buffer (Ambion) for 30 minutes at 42°C. Labeled DNA probe was denatured by boiling 5 minutes at 95°C and snap-freezing for 5 minutes. Denatured probe was added to the ULTRAhyb® Ultrasensitive hybridization buffer in the

hybridization bottle with the membrane and incubated overnight at 42°C. When using previously made hybridization solution, the probe was denatured by incubating the hybridization solution at 68°C for 10 minutes.

After overnight hybridization, two stringency washes (5 minutes each) were performed with 20 mL of Low Stringency Wash buffer 1 (2X SSC, 0.1% SDS) at 42°C and another two stringency washes (15 minutes each) were performed with 20 mL of High Stringency Wash buffer 2 (0.1X SSC, 0.1% SDS) at 42°C.

Immunochemical technique was used to detect the hybridization signal on the Northern blot and all the steps were done at 30°C. Instructions supplied with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) and CDP-star from Brightstar Biodetect Kit (Ambion) were followed to detect the signals.

The membrane was rinsed for 5 minutes in 20 mL Washing buffer (Appendix II) and incubated for 30 minutes in 20 mL of Blocking solution (Appendix II) followed by incubation for 30 minutes in 20 mL of Antibody solution with Anti-DIG Fab-AP (Appendix II). Then the membrane was washed twice (15 minutes each) in 20 mL of Washing buffer to remove any unbound antibodies and equilibrated in 20 mL of Detection buffer (Appendix II) for 10 minutes to adjust the pH to 9.5.

Membrane was placed in a plastic folder (facing the RNA side up) and 1 mL of CDP-star from Brightstar® Biodetect Kit (Ambion) was applied. The plastic folder was closed and opened several times to facilitate even distribution of CDP-star on the membrane and incubated at room temperature for 10 minutes. The excess CDP-star was removed from the membrane by pat drying with paper towels and the light produced by chemiluminescent substrate was recorded with CCD camera on MicroChemi imager.

2.16: Bioinformatics

Neurospora crassa sequences were retrieved from the FungiDB database (<http://fungidb.org/fungidb/>) (Stajich *et al.*, 2012) and yeast (*Saccharomyces cerevisiae*) sequences were retrieved from the *Saccharomyces* Genome Database (<https://www.yeastgenome.org/>) (Cherry *et al.*, 2012). Database searches for homologs were carried out using NCBI BLAST software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Madden 2013). Protein domains were identified using the NCBI Conserved Domain search tool (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) (Marchler-Bauer *et al.*, 2017). Pairwise alignments of protein sequences were made using EMBOSS Needle software (<http://www.ebi.ac.uk/Tools/psa/>) (Li *et al.*, 2015). Protein secondary structure predictions were made using SPIDER2 software (<http://sparks-lab.org/server/SPIDER2/>) (Heffernan *et al.*, 2015) and PHYRE2 Protein Fold Recognition Server (<http://www.sbg.bio.ic.ac.uk/phyre2/>) (Kelley and Sternberg 2009). Peptide hydrophobicity/hydrophilicity was analyzed using Peptide 2.0 software (https://www.peptide2.com/peptide_synthesis_hydrophobicity_hydrophilicity.php)

2.17: Microscopy

Expression of fluorescence from GFP- and RFP- tagged strains was observed in live cell cultures by fluorescence microscopy and confocal laser scanning microscopy. Cultures were grown on microscope slides covered with MA medium (Appendix II) with 3% agar and 100 μ M choline, under LL for 24 hours at 30°C or 48 hours at room temperature (22 °C) in 10 cm Petri dishes. Watman filter paper strips, wetted with sterile water were layered underneath

the microscope slides to prevent drying-out of the growth media on the microscope slide. 200 µg/mL inositol was added when growing strains requiring inositol.

Liquid medium (MA without agar) was added to the growing front under a cover-slip, and images were captured from actively growing hyphae using a 60x oil immersion objective in a Bio-Rad (Hercules, CA) MRC 600 confocal laser scanning microscope, 40x oil immersion objective in a Zeiss LSM 700 confocal microscope or Leica DMI 3000 inverted microscope equipped with an oil immersion objective (Leica, 100x, NA 1.40) with a piezo objective drive from Physik Instruments and attached to an Andor DSD2 confocal scanner. Leica DMI 3000 inverted microscope system was integrated and supplied by Quorum Technologies (Guelph, Ontario) and the images captured from this microscope were processed with Volocity software. Contrast of the images captured from Bio-Rad MRC 600 confocal laser scanning microscope and Zeiss LSM 700 confocal microscope were adjusted in Photoshop.

Table 2.1. List of mutations and their phenotypes.

Name	Abbreviation	Gene ID and location	Phenotype
Band (Ras-like protein)	<i>ras-1^{bd}</i> (formerly <i>bd</i>)	- NCU08823 - Linkage group –IV	Slower growth and conidial banding make the rhythm easier to assay. Prevents inhibition of conidiation by high concentration of CO ₂ in closed culture vessels and allows assays of rhythmicity in closed growth tubes.
Choline-1 (Phosphatidyle - thanolamine N-methyltransfer - ase)	<i>chol-1</i>	- NCU08045 - Linkage group –IV	Lengthens the period of circadian rhythm of conidiation. Unable to synthesis the lipid phosphatidylcholine. Carried in most of our lab strains to allow assay of the FRQ-less rhythms in choline dependent cultures. Requires choline supplementation for normal growth as well as rhythmicity and strains are indistinguishable from <i>chol</i> ⁺ strains. This allows us to assay FRQ-less rhythms that do not depend on choline depletion, such as heat pulse entrainment behavior.
Conidial separation -1	<i>csp-1</i>	- NCU02713 - Linkage group –I	Defective in separation of macroconidia and reduces contamination.
Frequency¹⁰ (Period clock protein FRQ)	<i>frq¹⁰</i>	- NCU02265 - Linkage group –VII	Null allele and arrhythmic under some conditions. An engineered gene deletion by gene replacement with

			hygromycine B (HygB ^R) under control of the <i>Aspergillus</i> trpC promoter (Aronson <i>et al.</i> ,1994).
Frequency⁹ (Period clock protein FRQ)	<i>frq⁹</i>	- NCU02265 - Linkage group –VII	Null allele, truncated protein, conditionally rhythmic. (Aronson <i>et al.</i> ,1994)
Histidine-3 (histidine biosynthesis trifunctional protein)	<i>his-3</i>	- NCU03139 - Linkage group –I	Requires histidine for normal growth. The <i>his-3</i> mutant strain contains a single mutation downstream of the <i>Sall</i> restriction site, in the gene encoding a multifunctional enzyme for histidine biosynthesis.
UV90 (hypothetical protein)	<i>UV90</i>	- NCU05950 - Linkage group –VI	Loss of FRQ-less conidiation rhythm in <i>chol-1</i> background, severe effect on heat entrainable FRQ-less rhythm, damping of amplitude of FRQ-WCC oscillator (Li <i>et al.</i> , 2011) , failure to produce PCR products from NCU05950-specific primers.

Table 2.2: PCR reaction to generate DIG labeled NCU05950 specific DNA probe.

Component	25 μ L Reaction	Master mixture for 3 reactions	Final Concentration
5X Q ⁵ reaction buffer	5 μ L	15.0 μ L	1X
1 mM DIG labeled dNTPs	5 μ L	15.0 μ L	200 μ M
10 μ M Forward primer-UV90Ex3F	1.25 μ L	3.75 μ L	0.5 μ M
10 μ M Reverse primer-UV90Ex3R	1.25 μ L	3.75 μ L	0.5 μ M
50 ng/ μ L Template DNA (pBM61-NCU05950 plasmid)	4 μ L	12.0 μ L	200 ng
Q ⁵ High-Fidelity DNA polymerase (2 units / μ L)	0.25 μ L	0.75 μ L	0.02 units / μ L
Nuclease free water	8.25 μ L	24.75 μ L	
Total volume	25 μ L		

Table 2.3: Thermo-cycling conditions used to generate DIG labeled NCU05950 specific DNA probe.

Step	Temperature	Time	
1. Initial DNA denaturing	98 °C	3 minutes	
2. (i) DNA denaturing	98 °C	15 seconds	30 cycles
(ii) Primer Annealing	69 °C	15 seconds	
(iii) Strand Extension	72 °C	1 minute	
3. Final Extension	72 °C	5 minutes	
4. Hold	4 °C	∞	

Table 2.4: PCR reaction to generate DIG labeled FRQ specific DNA probe.

Component	25 μ L Reaction	Master mixture for 3 reactions	Final Concentration
5X Q ⁵ reaction buffer	5 μ L	15.0 μ L	1X
1 mM DIG labeled dNTPs	5 μ L	15.0 μ L	200 μ M
10 μ M Forward primer- FrqF	1.25 μ L	3.75 μ L	0.5 μ M
10 μ M Reverse primer- FrqR	1.25 μ L	3.75 μ L	0.5 μ M
50 ng/ μ L Template DNA (Genomic DNA from SG#26)	8 μ L	24.0 μ L	400 ng
Q ⁵ High-Fidelity DNA polymerase (2 units / μ L)	0.25 μ L	0.75 μ L	0.02 units / μ L
Nuclease free water	4.25 μ L	12.75 μ L	
Total volume	25 μ L	μ L	

Table 2.5: Thermo-cycling conditions used to generate DIG labeled FRQ specific DNA probe.

Step	Temperature	Time	
1. Initial DNA denaturing	98 °C	3 minutes	
2. (i) DNA denaturing	98 °C	15 seconds	30 cycles
(ii) Primer Annealing	70 °C	15 seconds	
(iii) Strand Extension	72 °C	1 minute	
3. Final Extension	72 °C	5 minutes	
4. Hold	4 °C	∞	

Figure 2.1: Map and restriction sites of pBM61 plasmid. A. pBM61 is shown with all recognition sites for single-cutting restriction endonucleases indicated. Both the 5'-truncated and 3' *his-3* exons and the single *his-3* intron are indicated. The inner circle shows the sources of the fragments used to build pBM61, as well as vector primer sites and fusion junctions. The pBluescript KS(+) multiple cloning site is expanded below the map with recognition sites for single-cutting restriction endonucleases and primer sites indicated. Sites within the multiple cloning site that are duplicated elsewhere in the plasmid are not shown. B. All restriction endonucleases that cut pBM61 only once are listed along with their positions on the map. Coordinates run clockwise with position one at the SP6 primer site. C. All restriction endonucleases cutting only within the pSP72 fragment are indicated with their positions and number of cuts. These enzymes are useful for linearizing the plasmid in preparation for transformation. (adapted from Margolin *et al.*, 1997)

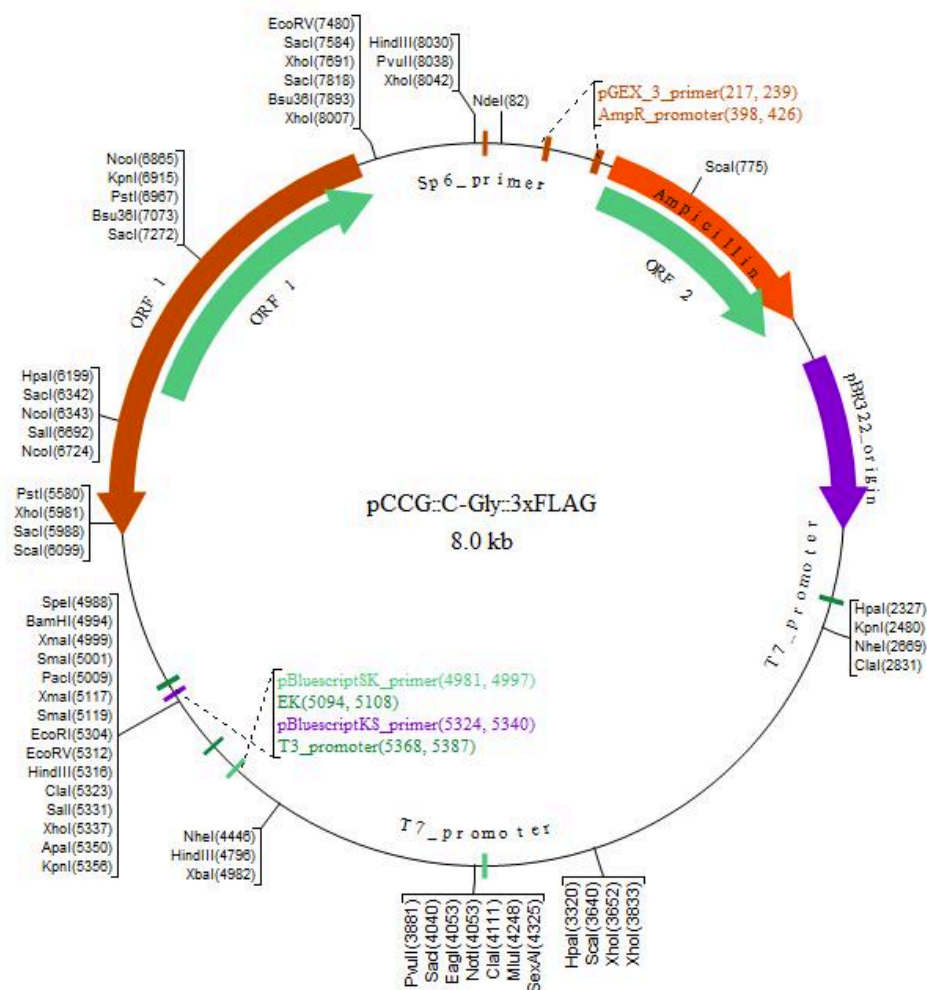


Figure 2.3: Map of *Neurospora* expression vector pCCG::C-Gly::3XFLAG (8049bp). Adapted from: http://www.biovisualtech.com/bvplasmid/pCCG_C-Gly_3xFLAG.htm in December 2014.

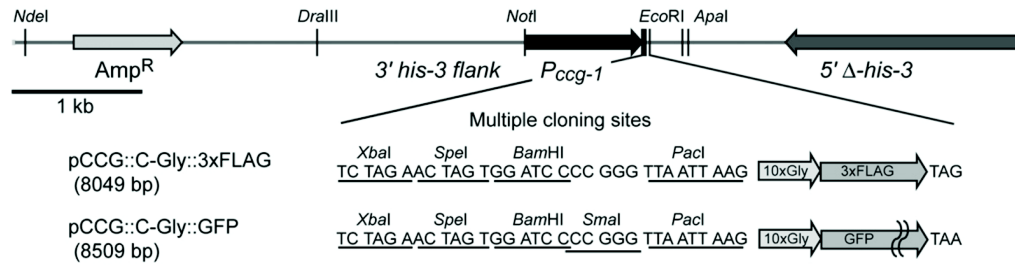


Figure 2.4: Partial maps of C-terminal epitope tag expression vectors pCCG::C-Gly::3xFLAG & pCCG::C-Gly::GFP and sequences of their multiple cloning sites (MCSs). Unique restriction sites are underlined and indicated by vertical bars. All vectors contain the *N. crassa* *cgg-1* promoter (*P_{cgg-1}*), a MCS, and a poly-glycine linker in the same frame and direction. Adapted from: Honda and Selker, 2009.

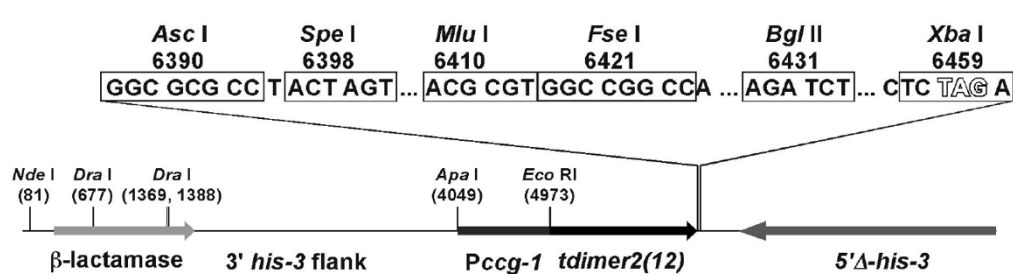


Figure 2.5: Partial map of pMF334 (*pccg-1-tdimerRedN*) plasmid (9177 bp). Adapted from: Freitag and Selker, 2005.

CHAPTER 03: Gene Identification

3.1: Introduction

Isolating mutants that affect the circadian rhythms in various organisms has opened the doors to unraveling the underlying molecular mechanisms. The first clock mutants that alter the period length of a rhythm and thereby affect the timing mechanism have been isolated in *Drosophila* in 1971 (Konopka & Benzer 1971; Lakin-Thomas *et al.*, 2011). Three mutants have been isolated with abnormal 24-hour rhythms of eclosion as well as the locomotor activity of individual flies by treating the male *Drosophila melanogaster* flies with ethyl-methane sulfonate, which is a chemical mutagen (Konopka & Benzer 1971). The genetic analysis of these three mutants revealed that three mutant alleles of one genetic locus in *Drosophila melanogaster*, named the *period* (*per*) locus, were responsible for these phenotype (Konopka & Benzer 1971; Konopka 1987). Clock mutants were isolated from *Chlamydomonas*, *Drosophila pseudoobscura*, *Neurospora crassa* and also additional clock mutants were isolated from *D. melanogaster*, both at the *period* locus as well as at other genetic loci such as *clock* (Konopka 1987).

Neurospora crassa was the second organism, after *Drosophila melanogaster*, in which mutations affecting the circadian rhythms were isolated (Lakin-Thomas *et al.*, 2011). New mutant strains of *Neurospora crassa* were isolated by mutagenizing the strains with *band* (*bd*) mutation with N-methyl-N'-nitro-N-nitrosoguanidine and these showed altered period lengths of their circadian rhythm of conidiation on solid agar medium (Lakin-Thomas *et al.*, 2011 ; Feldman & Hoyle 1973). The mutated locus was identified as *frequency* (*frq*) and it is one of the core component of the *Neurospora crassa* FWO (FRQ-WCC Oscillator) (Lakin-Thomas *et al.*, 2011 ; Feldman & Hoyle 1973).

Using this strategy our research group started searching for mutations affecting rhythmicity of FRQ-less strains, keeping the ultimate goal to identify the components of the FRQ-less oscillator (FLO) as well as to identify the molecular mechanisms that drive FRQ-less rhythms. In order to isolate mutations affecting the FLO, former lab members have conducted a mutagenesis screen to identify mutations affecting conidiation rhythms in the *frq* knockout background as the FLO does not require functional FRQ (Li *et al.*, 2011). By using UV light, uninucleate microconidial spores of a strain carrying both the *frq*¹⁰ knockout allele and *chol-1* mutation have been mutagenized (Li *et al.*, 2011). *Chol-1* mutation bearing strains exhibit conidiation rhythms in FRQ-less strains on choline deficient media and it is an example of a FLO (Li *et al.*, 2011). The parental strain (*csp-1* ; *chol-1 ras*^{bd} ; *frq*¹⁰) used for the mutagenesis showed conidiation rhythms on choline deficient media but is arrhythmic on high choline media (Li *et al.*, 2011). 600 colonies derived from mutagenized spores were arbitrarily numbered and those were individually analyzed on race tubes under high choline and choline deficient conditions (Li *et al.*, 2011). The 90th spore analyzed after UV mutagenesis produced an arrhythmic phenotype in the typical rhythmic *csp-1* ; *chol-1 ras*^{bd} ; *frq*¹⁰ strain on choline deficient conditions and this new mutation was named *uv90* (Li *et al.*, 2011; Lakin-Thomas *et al.*, 2011).

To identify the gene affected by the *uv90* mutation, initial mapping was done by crossing our lab generated *uv90* mutation bearing strain in the Oak Ridge background with known mutant strains and it was mapped to the right arm of linkage group (LG) VI (Li *et al.*, 2011). Former lab members mapped the gene affected by the *uv90* mutation as the NCU05950 gene in the *Neurospora* genome by using Cleaved Amplified Polymorphic Sequence

(CAPS) markers and screening gene knock out strains in this region for the growth phenotype.

Different strategies were used by former lab members to confirm that deletion of NCU05950, and only this gene, is responsible for the *uv90* phenotype. Assaying the circadian rhythm phenotype of the NCU05950 deletion strain has been conducted as the initial approach. FRQ-null mutation and the *chol-1* mutation were introduced to the NCU05950 knockout strain by crossing it with our laboratory strain *csp-1 ; chol-1 ras^{bd} ; frq¹⁰*. The conidiation rhythmicity under free running conditions was assayed and it was found that the phenotype of the NCU05950 deletion was very similar to the *uv90* mutation. These results were observed both in choline-supplemented and choline-deficient conditions as well as in the presence and absence of the FRQ-null mutation. Furthermore, the heat entrained rhythm of the NCU05950 deletion in the FRQ-null background has been assayed and the results were similar to *uv90* mutant and different from the UV90 wild-type strain.

3.2: Methods

3.2.1: Cloning the NCU05950 gene and rescue of the *uv90* mutant phenotype

In *Neurospora crassa* the *his-3* locus is a common target for ectopic gene expression by replacing the *his-3* mutation (Table 2.1) with wild type sequence through homologous recombination (Honda and Selker 2009). The plasmid carrying the 5'-truncated *his-3* gene fragment (5'-truncated *his-3* exon 1:: *his-3* intron :: *his-3* exon 2 -3') , the gene of interest and 3' *his-3* gene flanking region is integrated into the chromosome by a double crossover event (Aramayo and Metzenberg 1996). The first crossover event occurs between the

5'-truncated *his-3* gene fragment and the mutant allele of the *his-3* gene present in the chromosome (Aramayo and Metzenberg 1996). This event regenerates a functional copy of *his-3* gene, which confers histidine prototrophy on the transformant so that it does not need histidine in the media. Therefore, this property can be applied as a selection for transformants with *his*⁺ genotype. The second crossover event takes place somewhere between the 3' *his-3* gene-flanking region and the homologous region on the genome (Figure 3.1). The occurrence of double crossover events completes the integration of the *his-3* sequence to reconstruct the functional *his-3* gene and insert the gene of interest into an ectopic locus.

3.2.1.1: Cloning the NCU05950 gene into pBM61 plasmid

Cloning of the candidate gene NCU05950 into the *his-3* locus targeting 6.8 kb plasmid pBM61 (Figure 2.1) was performed as the first step (Margolin *et al.*, 1997). The genomic DNA sequence of the NCU05950 along with 1kb upstream and downstream (2883 bp in total) was isolated from genomic DNA of *Neurospora crassa* strain SG#80 with the genotype *csp-1* ; *chol-1 ras^{bd}* ; *frq¹⁰* (mat a) (Table 2.1, Appendix I & Appendix IX) by PCR using the primers UV90ApaIF and UV90NotIR (Appendix VI). pBM61 plasmid DNA was prepared and SspI and DraIII enzymes were used to confirm the identity of the plasmid. The plasmid and isolated gene fragment were digested with ApaI and NotI restriction enzymes and were gel purified. Ligation mixtures were transformed to chemically competent *E.coli*.

Colony PCR was carried out to analyze the transformed bacterial cells using the primers used to isolate the gene and the colonies which gave the desired size band (2883 bp) were subjected to further testing. Plasmid DNA was isolated from positive colonies and digested with ApaI-NotI as well as

DraIII-HindIII enzymes to confirm the presence of the insert on the recombinant plasmid named pBM61-NCU05950. The presence of the NCU05950 gene on the recombinant plasmid was further confirmed by sequencing (Appendix X).

3.2.1.2: Transformation of *Neurospora crassa*

NCU05950 knockout strain (carrying the deletion of NCU05950) SG #359 (*csp-1 his-3; chol-1 ras^{bd}; NCU05950^{KO} mat a*) was chosen for transformation. Fungal strain SG #359 grown on histidine and choline containing media was used to harvest conidiospores for transformation. Cloned plasmid (10-15 µg/ 15 µL) was linearized with DraIII and added to 40 µL of washed conidia. Conidiospores were transformed with the plasmid DNA by electroporation and plated on Top agar media without histidine supplement to select *his-3⁺* transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline and the transformants that showed healthy growth with bright orange spores were further analyzed.

3.2.1.3: Analysis of transformants and purification of heterokaryotic transformants by making microconidia

Race tubes containing maltose-arginine medium (100µM Choline present / Choline deficient) were inoculated with transformants, positive control SG # 26 (with genotype *csp-1; chol-1 ras^{bd} mat a*) and negative control SG # 361 (with genotype *csp-1; chol-1 ras^{bd}; NCU05950^{KO} mat a*) to check whether the transformants were showing the wild type phenotype, which is faster growth rate and clear bands. Growth rates and periods were calculated using

Mac Tau software. Some transformants were complemented and the positive results matched with the PCR results.

The PCR performed for transformants with pBM61 vector specific primers that are from the *his-3* locus gave a smaller sized band (964 bp) along with the expected band of 3026 bp. Since it was present on negative controls (SG # 26 with genotype *csp-1*; *chol-1 ras^{bd} mat a* and SG # 361 with genotype *csp-1*; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*), we can explain it as a product from the wild type *his-3* locus. The transformed macroconidia may have two or more nuclei and they have produced heterokaryons, which carry nuclei with *his-3* (untransformed) and *his-3⁺* (transformed). This explains the origin of the smaller sized unexpected PCR product. Therefore, heterokaryotic transformants were purified by making microconidia which are predominantly uninucleate (Horowitz and Macleod 1960; Baylis and DeBusk 1967).

In order to make microconidia, the method described in Ebbole and Sachs 1990 was used. This method uses a medium which contains Na-iodoacetate that induces stress, prevents the fungus from making aerial hyphae and forces the fungus to make microconidia. PCR and race tube experiments with or without 100µM choline containing media were performed with pure microconidia derived transformant strains. Silica stocks were prepared from positive homokaryotic transformants (SG#428, 429, 430, 431, 432).

3.2.1.4: Rescue of the *uv90* mutant phenotype

For the gene rescue experiment, the positive transformed strain with the genotype *csp-1 his-3⁺*:: NCU05950; *chol-1 ras^{bd}*; NCU05950^{KO} (*mat a*) (SG#430) was used as the male parental strain and a strain with the genotype *chol-1 ras^{bd}*; *uv90*; *frq9* (*mat A*) (SG#410) was constructed to act as female parent in a cross. The *csp-1* mutation is carried in most of our laboratory strains

but it was omitted from the female parental strain because *csp-1* makes a poor female parent. The null *frq⁹* allele was introduced instead of the *frq¹⁰* deletion to allow selection against the NCU05950^{KO} allele by hygromycin sensitivity; both the *frq¹⁰* and NCU05950^{KO} alleles confer hygromycin resistance and this would complicate the analysis of progeny. All progeny from the cross carried *chol-1* and *ras^{bd}* as these two mutations are present in both parents. Progeny were screened for the *csp-1* phenotype and for hygromycin sensitivity. Almost all *csp-1* progeny should carry *his-3⁺::NCU05950* which is the transgene because *csp-1* and *his-3* are linked. Hygromycin sensitivity test was performed in liquid media to test for the presence of NCU05950^{KO} alleles and hygromycin sensitive progeny were chosen as the original *uv90* mutant allele containing strains. All *csp-1* and *uv90* mutant (hygromycin sensitive) progeny were tested for rhythm phenotypes on race tubes with or without 100μM choline and the four expected classes of progeny were obtained: with or without the *frq⁹* mutant allele (*frq⁹* is arrhythmic on race tubes with choline) and with or without *his-3⁺::NCU05950* (identified by presence or absence of rhythmicity without choline). Furthermore, PCR analysis was performed with different primer pairs (UV90AFor-R2, pBM61F2- pBM61R2, NCU05950^{KO}F - NCU05950^{KO}R: sequence details are in Appendix III and Appendix VI) to confirm the presence of *his-3⁺::NCU05950* transgene on identified progeny. Silica stocks were prepared for strains with confirmed phenotype and genotype (SG#465,466,467: *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; uv90 ; frq⁹* and SG#468,469,470: *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; uv90*).

3.2.1.5: Southern blot analysis of strains obtained from gene rescue experiment

Digested DNA was separated by electrophoresis on 0.8% agarose gels, denatured and transferred to nylon membranes. The probe (816 bp) consisted of NCU05950 gene fragment, amplified from pBM61-NCU05950 plasmid DNA using the F2 and UV90BRev primers (Appendix III). The labeled probe was made using a digoxigenin labeling kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche) according to the manufacturer's instructions and was hybridized to the membrane. After washing the membrane, the signal was detected using an immunochemical method and chemiluminescence was recorded on film or by a CCD camera.

3.2.2: Southern blot analysis of *uv90* mutant strains

Digested samples were separated by electrophoresis on a 0.8% agarose gel. Separated DNA fragments were denatured and transferred to a nylon membrane. After overnight transfer DNA was cross-linked to the membrane by exposing to ultraviolet light. The probe (2883bp) consisted of the NCU05950 gene plus 1kb upstream and downstream sequence, amplified from pBM61-NCU05950 plasmid DNA using UV90ApaIF and UV90NotIR primers (Appendix IV). Digoxigenin-labeled probe was prepared by random-primed labeling using a digoxigenin labeling kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche) according to the manufacturer's instructions and allowed to hybridize to target DNA fragments. Immunochemical technique and chemiluminescence were used to detect the hybridization signal on the Southern blot. The light produced by chemiluminescent substrate was recorded on X-ray film or by a CCD camera.

3.2.3: Cloning of wild type NCU05950 gene to pUC19 vector

The wild type NCU05950 gene was cloned to a high copy number plasmid pUC19 (2686bp) for future experimental use. pUC19 is a high copy number plasmid and it yields a high quantity of the gene of interest without non-specific products, which is important for making probes. Furthermore, extracting plasmid DNA is a more rapid process than extracting genomic DNA from *Neurospora crassa*.

UV90BamHIF and UV90XbaIR primers that are around 1kb away (upstream and downstream) from the NCU05950 gene were used to isolate the expected size gene fragment of 2870bp from *N. crassa* strain SG # 80 (*csp-1*; *chol-1 ras^{bd}*; *frq¹⁰*) by PCR. pUC19 plasmid DNA was prepared and SspI and HindIII enzymes were used to confirm the identity of the plasmid. The plasmid and isolated gene fragments were digested with BamHI and XbaI restriction enzymes and were gel purified. Ligation mixtures were transformed to chemically competent *E.coli*. Several colonies were picked and colony PCR was carried out to analyze the transformed bacterial cells using the primers used to isolate the gene. Out of the colonies that gave the desired band of 2870bp size, six colonies were selected to isolate pUC19- NCU5950W (Wild type) plasmids and digested with BamHI-XbaI, HindIII, EcoRI enzymes. All the samples showed the presence of the insert. Plasmid DNA isolated from a selected pUC19- NCU5950W (Wild type) construct carrying colony was sent for sequencing to confirm the cloning and confirmed pUC19- NCU5950W construct carrying *E.coli* strains were stored in -80 °C freezer as glycerol stocks.

3.3: Results and Discussion

3.3.1: Confirming the identity of the UV90 gene

As the second strategy to confirm the identity of the UV90 gene ideally a gene rescue experiment should be performed on the *uv90* mutation bearing strain to check the restoration of the *uv90* mutant phenotype with the wild type copy of the putative gene. Therefore, NCU05950 gene along with 1kb upstream and downstream region was cloned to *Neurospora crassa his-3* locus targeting plasmid pBM61 and then it was transformed to NCU05950 knockout strain to confirm the functionality of the cloned gene.

Restriction enzyme digestion and sequencing of recombinant plasmid (pBM61-NCU05950) gave the expected results and confirmed the presence of the insert on the recombinant plasmid (Appendix X & Appendix XI). Linearized pBM61-NCU05950 plasmid was transformed into macrospores of NCU05950 knockout strain SG#359 and successful integration at the *his-3* locus was confirmed by extracting genomic DNA from all selected transformants and carrying out PCR with different combinations of pBM61 vector specific as well as NCU05950 gene specific primers (F2 - UV90BRev = 816 bp, pBM61F2 - UV90BRev = 1946 bp, F2 - pBM61R2 = 1897 bp and pBM61F2 - pBM61R2 = 3026 bp; See Appendix III & Appendix VI). Transformants that gave expected bands (Appendix XII) indicate successful transformation as well as integration at the *his-3* locus. Silica stocks were prepared from positive transformants (SG#420, 421, 422, 423, 424, 425).

Homokaryons were obtained by making microconidia from positive transformants and PCR as well as race tube experiments were performed with pure microconidia derived transformant strains. Clear banding patterns were observed on the race tube experiment and only one PCR derived band was

observed from some transformants with pBM61 vector specific primers which are specific to the *his-3* locus (Appendix XIII & Appendix XIV). The results confirmed that the strains are pure homokaryotic transformants with genotype *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; NCU05950^{KO} (mat a)* and the cloned gene is functional.

As described earlier positive identification of NCU05950 as *uv90* required demonstrating that the NCU05950 wild type gene could rescue the defective phenotype of the *uv90* mutant. The simplest method that we would have used to achieve this task was to transform a *his-3; uv90* strain which contains the original *uv90* mutation with the *his-3* targeting pBM61-NCU05950 recombinant plasmid, as I have done with the *his-3; NCU05950^{KO}* strain. Unfortunately, we were not able to use the standard transformation strategy due to the fact that the lab has been unable to construct the *his-3; uv90* double mutant strain (which carries the *uv90* original mutation and defective histidine biosynthesis gene) in spite of a number of attempts with various combinations of parental strains and the reason for this failure is still unknown. Therefore, we have used another approach to confirm the identity of the UV90 gene.

We have used the positive transformed strain with the genotype *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; NCU05950^{KO} (mat a)* (SG#430) as the male parental strain for the gene rescue experiment in order to introduce the NCU05950 sequence into the *uv90* mutant. A strain of genotype *chol-1 ras^{bd}; uv90; frq9 (mat A)* (SG#410) was constructed to act as female parent in a cross to a *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; NCU05950^{KO} (mat a)* transformant (Figure 3.2). The gene rescue experiment, which was used to introduce the wild type copy of NCU05950 into the original *uv90* mutant strain, both with and without FRQ-null mutation, demonstrated that the conidiation rhythm

phenotype under free running conditions was similar to the *uvr90*⁺ (wild type) strain (Figure 3.3).

To further confirm the identity of the NCU05950 gene as *uvr90*, heat entrained rhythms were assayed. Repeated pulses of high temperature can entrain FRQ-less strains and they behave as if they contain a functional heat-entrainable oscillator (Li *et al.*, 2011). If the rescue was successful, the progeny should give similar conidiation peak times and peak shapes for the heat pulse experiment as the *uvr90*⁺ (wild type) strain. Three FRQ-less strains (SG#465,466,467 with the genotype: *csp-1 his-3*⁺ :: NCU05950; *chol-1 ras*^{bd}; *uvr90* ; *frq*⁹) from the rescue experiment were used as test strains , SG#80 (Genotype: *csp-1*; *chol-1 ras*^{bd}; *frq*¹⁰) was used as the wild-type control and SG#213 (Genotype: *csp-1*; *chol-1 ras*^{bd}; *uvr90* ; *frq*¹⁰) was used as the *uvr90* mutant-type control. To entrain the conidiation rhythm to various T-cycles (T=total number of hours in the cycle) such as 16-hr, 20-hr and 24-hr, 2-hour heat pulses of 32°C on cultures growing at 22°C on race tubes containing maltose-arginine (MA) medium with 100 µM choline were used. Density traces were collected and analyzed as described on Lakin-Thomas, 2006c & Li and Lakin-Thomas, 2010. Changes in peak timing and peak shape in different T-cycles were monitored. Out of the three test strains two (SG#465 & SG#467) showed similar peak timing and peak shape as the wild-type strain SG#80. The *uvr90* mutant strain SG#213 showed a dramatic effect on the peak timing, shifting the major peak to a much earlier time. The peak timing and peak shape exhibited by SG#466 were neither similar to wild - type nor mutant type outcomes (Figure 3.4).

The phenotype of the gene rescued progeny are displayed in Figure 3.4D, where I have re-plotted heat pulse profiles to compare the effects of different T-cycles within each strain. The conidiation peaks in wild type and

rescued progeny occur at varying times after the heat pulse depending on the T-cycle, as expected from an entrained oscillator (Lakin-Thomas 2006c; Merrow *et al.*, 1999). In contrast, the main peaks in the *uvr90* mutant occur earlier in the cycle than the wild type and at nearly the same time, demonstrating that the loss of UV90 function disables the oscillator that is revealed by the heat pulse entrainment and the rescued progeny have gained back the lost function of UV90.

Since the SG#466 strain responded differently for the heat pulse experiment, to check whether any of parental strains or progeny are heterokaryons at the NCU05950 locus or at *his-3* locus, all the strains were grown on minimal medium as well as on minimal medium containing hygromycin and spore PCR was performed with NCU05950KO primers (UV90KO-F & UV90KO-R) and *his-3* locus specific primers (HIS-3F1 & HIS-3R) (Appendix V and Appendix VI). All the strains gave the expected size bands with the two tested primer pairs.

Therefore, Southern blot analysis was performed to look for chromosomal changes. Genomic DNA of wild type (SG#80), *uvr90* mutant type (SG#213), parental strain used for the rescue experiment (SG#430 with the genotype *csp-1 his-3⁺:: NCU05950; chol-1 ras^{bd}; NCU05950^{KO} mat a*) and transformants / rescue progeny (SG#465, SG#466, SG#467, SG#468, SG#469 & SG#470) were digested with KpnI restriction enzyme, which cuts within the gene. A 5295 bp long fragment was expected from wild type strain SG#80, which contain the NCU05950 gene at its native locus and a 4466 bp long fragment from transformants / rescue progeny, which contain the NCU05950 wild type gene at the *his-3* locus (Figure 3.5). The predicted results were observed from all tested strains, i.e.; 5295 bp long fragment from wild type

strain SG#80 and 4466 bp long fragment from transformants as well as rescue progeny (Appendix XV).

As there weren't any chromosomal changes in SG#466, I wanted to look for any sequence variations. Therefore, the NCU05950 gene at the *his-3* locus along with some flanking region (4137 bp) from all three transformants / rescue progeny (SG#465, SG#466 & SG#467) was amplified using HIS-3F1 & HIS-3R primers (Appendix VI) and sequences were obtained with 12 different primers lying across the amplified region. There were not any sequence variations among three tested strains.

Since two of the progeny strains exhibited positive results for all the conducted molecular and biochemical experiments as well as both the assays used to explore FRQ-less rhythms, we can confirm that the phenotype of the *uv90* mutant is due to a loss-of function (null) mutation in the NCU05950 gene in the *Neurospora crassa* genome and the observed *uv90* phenotype cannot be accounted by disruption of neighboring genes. Therefore, we confirmed that deletion of NCU05950 and only this gene is responsible for the *uv90* phenotype using two strategies: assaying the circadian phenotype of an NCU05950 deletion strain and rescuing the phenotype of the *uv90* mutant by transformation with a wild type copy of NCU05950.

3.3.2: Identifying the FLO-affecting mutation

To confirm the presence of a mutation in the NCU05950 locus in our *uv90* mutant strain and to discover the exact nature of the *uv90* mutation, we attempted to sequence this gene in the *uv90* mutant strain and compare the resultant sequences with the published Oak Ridge *Neurospora* genome sequence. According to data from previous *uv90* mutant gene sequencing attempts from our lab, several different primer pairs have been used when

attempting to isolate the mutated *uvr90* gene. Unfortunately, no PCR products were obtained from the tested *uvr90* strains (SG # 213: *csp-1; chol-1 ras^{bd}; uvr90; frq¹⁰* & SG# 227: *csp-1; ras^{bd}; uvr90*) for most of the primer pairs and some primer pairs have given a faint product that was not reproducible.

I tested *uvr90* mutant strains SG # 224 (genotype: *csp-1; chol-1 ras^{bd}; uvr90*) and SG # 213 (genotype: *csp-1; chol-1 ras^{bd}; uvr90; frq¹⁰*) with different primers pairs (Figure 3.6) at different annealing temperatures taking SG # 26 (genotype: *csp-1; chol-1 ras^{bd}*) and SG # 80 (genotype: *csp-1; chol-1 ras^{bd}; frq¹⁰*) as positive controls. At lower annealing temperatures *uvr90* mutant strains were able to produce the expected size product along with some non-specific products (Appendix XVI). Details of all the primers used are mentioned in Appendix III.

Since I was not able to find any changes within the candidate gene or the reason for failing to get the proper PCR product, I used Southern blot analysis to look for chromosomal changes. Genomic DNA of wild type (SG#26: genotype: *csp-1; chol-1 ras^{bd}* and SG#80: genotype: *csp-1; chol-1 ras^{bd}; frq¹⁰*) and *uvr90* mutant type (SG#224: genotype: *csp-1; chol-1 ras^{bd}; uvr90* and SG#213: genotype: *csp-1; chol-1 ras^{bd}; uvr90; frq¹⁰*) were isolated and digested with appropriate restriction enzymes, which cut within the gene. The DIG labelled probe (2883bp) consisted of the NCU05950 gene plus 1kb upstream and downstream sequence was used to hybridize with the Southern blot.

The experiment was performed with nine different enzymes (EcoRI, EagI, HindIII, BglII, DpnI, HpaII, AluI, XhoII, TaqI) and was able to detect clear differences between wild type and *uvr90* mutant type strains, i.e. the Southern blot analysis conducted using a probe for the wild-type NCU05950 gene showed a loss of DNA fragments in this region in the mutant (Appendix XVII,

Figure 3.7 & Figure 3.8). In order to check the repeatability of results, the southern blot analysis was performed for seven additional *uvr90* mutant strains obtained from different crosses (Progeny # 106, 140, 145, 160 with *csp-1*; *ras^{bd}*; *uvr90* genotype, SG#227: *csp-1*; *ras^{bd}*; *uvr90*, SG#370 & SG#427: *csp-1*; *chol-1 ras^{bd}*; *uvr90 wc-1^{KO}*) with XhoII enzyme. Clear banding pattern differences between the *uvr90* mutant progeny strains and wild type strains were observed and it indicates the differences always co-segregate with the *uvr90* phenotype. The results of southern blot analysis reveal an occurrence of major chromosomal change such as insertion, deletion, translocation, inversion or duplication in the entire NCU05959/UV90 gene-bearing region.

I carried out additional PCR in this region, walking in both directions from NCU05950 in approximately 1 kb increments (Details of all the primers used are mentioned in Appendix III). *uvr90* mutant strains (SG # 224: *csp-1*; *chol-1 ras^{bd}*; *uvr90* and SG # 213: *csp-1*; *chol-1 ras^{bd}*; *uvr90*; *frq¹⁰*) and wild type strains (SG # 26: *csp-1*; *chol-1 ras^{bd}* and SG # 80: *csp-1*; *chol-1 ras^{bd}*; *frq¹⁰*) were used for this experiment. PCR failed to give products from *uvr90* mutant strains until I reached genes NCU05957 upstream and NCU05947 downstream (Figure 3.9 & Figure 3.10). Long-range PCR across this region produced products shorter than predicted in the *uvr90* mutant but none in the wild type, likely due to the extreme length of the predicted products (Figure 3.9). I sequenced long-range PCR products obtained from the *uvr90* mutant strain SG# 213 using 17F21 – 17R7 as well as 17R22 - 17R8 primer pairs and found a deletion of 35,128 nt (Figure 3.18) of linkage group VI (3321068 to 3356197) (Details of the primers used to isolate the gene fragment and for sequencing are mentioned in Appendix III). This deletion removes 11 genes, including NCU05950 and two tRNAs, and partially deletes NCU05957 and NCU05947 (Appendix XVIII, Figure 3.10 & Figure 3.11). Even though UV mutagenesis

has deleted a 35,128 nt long fragment from the genome, our experiments show the phenotype of the *uvr90* mutant is due to a loss-of function (null) mutation in the NCU05950 gene and it cannot be accounted by disruption of other neighboring genes.

Table 3.1: Periods and growth rates of NCU05950 mutants and transformants (NCU05950⁺).

Genotype	Number of Isolates	+ choline		- choline	
		Period (h)	Growth rate (mm/h)	Period (h)	Growth rate (mm/h)
control	1	21.9 ± 0.2 (12)	1.38 ± 0.04 (12)	47.3 ± 1.8 (16)	0.54 ± 0.01 (16)
<i>frq</i> ^{null}	1	N.R.	1.46 ± 0.01 (4)	56.5 ± 4.3 (8)	0.50 ± 0.01 (8)
<i>uvr90</i>	1	22.1 ± 0.2 (12)	1.20 ± 0.02 (12)	N.R.	0.52 ± 0.01 (16)
<i>uvr90 frq</i> ^{null}	1	N.R.	1.28 ± 0.03 (12)	N.R.	0.57 ± 0.01 (16)
NCU05950 ^{KO}	1	23.1 ± 0.6 (8)	1.19 ± 0.02 (8)	N.R.	0.50 ± 0.02 (8)
NCU05950 ^{KO} <i>frq</i> ^{null}	1	N.R.	1.18 ± 0.01 (8)	N.R.	0.51 ± 0.01 (8)
<i>uvr90</i> NCU05950 ⁺	3	21.2 ± 0.1 (12)	1.39 ± 0.01 (12)	57.6 ± 3.0 (24)	0.48 ± 0.01 (24)
<i>uvr90 frq</i> ^{null} NCU05950 ⁺	3	N.R.	1.39 ± 0.02 (12)	61.2 ± 2.9 (23)	0.49 ± 0.01 (24)

Genotype abbreviations as for Figure 3.3.

All strains are also *csp-1*; *ras*^{bd} *chol-1*.

Data from transformants pooled from several independent isolates with the indicated genotype. Strains were grown with (+) or without (-) 100 µM choline in the medium.

Data reported as mean ± S.E.M. (N).

N.R.: Not rhythmic

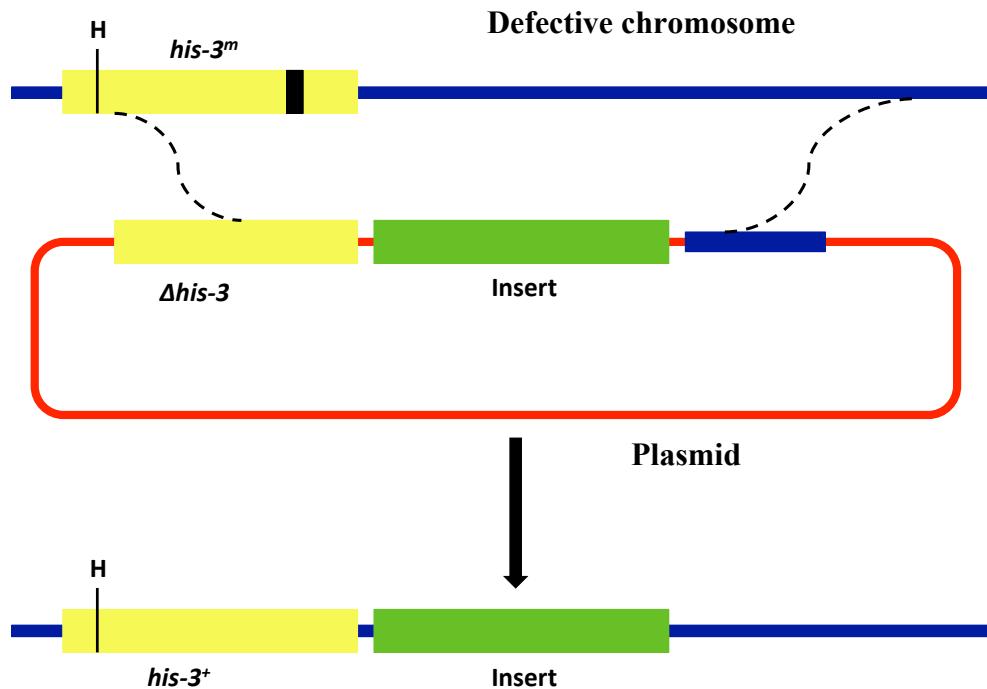


Figure 3.1: Integration of insert containing *His-3* locus targeting vector to the chromosome and reconstituting of *his⁺* genotype. Integration of *His-3* locus targeting vector with its insert occurs by double crossover event. During this process integration of the insert at *his-3* locus happens without duplication of the *his-3* sequence. In this case, only a linear fragment with homology to chromosomal DNA on both the ends is used. The thin vertical line marked H is a HindIII restriction site, at which the plasmid copy of *his-3* is truncated. The dark vertical within the chromosomal gene is the site of the 3' mutation, *his-3^m*. Recombination between the plasmid *his-3* sequence and the resident gene can reconstruct a wild type allele. Therefore, we can use media lacking histidine for selection of *His⁺* transformants.

Gene rescue experiment

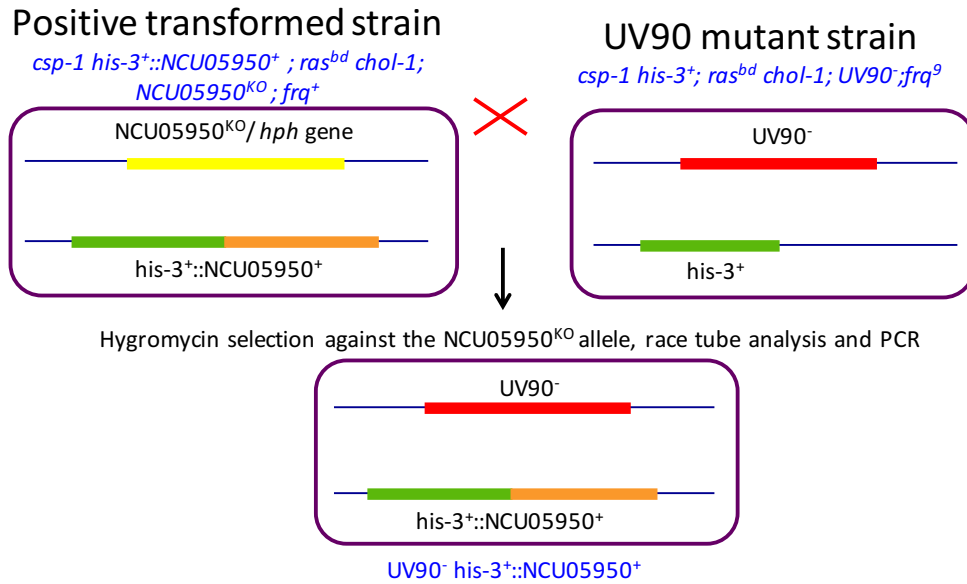


Figure 3.2: Schematic diagram showing the gene rescue experiment strategy.

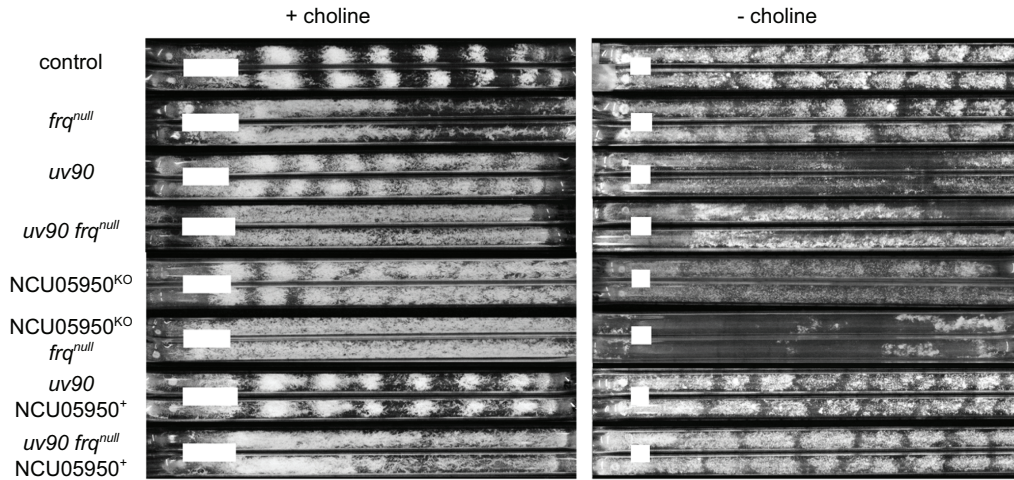
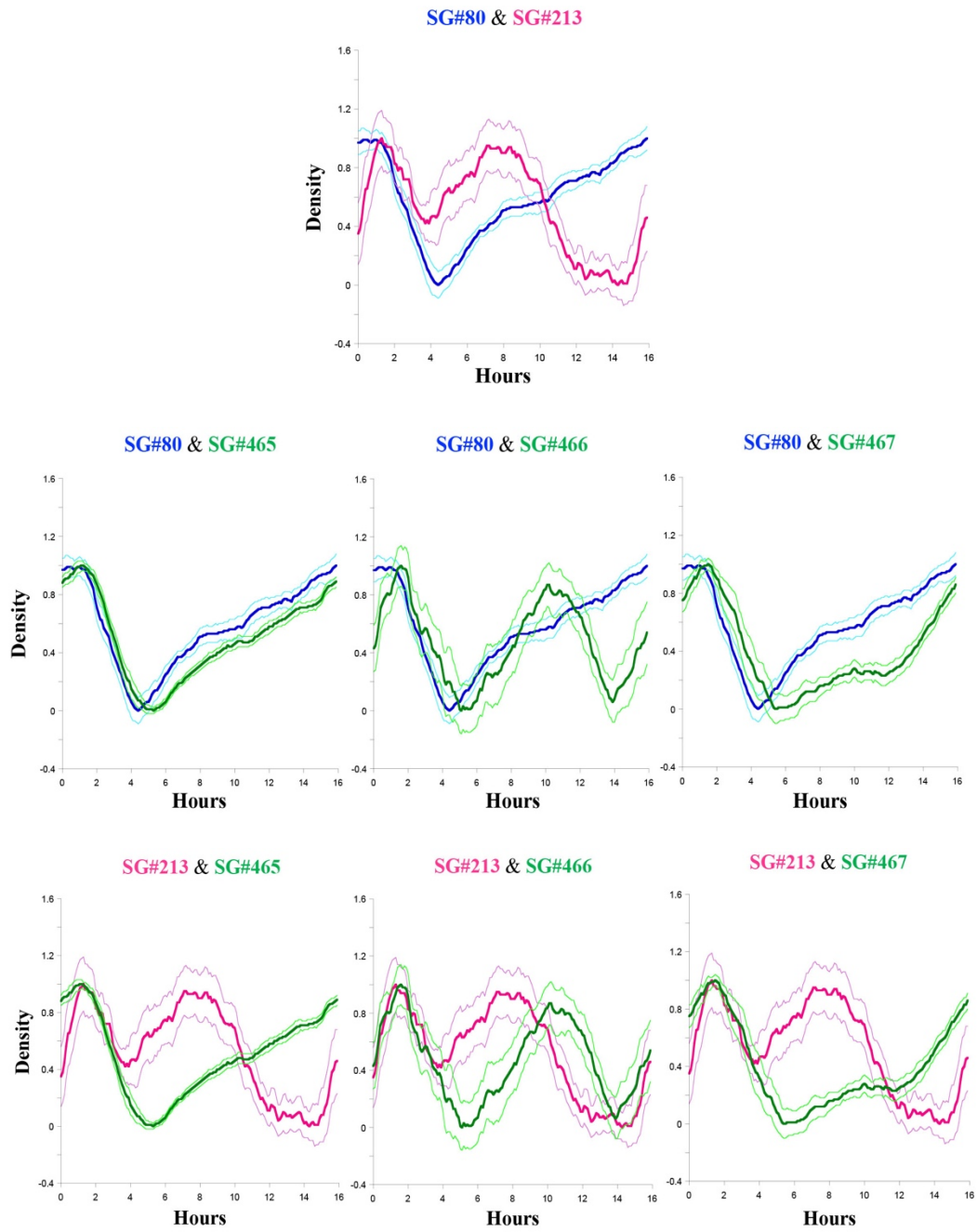
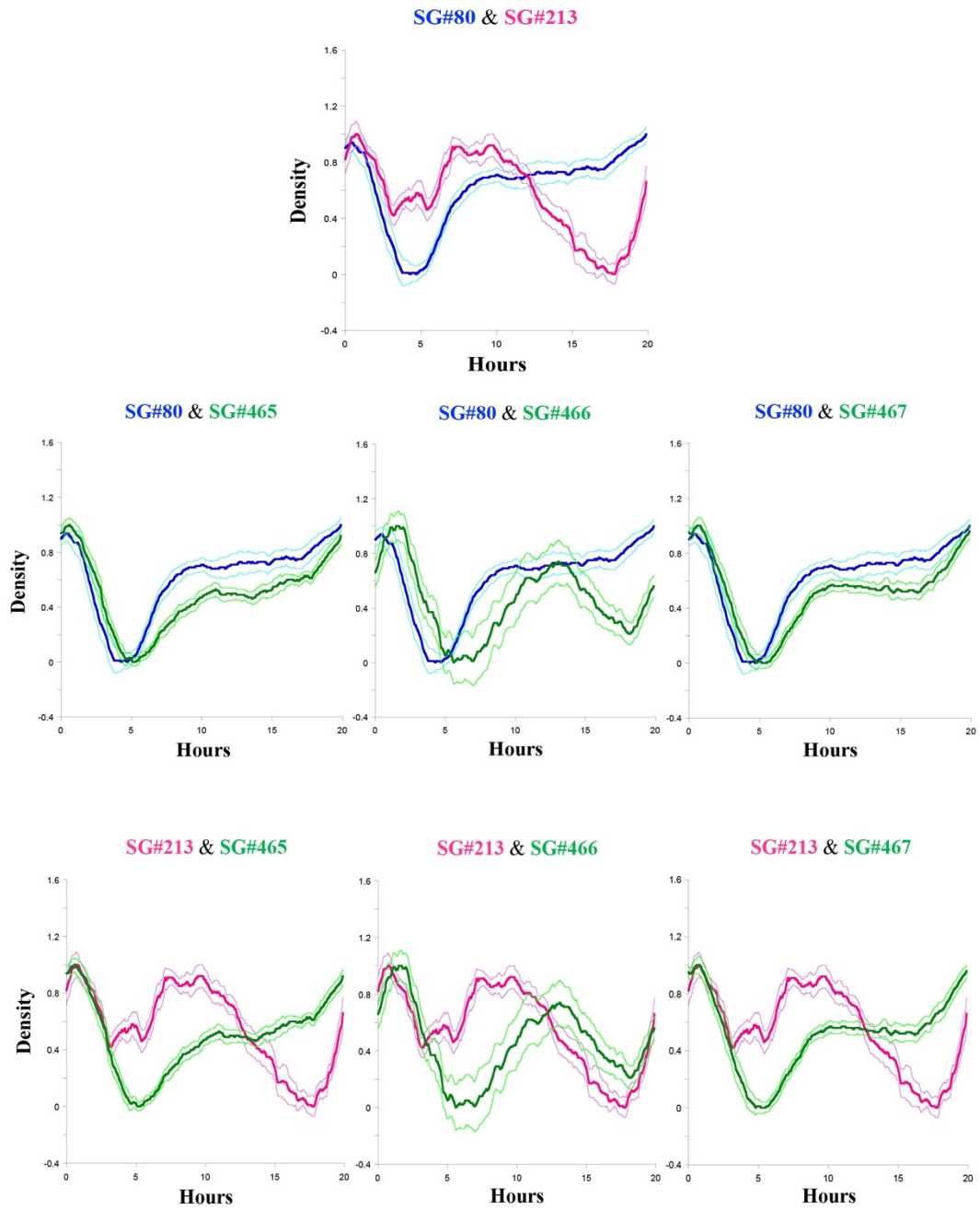


Figure 3.3: Free-running rhythmic phenotypes of NCU05950 mutants and transformants. “*frq*^{null}” indicates a null allele of *frq*. “NCU05950^{KO}” indicates deletion of the NCU05950 gene. NCU05950⁺ indicates transformant carrying a wild type copy of the NCU05950 gene at an ectopic location in the *his-3* locus. All strains, including “control”, are also *csp-1*; *ras*^{bd} *chol-1*. Strains were grown with (+, left panel) or without (-, right panel) 100 μM choline in the medium. Two representative replicate tubes for each condition are shown. Growth is from left to right. White bars indicate average growth in 24 hours. Periods and growth rates are reported in Table 3.1.

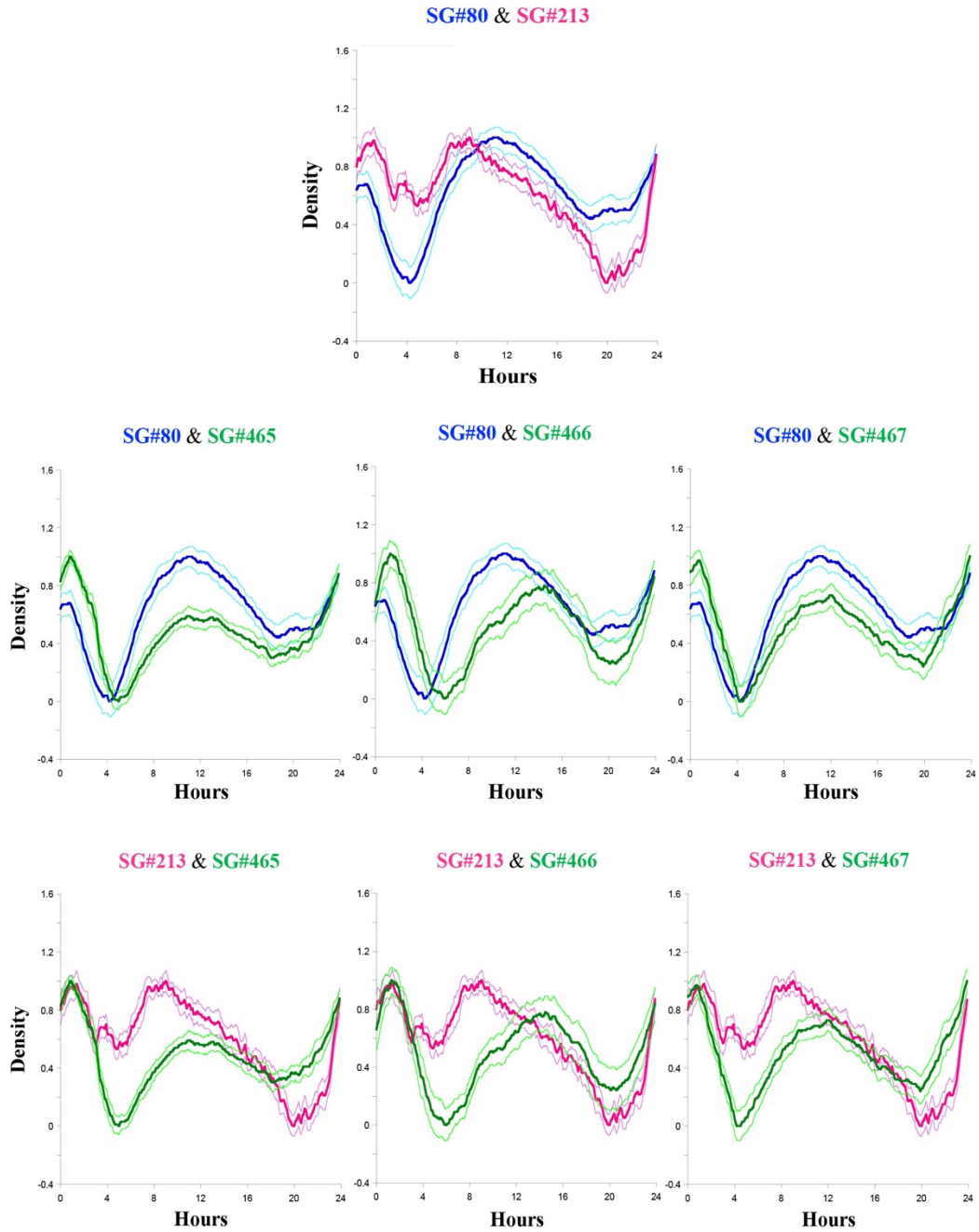
A. T=16 hrs



B. T=20 hrs



C. T=24 hrs



D.

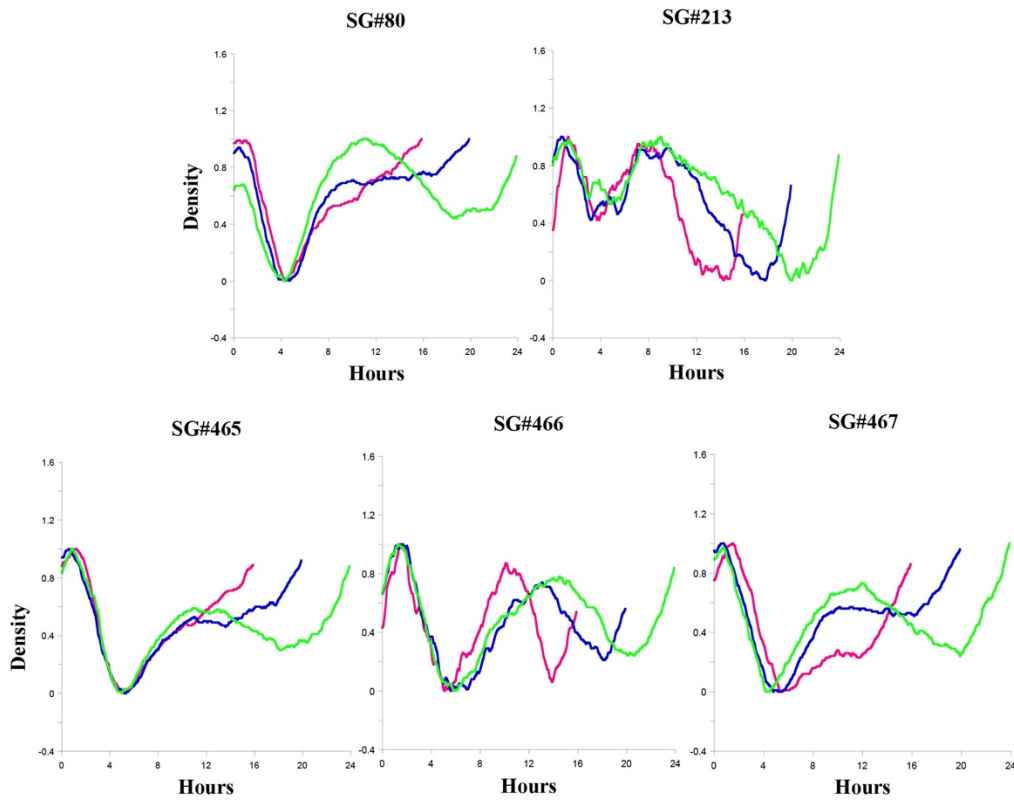
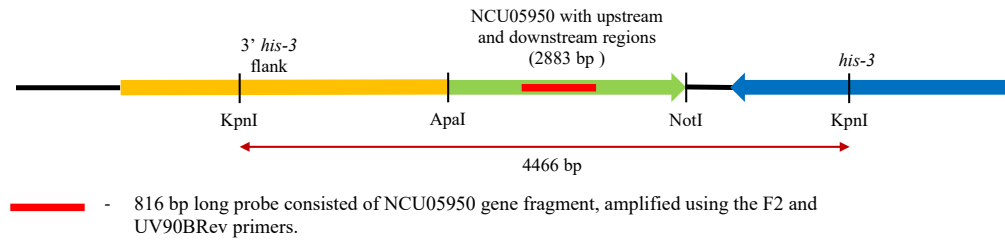


Figure 3.4: Rescue of the heat pulse entrainment phenotype of the *uv90* mutant by the NCU05950 gene. Comparison of NCU05950 wild type, *uv90* mutant and NCU05950 transformants. NCU05950 wild type control SG#80 (blue), *uv90* mutant SG#213 (pink) and NCU05950 transformants which are *uv90* mutants carrying a wild type copy of the NCU05950 gene (green) were grown at 22°C and entrained to repeated 2- hour pulses of 32°C at intervals of T hours. T is the length of one cycle from one pulse start to the next pulse start. The normalized conidiation density of an average “day” (one T-cycle) is plotted against time after the beginning of the heat pulse. T-cycles were 16 hrs (A), 20 hrs (B) or 24 hrs (C). Thin lines are ± 1 S.E.M. Five replicate tubes were averaged for each trace, 2 cycles from each tube, so N = 10 cycles averaged.

All strains, including control, are also *csp-1*; *ras^{bd}* *chol-1*; *frq^{null}*. Growth media contained 100 μ M choline to repair the *chol-1* defect, which is not relevant to this experimental design. (D). Comparison of different T-cycles for the heat pulse entrainment of NCU05950 wild type, *uvr90* mutant and NCU05950 transformants. Data are re-plotted from (A, B and C). T-cycles were 16 hrs (pink), 20 hrs (blue) or 24 h (green).

A.



B.

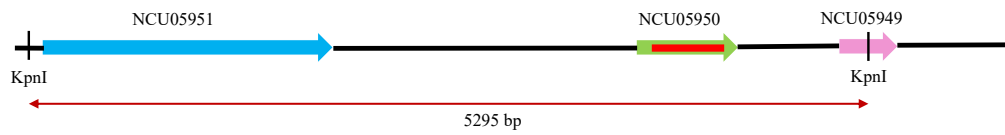


Figure 3.5: Schematic diagram of partial map of NCU05950 gene at *his-3* locus and its native locus. A. NCU05950 gene at *his-3* locus. B. NCU05950 gene at its own locus.

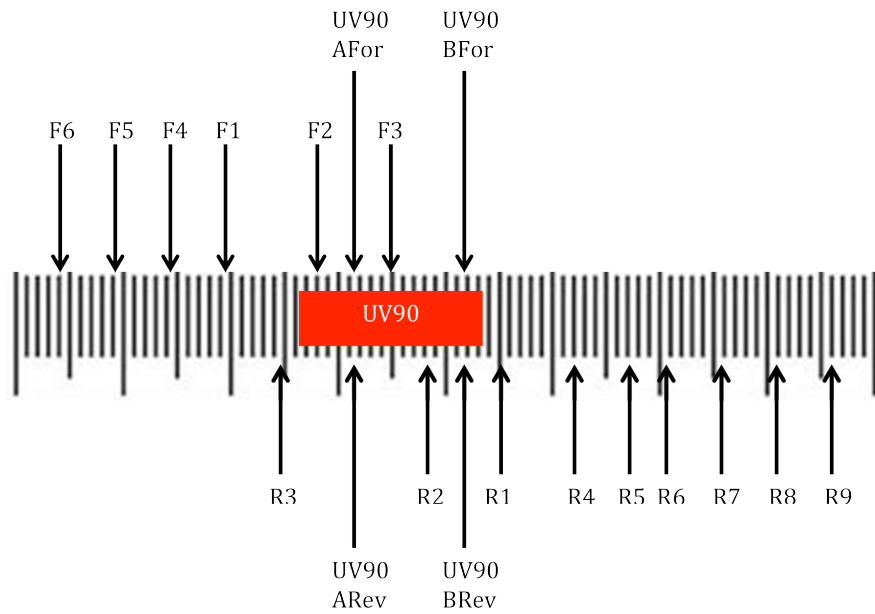


Figure 3.6: Schematic diagram of primers used to identify the mutation on UV90 gene , NCU05950. NCU05950 gene is located on *Neurospora crassa* Linkage Group VI at 3,347,867-3,348,756. Primer locations are listed on Appendix III.

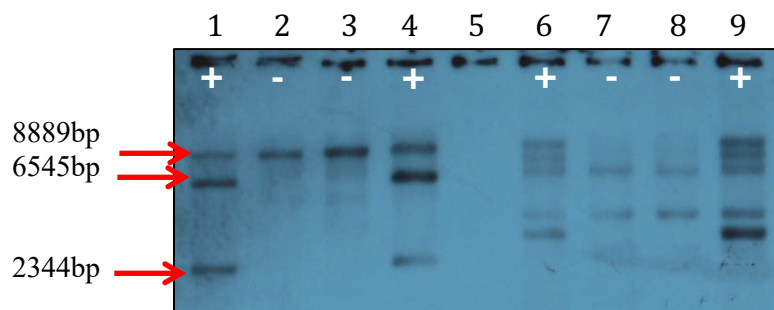


Figure 3.7: Representative Southern blot comparing *uv90* / NCU05950 wild type strains (SG#26 & SG#80) and *uv90* mutant type strains (SG#224 & SG#213). DNA from four strains, two *uv90* / NCU05950 wild type (+) and two *uv90* mutants (-), was cut with either EcoRI or BglII and the blot was hybridized with a DNA probe covering the NCU05950 locus plus upstream and downstream regions. Seven additional cross progenies with the *uv90* phenotype were analyzed with similar results.

Lane No.	Strain	Enzyme
Lane No.1	SG#26 - NCU05950 wild type (+)	EcoRI
Lane No.2	SG#224 - <i>uv90</i> mutants (-)	EcoRI
Lane No.3	SG#213 - <i>uv90</i> mutants (-)	EcoRI
Lane No.4	SG#80 - NCU05950 wild type (+)	EcoRI
Lane No.5	Empty	
Lane No.6	SG#26 - NCU05950 wild type (+)	EcoRI
Lane No.7	SG#224 - <i>uv90</i> mutants (-)	EcoRI
Lane No.8	SG#213 - <i>uv90</i> mutants (-)	EcoRI
Lane No.9	SG#80 - NCU05950 wild type (+)	EcoRI

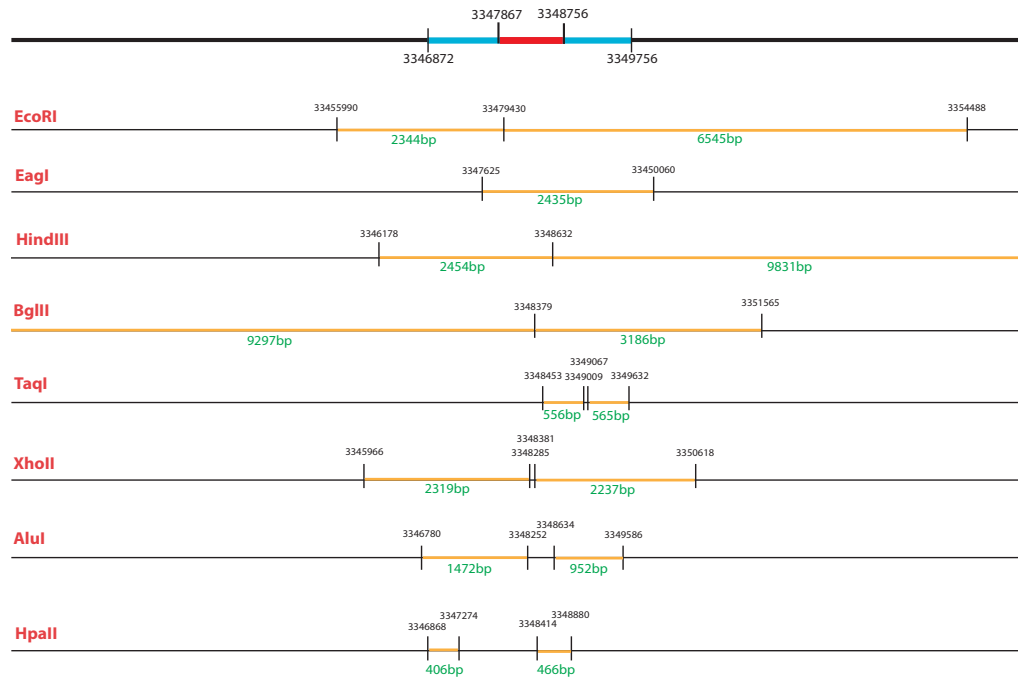


Figure 3.8: Positions of restriction enzyme cutting sites on NCU05950 gene and its neighborhood.

- - Fragments missing in *uv90* mutant (SG#213 & SG#224) strains.
- - NCU05950 gene.
- — — - Probe used during the Southern blotting.

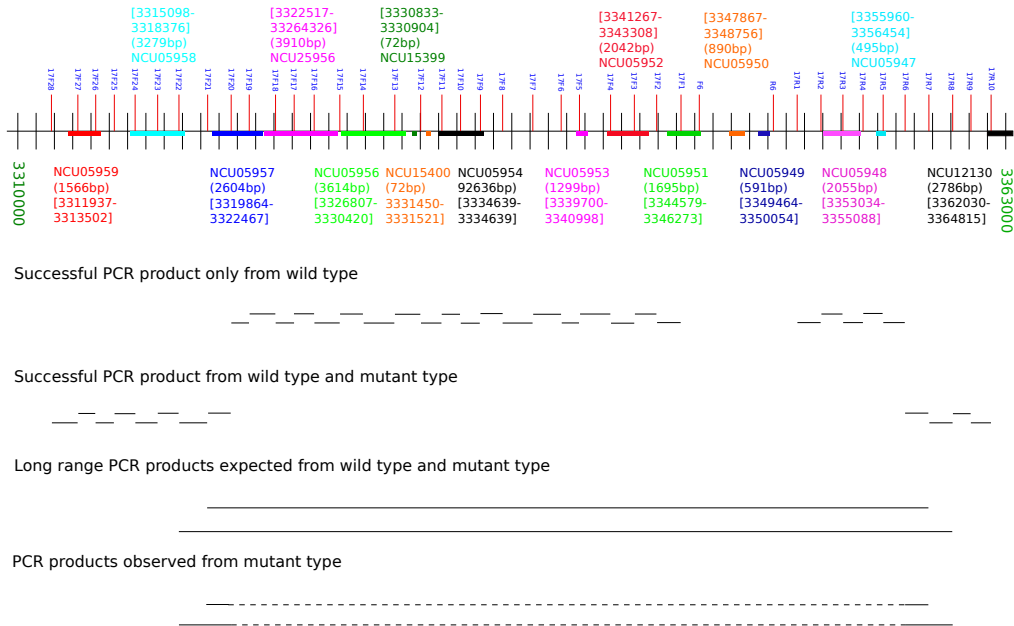
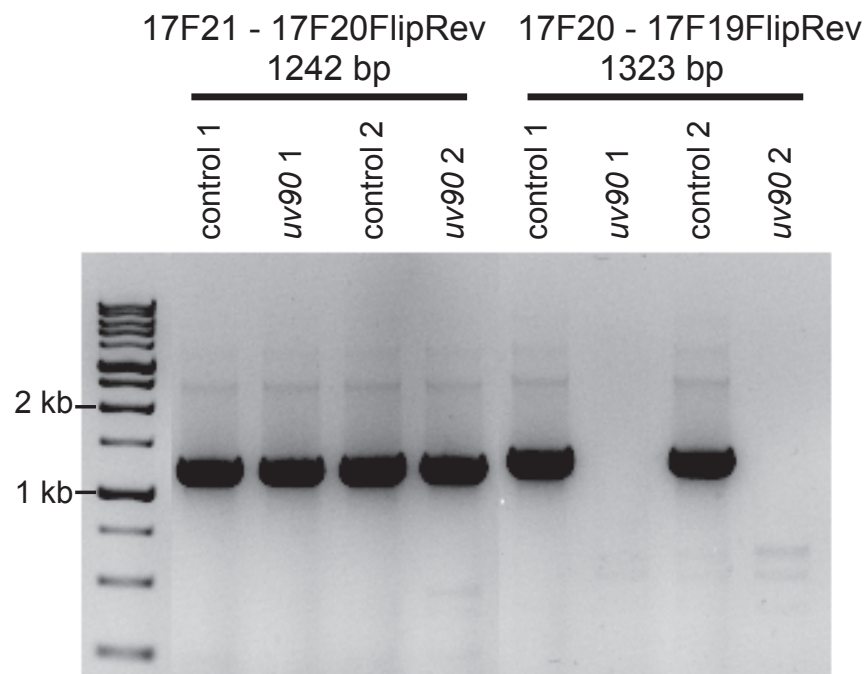
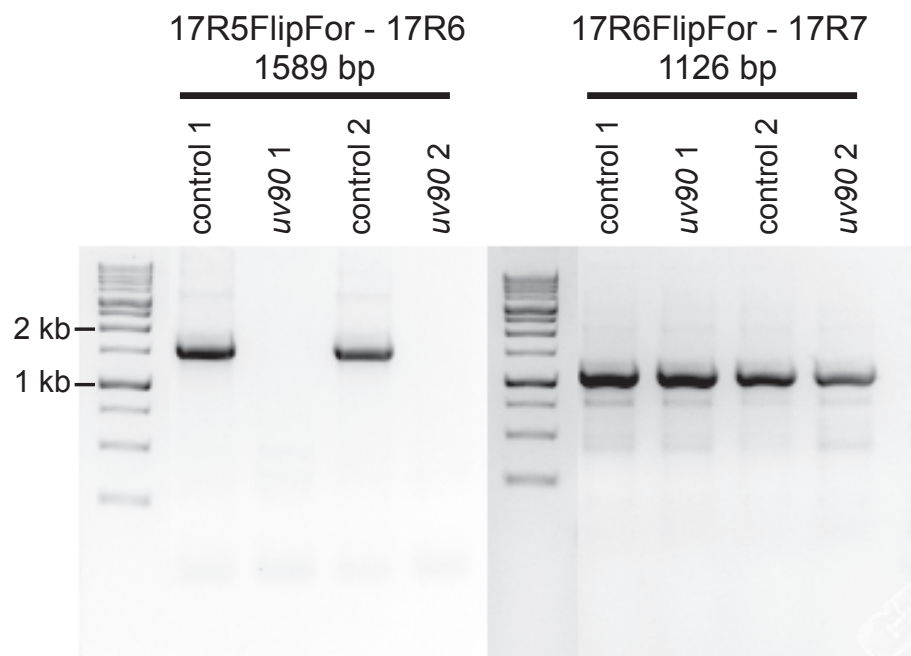


Figure 3.9: Map of PCR reactions across chromosome VI in the *uv90* / NCU05950 region. Nucleotide positions on chromosome VI and gene sizes are shown for genes. Marks are at 1 kb intervals. PCR primers shown above the chromosome are listed in Appendix III.

A.



B.



C.

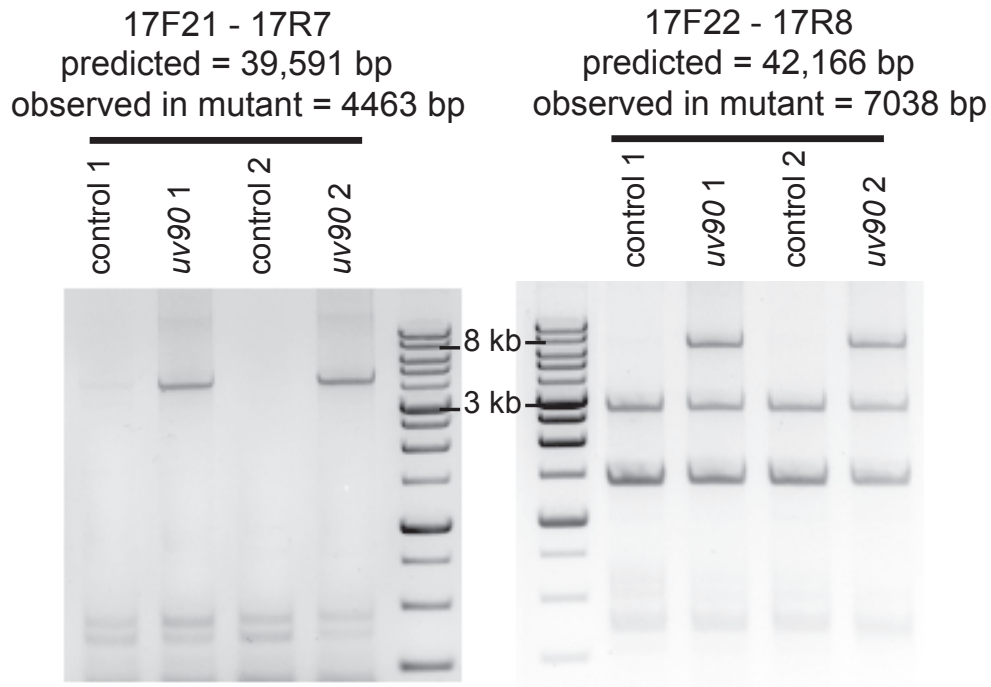


Figure 3.10: PCR of *uv90* mutant allele. Representative gels of PCR products derived from the regions around the *uv90* deletion. (A) & (B): Primer pairs on either side of the deletion breakpoints showing absence or presence of products in the *uv90* mutant. (C): Long- range PCR across the breakpoint showing presence of shorter products in the *uv90* mutant. Two strains of each genotype (*uv90* / NCU05950 wild-type “control” and *uv90* mutant) are shown. Genotypes are: control 1, *csp-1*; *chol-1 ras^{bd}*; control 2, *csp-1*; *chol-1 ras^{bd}*; *frq^{l0}*; *uv90* mutant 1, *csp-1*; *chol-1 ras^{bd}*; *uv90*; *uv90* mutant 2, *csp-1*; *chol-1 ras^{bd}*; *uv90*; *frq^{l0}*. PCR primers are listed in Appendix III and are mapped in Figure 3.16.

Figure 3.11: Partial sequences of genes upstream and downstream of the *uvr90* mutation. Highlighting indicates sequences bordering the deletion in the *uvr90* mutant.

NCU05957

3320919GGGGAATGGCTAACGCTCTGCCGGTCGACACATTCCCATTGCGAAG
ACGTGGACGCGGGCAGAATACCAGGACTTCTGGTTATTGACTGTATCACTGGC
TATATCGTCCCTATCCCACCTCGAACATAATGCTACCAATCAGGTGGCAAAC TA
TGTTACGCTCAGTTACCTTTGGGGCACAGCTGAAGCAACCGGCGAATCGGTTG
TTCAATCGACCAACTGCGATGGTGGTAGCGGTCTTGCTCTTCCAACCTCAGATA
CCCCTAGTCATCAGTGACGCGATACGGGTCTGTC AAGCAACTG

***uvr90* mutant**

3320919GGGGAATGGCTAACGCTCTGCCGGTCGACACATTCCCATTGCGAAG
ACGTGGACGCGGGCAGAATACCAGGACTTCTGGTTATTGACTGTATCACTGGC
TATATCGTCCCTATCCCACCTCGAACATAATGCTACCAATCAGGTGGCAAACAG
CATCAATTGAGTGCACATACCCTAATCCTTTTTTCGCCTTGTTGTTGTCTCCTT
CCTCTCTTTGCGTGTGCGCCCCACCGACTTTTGCTAGGCACTTCCAGTACGGG
ACTTGGCTGGGCCAGGTTGCCCATCAAGGAGAAACACGCAGA3356346

NCU05947

GTACATGTTCCCTTGTTCTCTCCATTCCCTCGTGGACCTTGCATACACGCGGAC
CCCTGTTGCTATGGTCATGTTATTACGACCCACTCGGGATGGTGCCTGAGTGT
TGGGGAGTTGGAAGAAGTATCTGAACGGTGAGGGTGGTGTAGACAGCATCAAT
TGAGTGCACATACCCTAATCCTTTTTTCGCCTTGTTGTTGTCTCCTTCTCTCT
TTGCGTGTGCGCCCCACCGACTTTTGCTAGGCACTTCCAGTACGGGACTTGGC
TGGGCCAGGTTGCCCATCAAGGAGAAACACGCAGA3356346

CHAPTER 04: Functional annotation and structure

elucidation of the NCU05950 gene product

4.1: Introduction

Fungi are estimated to represent about 25% of the world's biomass and include nearly as many as 5 million species (Ellison *et al.*, 2014). Most fungal genomes are poorly annotated and assigning genes to phenotypes on a genomic scale is important for industrial, biomedical as well as other fields (Ellison *et al.*, 2014). The budding yeast *Saccharomyces cerevisiae* is one of the well-studied eukaryotic model organisms that provides information about cellular and molecular processes and genetic basis of fungal growth, the coordination of nutrient acquisition, transport, and metabolism (Ellison *et al.*, 2014) and it is also important for industry. The *Saccharomyces cerevisiae* genome is approximately 12 Mb in size and organized into 16 chromosomes and has been extensively annotated (Engel *et al.*, 2014).

The filamentous fungus *Neurospora crassa* genome is approximately 40-megabase in size, and consists of 7 chromosomes (Linkage groups) and about 10000 protein-coding genes (Galagan *et al.*, 2003; Roche *et al.*, 2014). It has been sequenced and is available at the FungiDB website (<http://fungidb.org/fungidb/>) (Stajich *et al.*, 2012; Galagan *et al.*, 2003; Lakin-Thomas *et al.*, 2011). However, 40% of the predicted genes in this genome remain to be annotated with no known function, because filamentous fungi harbor thousands of genes that are absent in unicellular yeast (Ellison *et al.*, 2014).

Traditionally, functions of uncharacterized genes have often been discovered using deletion mutants (Ellison *et al.*, 2014; Colot *et al.*, 2006;

Kasuga *et al.*, 2009). Genes or proteins of organisms that have similar evolutionary history may perform similar biological functions in a biological pathway and those genes or proteins can have similar architecture, sequence similarities, common structural motifs, gene order, gene fusion events, similarities in gene expression as well as similar domain structures. Therefore, computational tracking of complementary architectural components of a gene or protein could provide clues to their function and the biological pathway that these genes or proteins participate in.

4.2: Results & Discussion

4.2.1: Functional annotation of UV90 gene

The NCU05950 gene is located on *Neurospora crassa* chromosome VI (Location: CM002241: (+) 3,347,867...3,348,756). The 890 bp transcript of NCU05950 consists of 3 exons and 2 introns. The predicted mRNA sequence of NCU05950 is 695 bp and the predicted protein sequence is 158 amino acids (Appendix IX) (FungiDB, <http://fungidb.org/fungidb/>; Stajich *et al.*, 2012).

The UV90 / NCU05950 gene does not have a functional annotation in the *Neurospora crassa* genome database (FungiDB, <http://fungidb.org/fungidb/>; Stajich *et al.*, 2012) and it is listed as a “Hypothetical protein”. In order to find homologous proteins in other organisms, bioinformatics analyses were carried out using the 158 amino acids translated sequence retrieved from the *Neurospora crassa* genome database (FungiDB, <http://fungidb.org/fungidb/>; Stajich *et al.*, 2012). NCBI BLAST (Basic Local Alignment Search Tool: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) software was used for database searches for homologous proteins and NCBI DELT-BLAST (Domain Enhanced Lookup Time Accelerated BLAST)

as well as NCBI conserved domain search tool ([https:// www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi](https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)) were used to identify the protein domains (Madden 2013; Marchler-Bauer *et al.*, 2017; Geer *et al.*, 2002). Database searches using these programs retrieved a number of similar proteins. These similar proteins are found in other filamentous fungi and none of these had functional annotations (Table 4.1).

I performed a search for putative conserved domains and identified a conserved domain in the LAMTOR family with the domain architecture ID 10634440, which includes human p18/LAMTOR1 and yeast EGO1/Meh1p/Gse2p (Figure 4.1 and Table 4.2). About 853 proteins with LAMTOR domain were found, which includes 411 animal proteins, 439 fungi proteins, 1 bacterial protein and 1 virus protein.

LAMTOR (Late endosomal/lysosomal adaptor and MAPK and MTOR activator) is a family of eukaryotic proteins. Human p18/LAMTOR1 is found in mammalian “Ragulator” complex, which interacts with the Rag complex and localizes it to the surface of late endosomes and lysosomes (Sancak *et al.*, 2010). Ragulator plays positive roles in the MAPK pathway by interacting with the Rag GTPases, recruiting them to lysosomes, and bringing about mTORC1 activation. In yeast, EGO1 is localized to the vacuolar membrane and serves as an anchor for the EGO complex (Loewith and Hall 2011; Binda *et al.*, 2009). The EGO complex and “Ragulator” complex function upstream of TORC1 and transmit nutritional signals, specifically amino acid sufficiency signals, to TORC1.

TOR (Target of rapamycin) cell signaling pathway integrates responses from both the intracellular and extracellular environment to coordinate cell growth, via regulating several processes such as protein synthesis, cell cycle/cell size, stress response, aging, autophagy, nutrient sensing and

intermediary metabolism (Loewith and Hall 2011; Laplante and Sabatini 2009). TOR and its mammalian ortholog mTOR are serine-threonine kinases that sense nutrients (glucose, amino acids) or oxygen status or growth factors such as insulin in mammals via a complex of other proteins (EGO complex in yeast or “Ragulator” complex in mammals) and promote appropriate changes in cell growth, proliferation or cell survival by activating the mechanisms of protein synthesis. Therefore, TOR signaling has key roles in multiple physiologic and disease processes such as cellular metabolism, bone formation, diabetes, neurodegenerative disorders, dementia, cancer and autophagy (Loewith and Hall 2011; Maiese 2017).

4.2.2: Elucidating the structure of NCU05950 gene product

I have identified human p18/LAMTOR 1 and yeast EGO1/Meh1p/Gse2p as orthologs of the UV90 / NCU05950 gene product by using the Conserved Domain Architecture Retrieval Tool (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>). In addition to the LAMTOR domain, the predicted amino acid sequence of NCU05950 contains elements that support the identification of the protein product as a member of the LAMTOR family (Figure 4.1).

NCU05950 is predicted to encode a 17.7kDa protein and yeast EGO1/Meh1p/Gse2p and human p18 are encoding approximately similar size proteins (20.2 kDa and 17.7kDa respectively) (Table 4.3).

In order to determine a possible structure for the protein product of NCU05950 gene, the 158 amino acids translated sequence retrieved from the *Neurospora crassa* genome database (FungiDB, <http://fungidb.org/fungidb/>; Stajich *et al.*, 2012) was run through a program called PHYRE2 Protein Fold Recognition Server (Kelley and Sternberg 2009) and SPIDER2 software

(<http://sparks-lab.org/server/SPIDER2/>) (Heffernan *et al.*, 2015). The secondary structure prediction by PHYRE2 is shown in Figure 4.2. Both human p18/LAMTOR1 and yeast EGO1/Meh1p/Gse2p are predicted to be helix-rich (Kogan *et al.*, 2010) as is NCU05950 (Table 4.3).

Similar to LAMTOR1 and EGO1, the N-terminus of NCU05950 has two cysteines that may be sites for S-acylation (palmitoylation) and an N-terminal glycine that is a potential site for myristoylation (Figure 4.1). Some proteins with dual acylations are known to localize to lipid rafts (Farazi *et al.*, 2001). The human LAMTOR1 protein has been found in lipid rafts in late endosomes and these N-terminal putative acylation sites are essential for this localization (Nada *et al.*, 2009).

The NCU05950 gene contains a sequence (DEAQHL) near the N-terminus that fits a localization signal [DE]XXXL[LI] for sorting proteins to the endosomal/lysosomal compartments in mammals, yeast, and many other organisms (Bonifacino Traub 2003). Both human LAMTOR1 and yeast EGO1 contain similar localization sequences (Figure 4.1).

Pairwise protein sequence alignments using the EMBOSS Needle software were used to identify regions of similarity that may indicate functional, structural and/or evolutionary relationships between proteins from *N. crassa* and yeast, and *N. crassa* and human, in the N-terminal acylation sites, the lysosomal localization sequences, and the LAMTOR domains (Figure 4.3). The hydrophobicity index is a measure of the relative hydrophobicity or how soluble an amino acid or protein is in water. Peptide hydrophobicity / hydrophilicity of NCU05950 protein was analyzed using Peptide 2.0 software and the results indicated NCU05950 protein is 39.24% hydrophobic (Table 4.4).

Table 4.1: Top ten sequences similar to NCU05950*.

NCBI/Gene bank sequence reference number	Protein function and name	Organism	E value
XP_009854653.1	Hypothetical protein NEUTE1DRAFT_69638	<i>Neurospora tetrasperma</i>	8e-114
XP_003349138.1	Uncharacterized protein SMAC_06974	<i>Sordaria macrospora</i>	2e-103
XP_003650216.1	Hypothetical protein THITE_2063314	<i>Thielavia terrestris</i>	3e-60
KXX83062.1	Hypothetical protein MMYC01_200469	<i>Madurella mycetomatis</i>	1e-58
XP_001227334.1	Hypothetical protein CHGG_09407	<i>Chaetomium globosum</i>	1e-56
XP_006696736.1	Hypothetical protein CTHT_0064310	<i>Chaetomium thermophilum</i>	3e-56
XP_003663911.1	Hypothetical protein MYCTH_2315477	<i>Thermothelomyces thermophila</i>	2e-55
XP_001906340.1	Hypothetical protein PODANS_4_5160	<i>Podospira anserina</i>	4e-48
XP_008095402.1	Hypothetical protein GLRG_06526	<i>Colletotrichum graminicola</i>	7e-39
KZL63082.1	Hypothetical protein CI238_05869	<i>Colletotrichum incanum</i>	3e-38

*The NCU05950 protein sequence retrieved from the *Neurospora crassa* genome database (FungiDB, <http://fungidb.org/fungidb/>; Stajich *et al.*, 2012) was used as the query for a protein BLAST search in the NCBI database of non-redundant coding sequences from GenBank.

Table 4.2: Some of the proteins with similar domain architecture to LAMTOR superfamily.

NCBI/Gene bank sequence reference number	Protein function and name	Organism
NP_079881.2	Ragulator complex protein LAMTOR1	<i>Mus musculus</i>
NP_060377.1	Ragulator complex protein LAMTOR1	<i>Homo sapiens</i>
XP_719486.1	Hypothetical protein CAALFM_C200870WA	<i>Candida albicans</i>
XP_451128.1	Uncharacterized protein KLLA0_A02937g	<i>Kluyveromyces lactis</i>
XP_750803.1	Conserved hypothetical protein	<i>Aspergillus fumigatus</i>
NP_649173.1	Uncharacterized protein Dmel_CG14184, isoform A	<i>Drosophila melanogaster</i>
NP_954533.1	Ragulator complex protein LAMTOR1	<i>Rattus norvegicus</i>
NP_012932.1	Meh1p	<i>Saccharomyces cerevisiae</i>
XP_958794.1	Hypothetical protein NCU05950	<i>Neurospora crassa</i>
NP_001080198.1	Ragulator complex protein LAMTOR1	<i>Xenopus laevis</i>
ELQ43503.1	Hypothetical protein OOU_Y34scaffold00148g6	<i>Magnaporthe oryzae</i>
NP_989303.1	Ragulator complex protein LAMTOR1	<i>Xenopus tropicalis</i>
XP_659366.1	Hypothetical protein AN1762.2	<i>Aspergillus nidulans</i>
XP_011323877.1	Hypothetical protein FGSG_05355	<i>Fusarium graminearum</i>

Table 4.3: Sequence comparisons of LAMTOR family proteins.

Protein	MW (kDa)	LAMTOR domain e Value	% Alpha Helix
Human LAMTOR1	17.7	8.41e-18	37.3
Yeast EGO1	20.2	1.07e-18	39.1
<i>N. crassa</i> NCU05950	17.7	3.22e-05	29.1

Table 4.4: Peptide Hydrophobicity/Hydrophilicity Analysis results.

Peptide:	MGNFCSTCFGGRRSDDYDEEDEAQHLFDENN MHYGSFDQQHMMNQEDPQETEREIAALQGVV ERTSNNMVDIYDMVPHDKPMQDAPAPYGFAN • Green: hydrophobic uncharged residues, like F I L M V W A and P • Red: acidic residues, like D E and C-terminal -COOH • Blue: basic residues, like R K H and N-terminal -NH₂ • Black: other residues, like G S T C N Q and P
Hydrophobicity/ Hydrophilicity:	• Hydrophobic: 39.24% • Acidic: 18.99% • Basic: 10.76% • Neutral: 31.01%

(https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php)

***Neurospora crassa* → NCU05950**

1 2 3 4 5
 MGNF **C**ST**C**FGGRRSDDYDEED **DEAQHL**FDENNMHYGSFDQQHMMNQEDPQE
 TEREIAALQGVVERTSNNMVDIYDMVPHDKPMQDAPAPYGFANQRYNALL
 SKLSTHDDMAAVARVDWGTPEDDSMEMLRKASLPTIPIKAEGGEALVGNF
 TDAAMR

***Saccharomyces cerevisiae* (Yeast) → EGO1/MEH1/Gse2p**

1 2 3 4 5
 MGAVLSCCRNHSGE **ENEALL**REQQAGYGSQGNANDEYDAEQMRLKEHEHE
 QKLLAREQELRDIVANTNDKLIDISMINNSGIVIQGTDLQEALDKRQQEE
 GGDSREDERSAGDDNLSGHSVPSSGSAQATTHQTAPRTNTFTLLTSPDSA
 KISKEQLKKLHSNILNEIFSQSQVNKPGPLTVPF

***Homo sapiens* (Human) → LAMTOR1/p18**

1 2 3 4 5
 MGCCYSSSENE~~SDQDR~~ **EERKLL**LDPSSPPTKALNGAEPNYHSLPSARTDE
 QALLSSILAKTASNIIDVSAADSQGM**EQHEYMD**RARQYSTR LAVLSSSLT
 HWKKLPPLPSLTSQPHQVLASEPIPFSDLQQVSRIAAYAYSALSQIRVDA
 KEELVVQFGIP

KEY:

Conserved LAMTOR domains: **blue**

Potential myristoylation sites: **yellow**

Potential palmitoylation sites: **green**

Potential lysosomal/vacuolar localization signals: **underlined bold**

Figure.4.1: Predicted NCU05950 amino acid sequence. The NCU05950 sequence is compared to the sequences of the yeast protein EGO1/Meh1p/Gse2p (UniProt accession code Q02205) and the human protein p18/LAMTOR1 (UniProt accession code Q6IAA8). LAMTOR domains identified by NCBI Conserved Domain searches are blue; potential myristoylation sites are yellow; potential palmitoylation sites are green; potential lysosomal or vacuolar localization signals are underlined in bold. E-values for LAMTOR domains are 2.63e-09 (NCU05950), 4.25e-09 (yeast) and 1.88e-16 (human).

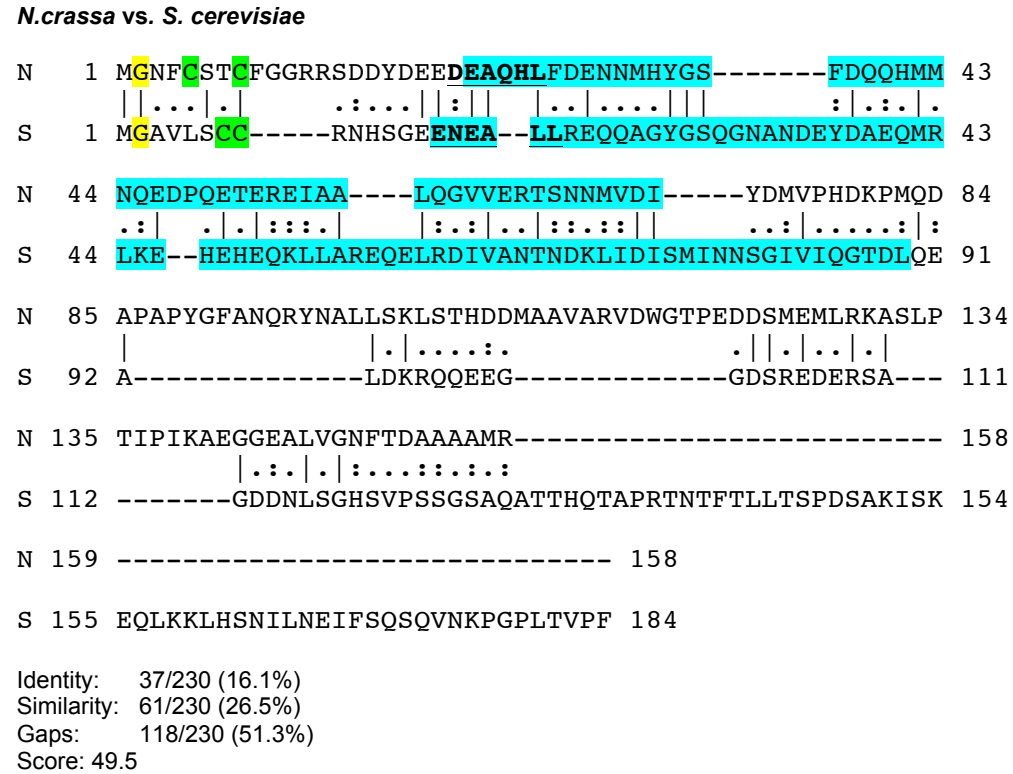


Figure 4.2: PHYRE2 secondary structure and disorder prediction for protein product of *Neurospora crassa* gene NCU05950.

Confidence Key represents the probability of NCU05950 protein product being homologous to the template.

(http://www.sbg.bio.ic.ac.uk/phyre2/phyre2_output/1db8c2b801c9d942/summary.html)

Figure 4.3: Sequence alignments of LAMTOR-related proteins.



N. crassa* vs. *H. sapiens

```

N   1  MGNFSTCFGGRRSDDYDEEDEAQHLFDENN----MHYGSFDQQHMMNQE 46
      ||| | | | :.....|.....|...|...|...|:.....|:
H   1  MG--C--CYSSENEDSDQDREERKLLLDPSPPTKALNGAEPNYHSL--- 43

N  47  DPQETEREIAALQGVVERTSNNMVDI-----YDMVPHDKPMQDAPAPYGF 91
      |.....|.|.|.....|::|::|:.....|...|...|:
H  44  -PSARTDEQALLSSILAKTASNIIDVSAADSQGMEQHE-----YMD 83

N  92  ANQRYN---ALLSKLSTHDDMAAVARVDWGTPEDDSMEMLRKASLPTIP- 137
      ...:|: |::|...|| | | | | | | | | | | | | | | | | |
H  84  RARQYSTRLAVLSSSLTH-----W-----KKLPPLPS 110

N 138  IKAEGGEALVGN---FTDAAAAMR----- 158
      :.....:|... |::|.....|
H 111  LTSQPHQVLASEPIPFSDLQQVSRIAAYAYSALSQIRVDAKEELVVQFGI 160

N 159 - 158

H 161 P 161

```

Identity: 34/201 (16.9%)
 Similarity: 61/201 (30.3%)
 Gaps: 83/201 (41.3%)
 Score: 55.5

KEY:

Conserved LAMTOR domains: **blue**
 Potential myristoylation sites: **yellow**
 Potential palmitoylation sites: **green**
 Potential lysosomal/vacuolar localization signals: **underlined bold**
 | Identity
 . : Similarities

CHAPTER 05: Subcellular localization of NCU05950

gene product

5.1: Introduction

NCU05950 is predicted to be a component of the TOR signaling pathway as it is homologous to human p18/LAMTOR1 and yeast EGO1/Meh1p/Gse2p. In yeast, EGO1 is localized to the vacuolar membrane and serves as an anchor for the EGO complex (Loewith and Hall 2011; Binda *et al.*, 2009). In humans, p18/LAMTOR1 is localized to the surface of late endosomes/lysosomes to anchor the “Ragulator” complex (Sancak *et al.*, 2010). Fluorescent proteins are powerful tools in studies of living cells and to determine the subcellular localization of the NCU05950 protein, a green fluorescent protein (GFP) fusion protein was used. *Neurospora crassa ccg-1* promoter was used to achieve strong expression of the GFP fusion protein.

As mentioned in Chapter 04, the NCU05950 gene contains a sequence (DEAQHL) near the N-terminus that fits a localization signal [DE]XXXL[LI] for sorting proteins to the endosomal/lysosomal compartments in mammals, yeast, and many other organisms (Bonifacino Traub 2003) . Apart from that feature, it has two cysteines that may be sites for S-acylation (palmitoylation) and an N- terminal glycine that is a potential site for myristoylation , which may be essential for its localization. Therefore, tagging the NCU05950 protein at the N-terminus may block the normal function and localization of the NCU05950 protein. During this study, strains expressing NCU05950 protein fused with C-terminal GFP tag were constructed and used for live-cell imaging.

5.2: Methods

5.2.1: Construction of strains expressing NCU05950-GFP fusion protein

pCCG::C-Gly::GFP plasmid DNA was prepared and EcoRI and DraIII enzymes were used to confirm the identity of the plasmid. To construct a highly expressed fusion protein of NCU05950 and GFP, coding sequence of the candidate gene NCU05950 without regulatory sequences (672bp) was isolated from previously constructed pBM61-NCU05950 plasmid by PCR using UV90tagXbaIF and UV90tagPacIR primers (Appendix IV) and inserted into the XbaI-PacI site of the *his-3* locus targeting pCCG::C-Gly::GFP plasmid between the highly expressed *cgg-1* promoter and the 10X Gly linker upstream of the GFP sequence. To construct a fusion protein expressed from the native NCU05950 promoter, the coding sequence along with upstream regulatory region (1651bp total) was amplified from pBM61-NCU05950 plasmid using UV90tagNotIF and UV90tagPacIR primers (Appendix IV) and inserted into the NotI-PacI site of the *his-3* locus targeting pCCG::C-Gly::GFP plasmid by removing the *cgg-1* promoter sequence from the plasmid and inserting the NCU05950 upstream regulatory region along with coding sequence upstream of the 10X Gly linker and GFP sequence.

The plasmid and isolated gene fragments were digested with NotI-PacI (NCU05950 gene along with its native promoter) or XbaI-PacI (to obtain highly expressed fusion protein of NCU05950 and GFP from the *cgg-1* promoter) restriction enzymes and were gel purified. Ligation mixtures were transformed to chemically competent *E.coli*. Several colonies were picked and colony PCR was carried out to analyze the transformed bacterial cells using the primers used to isolate the gene. Several colonies gave the desired bands for two constructs and those colonies were selected for further testing. Plasmid DNA isolated from

PCR positive colonies was digested with different restriction enzymes to confirm the presence of the inserted candidate gene on the recombinant plasmids (Appendix XIX-A). Recombinant plasmids with NCU05950 promoter along with NCU05950 gene coding sequence were named as pGN(1-10) and recombinant plasmids with *ccg-1* promoter followed by NCU05950 gene coding sequence were named as pGX(1-10).

After confirming the cloning by sequencing, pGN8 and pGX10 recombinant plasmids were selected for transformation. pGN8 and pGX10 plasmid DNA was transformed to the spores of NCU05950 knockout strain (carrying the deletion of NCU05950) SG #359 (*csp-1 his-3; chol-1 ras^{bd}; NCU05950^{KO} mat a*). Fungal strain SG #359 grown on histidine and choline containing media was used to harvest conidiospores for transformation. Cloned plasmid (10-15 µg/ 15 µL) was linearized with NdeI and added to 40 µL of washed conidia. Conidiospores were transformed with the plasmid DNA by electroporation and plated on Top agar media without histidine supplement to select *his-3⁺* transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline and the transformants that showed healthy growth with bright orange spores were further analyzed.

Genomic DNA was isolated from all selected transformants and PCR was carried out with different combinations of *his-3* locus specific and NCU05950 gene specific primers. Some transformants gave expected bands and it indicates successful transformation as well as integration at the *his-3* locus. The PCR performed for transformants with *his-3* locus specific primers (HIS-3F1 & HIS-3R and pBM61F2 & pBM61R2- Details are mentioned in Appendix VI) gave a smaller sized band along with the expected band. Since it was present on negative controls (SG # 26 with genotype *csp-1; chol-1 ras^{bd} mat a* and SG # 361 with genotype *csp-1; chol-1 ras^{bd}; NCU05950^{KO} mat a*), I

can explain it as a product from the wild type *his-3* locus. The transformed macroconidia may have two or more nuclei and are heterokaryons, which carry nuclei with *his-3* (untransformed) and *his-3*⁺ (transformed). This explains the origin of the smaller sized unexpected PCR product. Therefore, heterokaryotic transformants were purified by making microconidia which are predominantly uninucleate (Horowitz and Macleod, 1960; Baylis and DeBusk, 1967).

In order to make microconidia, the method described in Ebbole and Sachs 1990 was used as described in section 2.7. PCR analysis with *his-3* locus specific primers and race tube experiments with or without 100µM choline containing media were performed with pure microconidia derived transformant strains.

Strains expressing GFP fusion protein from the native NCU05950 promoter were named as GN strains and had the genotype; *csp-1 his-3*⁺:: Native promoter -NCU05950::Gly::GFP; *chol-1 ras*^{bd}; NCU05950^{KO} (*mat a*). Silica stocks were prepared from positive homokaryotic transformants of GN strains (SG# 448 - 453). Strains expressing GFP fusion protein from the high-expression *ccg-1* promoter were named as GX strains and had the genotype; *csp-1 his-3*⁺:: *pccg-1*::NCU05950::Gly::GFP; *chol-1 ras*^{bd}; NCU05950^{KO} (*mat a*). Silica stocks were prepared from positive homokaryotic transformants of GX strains (SG# 454 - 464). Details of strain identification names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

5.2.2: Testing the expression of NCU05950-GFP fusion protein.

To confirm the expression of the fusion proteins, the GN strains (strains expressing GFP fusion protein from the native NCU05950 promoter) and GX strains (strains expressing GFP fusion protein from the high-expression *ccg-1*

promoter) were grown in 24-well microtiter plates containing 1 mL of liquid minimum media containing 1xVogel's salts (Vogel, 1956), 2% glucose along with 100 μ M choline at room temperature for 3 days under constant light (LL) as well as constant dark (DD) conditions. Protein extraction, Western blotting and immunodetection were performed as described in section 2.14.

5.2.3: Construction of RFP-VAM-3 strain

pRFP-vam-3 plasmid (10.1kb) was obtained from FGSC and using SpeI – XbaI restriction enzymes, the identity of the plasmid was confirmed (Details about the plasmid are mentioned in section 2.9.1.4 and Figure 2.5). pRFP-vam-3 plasmid was linearized with NdeI enzyme and transformed to conidiospores of SG#274 (*csp-1 his-3: chol-1 ras-1^{bd} mat a*) by electroporation. Transformed conidiospores were plated on Top agar media without histidine supplement to select *his-3*⁺ transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline. The transformants that showed healthy growth with bright orange spores were further analyzed by PCR and monitoring live cell cultures under fluorescence microscopy.

The PCR performed for transformants with vector specific primers that are from the *his-3* locus (HIS-3F1 & HIS-3R- Appendix VI) gave a smaller sized band along with the expected band. Since it was present on untransformed controls, I can explain it as a product from wild type *his-3* locus. The transformed macroconidia may have two or more nuclei, which carry nuclei with *his-3* (untransformed) and *his-3*⁺::*RFP-vam-3* (transformed). This may explain the origin of the smaller sized unexpected PCR product. Therefore, several attempts were taken to purify the nuclei of transformed strains by making microconidia which are predominantly uninucleate (Horowitz and Macleod, 1960; Baylis and DeBusk, 1967) using the method described in

section 2.7. Unfortunately, I was not able to purify the transformants to get a homokaryon and a silica stock (SG#514: *csp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺: chol-1 ras-1^{bd} mat a*) was made from heterokaryon expressing RFP-VAM-3 protein.

5.2.4: Construction of heterokaryons expressing the RFP-VAM-3 and NCU05950-GFP proteins

Co-inoculating both types of strains into one culture tube made heterokaryons between the RFP-VAM-3 and NCU05950-GFP proteins expressing strains. Both GN strains (strains expressing GFP fusion protein from the native NCU05950 promoter) and GX strains (strains expressing GFP fusion protein from the high-expression *cgc-1* promoter) were used to make heterokaryons and several heterokaryon cultures were made by inoculating different as well as equal amount of spores from both strains into culture tubes. The best heterokaryon cultures were screened by confocal microscopy and cultures with approximately equal fluorescence intensity in green and red channels were chosen for observation of co-localization. Silica stocks (SG#515-518) were made from these heterokaryons and details are mentioned in Appendix I.

5.3: Results & Discussion

5.3.1: Construction of strains expressing NCU05950-GFP fusion protein

Identification of the subcellular localization of the NCU05950 gene product is a major aspect of the gene characterization. In order to achieve this, the candidate gene was cloned to the *his-3* locus with the *Neurospora crassa* targeting expression vector pCCG::C-Gly::GFP→8509bp (Figure 2.2 & Figure

2.4). This vector contains C-terminal GFP tag with a flexible poly-glycine linker, high-level gene expression *Neurospora* promoter *cgc-1* and sequences for targeting to the *his-3* locus in *Neurospora* (Honda and Selker 2009). (Details about the vector are available in section 2.9.1.2 and details about the ectopic gene expression strategy used for *Neurospora crassa* are available in section 3.2.1) To determine the intracellular localization of the NCU05950 protein, a GFP fusion protein expressed from the high-expression *cgc-1* promoter as well as a fusion protein expressed from the native NCU05950 promoter were constructed as described in Methods section 5.2.1.

Restriction enzyme digestion and sequencing of recombinant plasmids with NCU05950 promoter along with NCU05950 gene coding sequence (pGN) as well as recombinant plasmids with *cgc-1* promoter followed by NCU05950 gene coding sequence (pGX) gave the expected results and confirmed the presence of the insert on the recombinant plasmid (Appendix XIX – A and Appendix XIX - B). Linearized pGN and pGX plasmids were transformed into macrospores of NCU05950 knockout strain SG#359 and successful integration at the *his-3* locus was confirmed by carrying out PCR with different combinations of pCCG::C-Gly::GFP vector specific, *his-3* locus specific as well as NCU05950 gene specific primers. Transformants that gave expected bands indicate successful transformation as well as integration at the *his-3* locus.

Homokaryons were obtained by making microconidia from positive transformants and PCR as well as race tube experiments were performed with pure microconidia derived transformant strains. Clear banding patterns were observed on the race tube experiment and only one PCR derived band was observed from some transformants with *his-3* locus specific primers (Appendix XX - A, Appendix XX - B & Appendix XX - C). The PCR results confirmed

that the strains are pure homokaryotic transformants with the correct genotype and as shown by the rescue of the rhythmic phenotype in an NCU05950 deletion strain on race tubes, the cloned gene produced a functional fusion protein.

Furthermore, strains expressing NCU05950::GFP fusion protein from both the native NCU05950 promoter and the high expression *ccg-1* promoter can rescue the rhythmic phenotype in the NCU05950 deletion mutant background (Appendix XX – B & Appendix XX - C). This indicates that the native promoter is not required for the clock function of NCU05950. Therefore, NCU05950 does not need to be rhythmic to rescue the phenotype of NCU05950 deletion mutant.

Western blotting using anti-GFP antibodies confirmed both the overexpressing GX strains with *pccg-1*::NCU05950::Gly::GFP and native promoter GN strains with Native promoter -NCU05950::Gly::GFP produced a band corresponding to 44.6 kDa, the predicted size of the NCU05950-GFP fusion protein (Figure 5.1).

5.3.2: NCU05950 localizes to the vacuolar membrane

Expression of fluorescence was monitored from live cell cultures under fluorescence microscopy and confocal laser scanning microscope (Details are mentioned in section 2.17). Different organelle GFP and/or RFP marker strains (Vacuoles: *vma-5* with GFP tag & *vam-3* with RFP tag, Mitochondria: *arg-4* with GFP tag, Golgi: *vps-52* with GFP tag, Endoplasmic reticulum: *nca-1* with GFP tag, nuclei: *hH1* with RFP tag) which were constructed by Bowman *et al.*, 2009 and untagged strains (SG#26 & SG#259) as negative controls were used to compare to the test specimens (Details of all the strains are mentioned in Appendix I). Fluorescence expression was detected from strains expressing

NCU05950-GFP fusion protein from both *ccg-1* and the endogenous promoter. Localization of NCU05950-GFP fusion protein was different from localization of mitochondrial, Golgi, endoplasmic reticulum and nuclei markers. NCU05950-GFP fusion protein was detected near the growing tip of the hyphae on some fibrous structures, on the limiting membrane of young vacuoles and inside of the old vacuoles (Figure 5.2) as the vacuolar markers. Spherical shaped vacuoles are prominent in older parts of the hypha. In the growing tip of the hypha, vacuoles take the form of a dense network of interconnected tubules (Bowman *et al.*, 2015).

As a control, a strain carrying an RFP-tagged vacuolar marker, *vam-3* (NCU06777) (Bowman *et al.*, 2009) was constructed by transforming the pRFP-*vam-3* plasmid (See 2.9.1.4 & Figure 2.5) into a strain of genotype *ccp-1 his-3::chol-1 ras-1^{bd} mat a* (SG#274). This strain had genotype *ccp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺; chol-1 ras^{bd} (mat a)* and it expresses the RFP::VAM-3 fusion protein from the high expression *ccg-1* promoter. The transformants were observed under live cell fluorescence microscopy and as previously reported in Bowman *et al.*, 2009, I found the RFP-VAM-3 protein in filamentous structures behind the tip, and inside large spherical vacuoles in older hyphal regions that were filled with large vacuoles (Figure 5.3). Localization inside the vacuole may be the result of turnover of vacuolar membrane proteins and normal degradation processes (Bowman *et al.*, 2009).

A heterokaryon strain harboring NCU05950-GFP fusion protein and RFP- vacuolar marker protein was made to confirm the subcellular localization of the NCU05950 gene product by co-localization. In heterokaryons between GFP and RFP strains, the NCU05950-GFP protein co-localized with RFP-VAM-3 in hyphal tips. In older hyphae, NCU05950-GFP was found on the vacuolar membrane. These results localize the NCU05950 protein to the

vacuole, and specifically the vacuolar membrane (Figure.5.3). As predicted based on amino acid sequence data and localization of the orthologous mammalian p18/LAMTOR1 and yeast EGO1/Meh1p/Gse2p proteins, NCU05950-GFP fusion protein localized to the vacuolar membrane. This observation is consistent with the participation of the NCU05950 gene product as a component of the TOR signaling network.

Since the NCU05950 gene is homologous to yeast EGO1 and mammalian p18/LAMTOR1, and the protein is found on the vacuolar membrane, I propose naming this gene as “*vta*”, vacuolar TOR-associated protein.

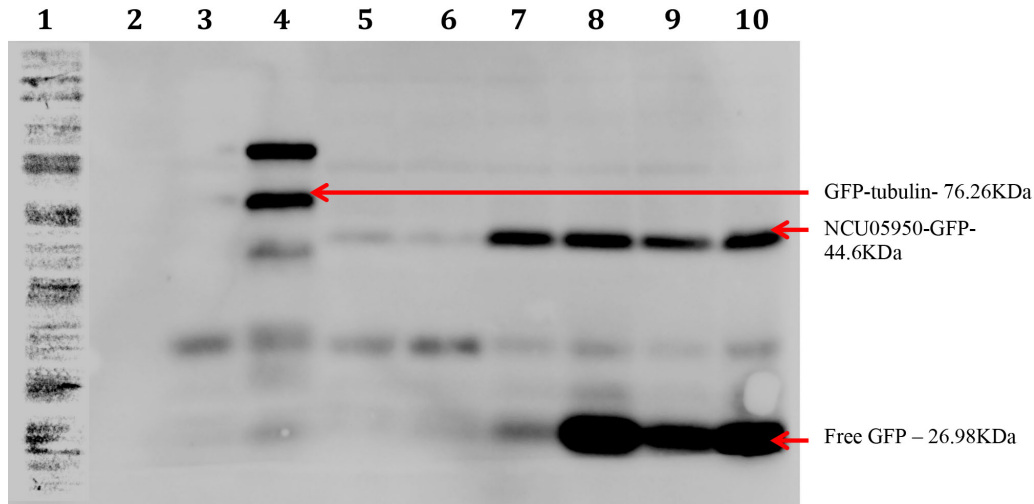


Figure 5.1: Expression of GFP tagged NCU05950 protein.

Lane No.1 - Molecular weight marker.

Lane No.2 - Blank.

Lane No.3 - SG#26 (*csp-1; ras^{bd} chol-1*) grown in LL (Negative control).

Lane No.4 - SG#198 (*csp-1; ras^{bd} chol-1; inl Bml-GFP*) grown in LL (Positive control).

Lane No.5 - SG#448 (*csp-1 his-3⁺:: Native promoter -NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} (mat a)*) grown in LL.

Lane No.6 - SG#448 (*csp-1 his-3⁺:: Native promoter -NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} (mat a)*) grown in DD.

Lane No.7 - SG#454 (*csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} (mat a)*) grown in LL.

Lane No.8 - SG#454 (*csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} (mat a)*) grown in DD.

Lane No.9 - SG#462 (*csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} (mat a)*) grown in LL.

Lane No.10 - SG#462 (*csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} (mat a)*) grown in DD.

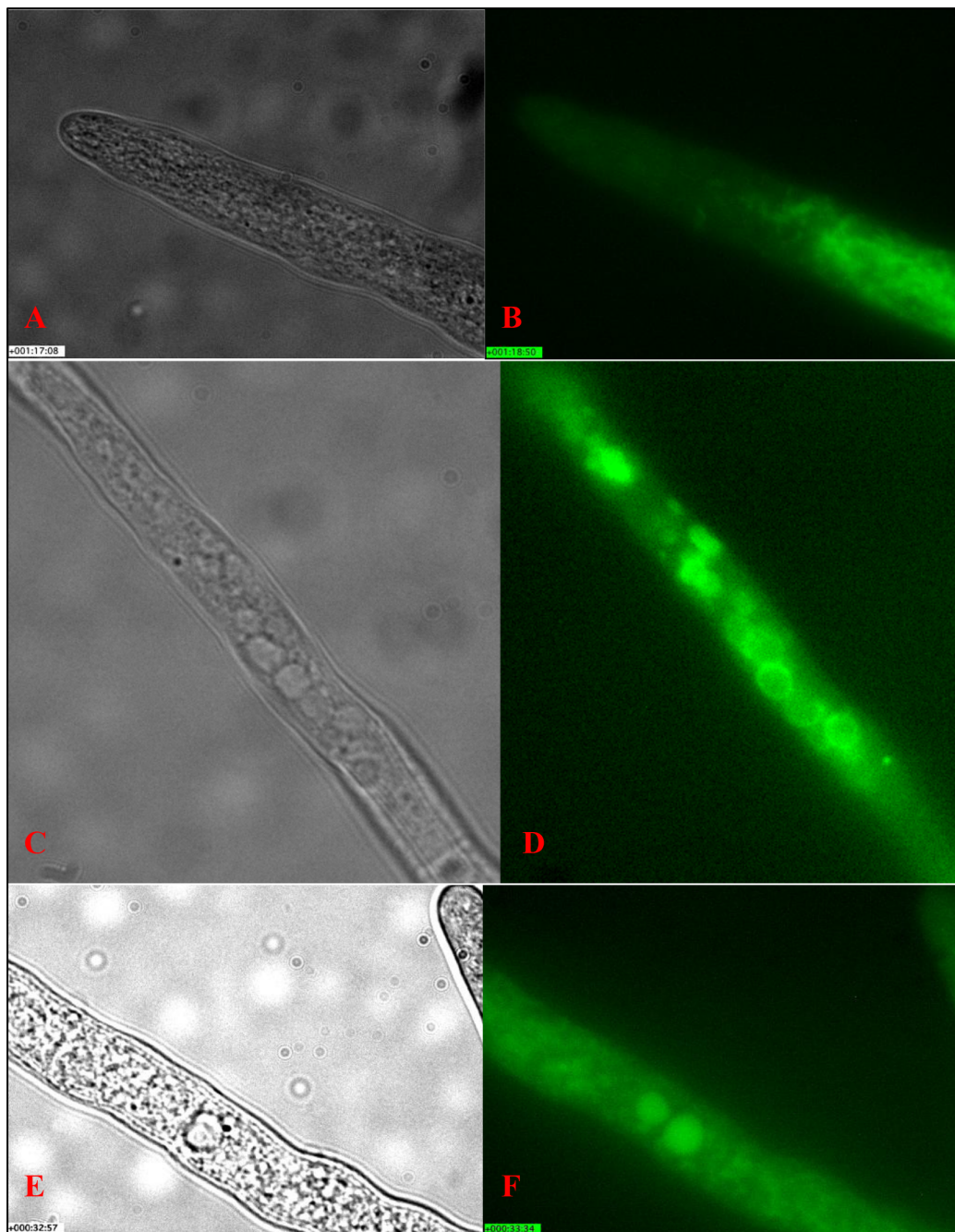


Figure 5.2: NCU05950 gene product is localized in the vacuole.

Neurospora crassa expressing NCU05950::GFP under the control of high expressing promoter *cgc-1* and analyzed by fluorescence microscopy.

- (A) Growing tip under the bright field.
- (B) Growing tip under the GFP filter.
- (C) Trunk of hyphae with young vacuoles under the bright field.
- (D) Trunk of hyphae with young vacuoles under the GFP filter.
- (E) Trunk of senescent fungal hyphae with old vacuoles under the bright field.
- (F) Trunk of senescent fungal hyphae with old vacuoles under the GFP filter.

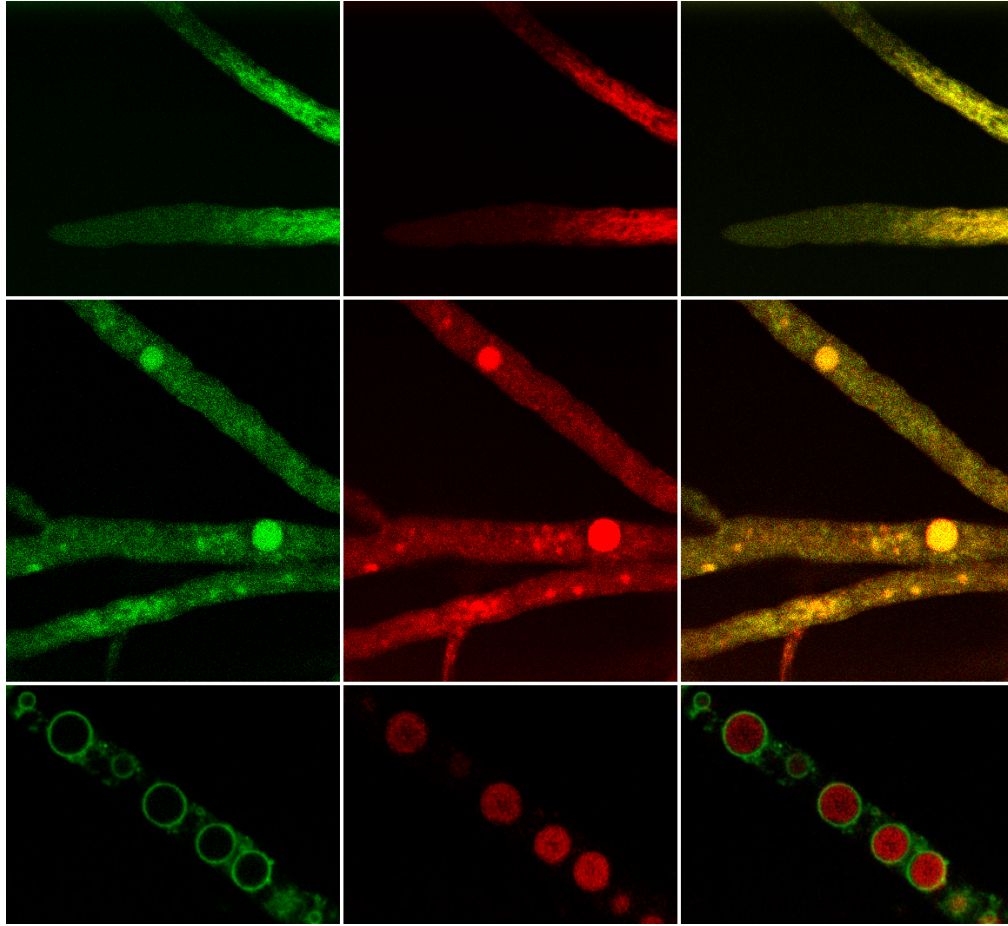


Figure 5.3: Subcellular localization of NCU05950 protein.

Using the RFP-vacuolar marker strain with the genotype *csp-1 his-3⁺::Pccg-1::tdimer2(12)::vam-3⁺; chol-1 ras^{bd}* that expresses the RFP::VAM-3 fusion protein from the a high-expression *cpg-1* promoter and NCU05950::GFP strain with the genotype *csp-1 his-3⁺::pccg-1::NCU05950⁺::GFP; chol-1 ras^{bd}; NCU05950^{KO}* a that expresses the wild-type NCU05950::GFP fusion protein from *cpg-1* promoter , a heterokaryon was made for microscopic observation. Cultures were grown on agar-covered slides with maltose-arginine medium and living hyphae were observed by confocal microscopy.

Top row: the tips of growing hyphae.

Middle and bottom rows: older hyphae containing large round vacuoles.

Left column: green channel, GFP.

Middle column: red channel, RFP.

Right column: merged channel, red + green.

CHAPTER 06: Molecular rhythmicity of NCU05950

gene product / VTA at protein and RNA level

6.1: Introduction

The *uvr90* mutation was found to affect two FRQ-less rhythms under two conditions: the free-running long-period rhythm under low-choline conditions in *chol-1* strains and the heat-entrained rhythm in choline-sufficient conditions in the *frq¹⁰* knockout (Li *et al.*, 2011). In a FRQ-sufficient background, the *uvr90* mutation causes damping of the free-running conidiation rhythm, reduction of the amplitude of the FRQ protein rhythm, and increased phase-resetting responses to both light and heat pulses (Li *et al.*, 2011). Therefore, the wild-type NCU05950 gene product appears to be required for a functional FLO and also for sustained, high-amplitude rhythms in FRQ-sufficient conditions. The NCU05950 gene product has been considered as a component of the FRQ-less oscillator.

In *Neurospora crassa*, as a result of the transcription - translation autoregulatory negative feedback loop, *frq* mRNA and FRQ protein exhibit rhythmic expression levels under constant conditions (Liu and Bell-Pedersen 2006; Baker *et al.*, 2012). These *frq* mRNA and FRQ protein oscillations are critical for the normal circadian behavior (Liu and Bell-Pedersen 2006). The key clock gene products (mRNA and protein) that control circadian clocks in other organisms such as PER & TIM in *Drosophila* and PER (1-3) & CRY (1-2) in mammals also have rhythmic expression levels under constant conditions (Tataroglu and Emery 2015; Kriegsfeld and Silver 2006).

Understanding the expression pattern of the NCU05950 gene product / VTA is important to discover its possible functions. Therefore, to examine

the NCU05950 / VTA protein levels, Western blotting of cell extracts from cultures harvested at different clock times was used. Since there was no antibody specific to NCU05950 protein to carry out immunodetection, strains expressing a fusion protein of NCU05950 with a FLAG epitope tag at the C-terminal were generated. To assay the mRNA levels, Northern blotting was carried out using a NCU05950 specific DNA probe.

6.2: Methods

6.2.1: Construction of NCU05950-FLAG fusion protein expressing strains

The candidate gene was cloned to the *his-3* locus targeting *Neurospora crassa* expression vector pCCG::C-Gly::3xFLAG (Figure 2.3) , which can be used to detect and isolate proteins and protein complexes. This vector contains C-terminal 3xFLAG tag with a flexible poly-glycine linker, high-level gene expression *Neurospora* promoter *cgc-1* and sequences for targeting to the *his-3* locus in *Neurospora* (Honda and Selker 2009). (Details about the vector are available in section 2.9.1.3 and details about the ectopic gene expression strategy used for *Neurospora crassa* are available in section 3.2.1)

pCCG::C-Gly::3xFLAG plasmid DNA was prepared and EcoRI and DraIII enzymes were used to confirm the identity of the plasmid. To construct a highly expressed fusion protein of NCU05950 and 3xFLAG, coding sequence of the candidate gene NCU05950 without regulatory sequences (672bp) was isolated from previously constructed pBM61-NCU05950 plasmid by PCR using UV90tagXbaIF and UV90tagPacIR primers (Appendix IV) and inserted into the XbaI-PacI site of the *his-3* locus targeting pCCG::C-Gly::3xFLAG plasmid between the highly expressed *cgc-1* promoter and the 10X Gly linker upstream of the 3xFLAG sequence. To construct a fusion protein expressed

from the native NCU05950 promoter, the coding sequence along with upstream regulatory region (1651bp total) was amplified from pBM61-NCU05950 plasmid using UV90tagNotIF and UV90tagPacIR primers (Appendix IV) and inserted into the NotI-PacI site of the *his-3* locus targeting pCCG::C-Gly::3xFLAG plasmid by removing the *ccg-1* promoter sequence from the plasmid and inserting the NCU05950 upstream regulatory region along with coding sequence upstream of the 10X Gly linker and 3xFLAG sequence.

The plasmid and isolated gene fragments were digested with NotI-PacI (NCU05950 gene along with its native promoter) or XbaI-PacI (to obtain highly expressed fusion protein of NCU05950 and 3xFLAG from the *ccg-1* promoter) and were gel purified. Ligation mixtures were transformed to chemically competent *E.coli*. Several colonies were picked and colony PCR was carried out to analyze the transformed bacterial cells using the primers used to isolate the gene. Several colonies gave the desired bands for two constructs and those colonies were selected for further testing. Plasmid DNA isolated from PCR positive colonies was digested with different restriction enzymes to confirm the presence of the inserted candidate gene on the recombinant plasmids (Appendix XXI - A). Recombinant plasmids with NCU05950 promoter along with NCU05950 gene coding sequence were named as pFN (1,8,9,10,11,15,16,17,19,31,33,35) and recombinant plasmids with *ccg-1* promoter followed by NCU05950 gene coding sequence were named as pFX(1-10).

After confirming the cloning by sequencing, pFN33 and pFX8 recombinant plasmids were selected for transformation. pFN33 and pFX8 plasmid DNA was transformed to the spores of NCU05950 knockout strain (carrying the deletion of NCU05950) SG #359 (*csp-1 his-3; chol-1 ras^{bd}; NCU05950^{KO} mat a*). Fungal strain SG #359 grown on histidine and choline

containing media was used to harvest conidiospores for transformation. Cloned plasmid (10-15 µg/ 15 µL) was linearized with NdeI and added to 40 µL of washed conidia. Conidiospores were transformed with the plasmid DNA by electroporation and plated on Top agar media without histidine supplement to select *his-3*⁺ transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline and the transformants that showed healthy growth with bright orange spores were further analyzed.

Genomic DNA was isolated from all selected transformants and PCR was carried out with different combinations of *his-3* locus specific and NCU05950 gene specific primers. Some transformants gave expected bands and it indicates successful transformation as well as integration at the *his-3* locus. The PCR performed for transformants with *his-3* locus specific primers (HIS-3F1 & HIS-3R and pBM61F2 & pBM61R2- Details are mentioned in section Appendix VI) gave a smaller sized band along with the expected band. Since it was present on negative controls (SG # 26 with genotype *csp-1; chol-1 ras^{bd} mat a* and SG # 361 with genotype *csp-1; chol-1 ras^{bd}; NCU05950^{KO} mat a*), I can explain it as a product from the wild type *his-3* locus. The transformed macroconidia may have two or more nuclei and they have produced heterokaryons, which carry nuclei with *his-3* (untransformed) and *his-3*⁺ (transformed). This explains the origin of the smaller sized unexpected PCR product. Therefore, heterokaryotic transformants were purified by making microconidia which are predominantly uninucleate (Horowitz and Macleod, 1960; Baylis and DeBusk, 1967).

In order to make microconidia, the method described in Ebbole and Sachs 1990 was used as described in section 2.7. PCR analysis with *his-3* locus specific primers and race tube experiments with or without 100µM choline

containing media were performed with pure microconidia derived transformant strains.

Strains expressing 3xFLAG fusion protein from the native NCU05950 promoter were named as FN strains and had the genotype; *csp-1 his-3⁺::* Native promoter -NCU05950::*Gly::3xFLAG*; *chol-1 ras^{bd}*; NCU05950^{KO} (*mat a*). Silica stocks were prepared from positive homokaryotic transformants of FN strains (SG# 444-447). Strains expressing 3xFLAG fusion protein from the high-expression *ccg-1* promoter were named as FX strains and had the genotype; *csp-1 his-3⁺::* *pccg-1::*NCU05950::*Gly::3xFLAG*; *chol-1 ras^{bd}*; NCU05950^{KO} (*mat a*). Silica stocks were prepared from positive homokaryotic transformants of FX strains (SG# 439-443). Details of strain identification names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

6.2.2: Construction of strains expressing NCU05950-FLAG fusion protein in *frq¹⁰* background

In order to create a strain expressing NCU05950::*3xFLAG* fusion protein in *frq¹⁰* background, a genetic cross was made between a strain expressing 3xFLAG fusion protein from the native NCU05950 promoter (Male parent- SG#444 - *csp-1 his-3⁺::* Native promoter -NCU05950::*Gly::3xFLAG*; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*) and a strain with *frq¹⁰* background (Female parent-SG#59 *ras-1^{bd}*; *frq¹⁰* *mat A*). 188 spores were picked after 4 weeks and 116 showed healthy growth. Genotypes of the strains were tested by tube tap test for *csp-1*, spore PCR with FRQ primers & race tube assay for *frq¹⁰*, spore PCR with *his-3* locus specific primers (HIS-3F1 & HIS-3R- Appendix VI) for *his-3⁺::* Native promoter -NCU05950::*Gly::3xFLAG*, spore PCR with

NCU05950^{KO} primers (UV90KO-F & UV90KO-R - Appendix V) for NCU05950^{KO} and race tube assay for *chol-1* mutation. Silica stocks were made from selected progeny strains (SG#572-577 - *csp-1 his-3⁺*:: Native promoter - NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; *frq¹⁰*; NCU05950^{KO} and SG#578-581 - *csp-1 his-3⁺*:: Native promoter -NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; *frq¹⁰*). Details of strain identification names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

6.2.3: Preliminary assay of NCU05950-FLAG fusion protein expression

To optimize the Western blotting protocols, the following experiments were conducted. Strains expressing 3xFLAG fusion protein from the high-expression *ccg-1* promoter with the genotype; *csp-1 his-3⁺*:: *pccg-1*::NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a* (SG#439-SG#443) and strains expressing 3xFLAG fusion protein from the native NCU05950 promoter with the genotype; *csp-1 his-3⁺*:: Native promoter - NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a* (SG#444 - SG#447) were used for preliminary experiments.

Strains expressing NCU05950-FLAG fusion protein as well as the control strains (Negative control – SG#26: *csp-1: chol-1 ras-1^{bd} mat a* , Negative control – SG#80: *csp-1: chol-1 ras-1^{bd} ; frq¹⁰ mat a* & Positive control SG#438 : FLAG::*pcna inl⁺*) were grown in 24-well microtiter plates containing 1 mL of liquid minimum media containing 1xVogel's salts , 2% glucose , 100 µM choline at 22°C for 2 days under LL or DD conditions. Mycelia were harvested as described in section 2.14.1 and frozen samples were stored at -80°C until protein extraction was performed. Protein extraction, protein quantification, SDS-PAGE, Western blotting and immunodection were

performed as described in section 2.14. Mouse monoclonal M2-Anti-FLAG (DYKDDDDK) antibodies were used as the primary antibody.

6.2.4: Optimizing SDS-PAGE, Western blotting and Immunodetection protocols to assay FRQ protein levels

For initial experiments 100 µg of total protein containing samples (SG#444) were used on 7.5% acrylamide gels. The following blocking buffers/antibody buffers were tested; A) 5% skimmed milk in PBS, B) 5% BSA & 5% skimmed milk in TBST, C) 5% BSA in TBST and D) 10% BSA in TBST. Blocking buffer with 5% BSA and 5% skimmed milk in TBST gave a clear band corresponding to FRQ protein (108.25 kDa) along with the least number of non-specific bands. Therefore, blocking buffer with 5% BSA and 5% skimmed milk in TBST was used in subsequent experiments.

In order to optimize protein quantity needed for detection with anti-FRQ-antibodies, the following amount of total protein containing samples from SG#444 and SG#80 were tested; 10 µg, 20 µg, 30 µg, 40 µg, 50 µg and 100 µg and samples with 50 µg of total protein gave sufficient signal. Hence, 50 µg of total protein was used when using anti-FRQ antibodies in latter experiments.

6.2.5: Preliminary tests to optimize Northern-blotting technique for NCU05950 mRNA

Digoxigenin-labeled DNA probe was used to detect NCU05950 transcript. UV90Ex3F (3' ATCTATGATATGGTCCCGCATG 5') and UV90Ex3R (3'GCAGCGGCATCGGTAAAGTT 5') primers were used to generate the 251 bp long DNA probe specific to the 3rd exon of the NCU05950 gene. Previously constructed pBM61-NCU05950 plasmid was used as the

template to generate the DIG-labeled probe and details are given in section 2.15.

24 well plates containing 1 mL (on each well) Vogel's minimum liquid medium with 2% glucose & 100 μ M choline were inoculated with SG#26 & SG#361 strains and allowed to grow for 2 days at room temperature (22°C) in constant light (LL) condition. RNA was extracted using Trizol reagent protocol from one well of each strain and remaining fungus along with extracted RNA were stored at -80°C. From each sample 20 μ g and 40 μ g of RNA were run on 1.2% formaldehyde-agarose gel and transferred to positively charged nylon membrane using BrightStar-Plus protocol. After the transfer, RNA was cross-linked to the membrane by exposing to ultraviolet light. Digoxigenin-labeled DNA probe prepared by random-primed labeling was allowed to hybridize to target RNA. Immunochemical technique was used to detect the hybridization signal on the northern blot. The light produced by chemiluminescent substrate was recorded with MicroChemi imager.

6.3: Results & Discussion

6.3.1: Rhythmicity of NCU05950 protein

6.3.1.1: Construction of NCU05950-FLAG fusion protein expressing strains

To determine whether the NCU05950 protein product is regulated by the circadian system, a fusion protein with a FLAG epitope tag, driven by the high-expression *ccg-1* promoter as well as a fusion protein expressed from the native NCU05950 promoter was constructed and introduced into the NCU05950 deletion strain as described in Methods section 6.2.1.

Restriction enzyme digestion and sequencing of recombinant plasmids with NCU05950 promoter along with NCU05950 gene coding sequence (pFN) as well as recombinant plasmids with *cgc-1* promoter followed by NCU05950 gene coding sequence (pFX) gave the expected results and confirmed the presence of the insert on the recombinant plasmid (Appendix XXI – A & Appendix XXI - B). Linearized pFN and pFX plasmids were transformed into macrospores of NCU05950 knockout strain SG#359 and successful integration at the *his-3* locus was confirmed by carrying out PCR with different combinations of pCCG::C-Gly::3xFLAG vector specific, *his-3* locus specific as well as NCU05950 gene specific primers. Transformants that gave expected bands indicate successful transformation as well as integration at the *his-3* locus.

Homokaryons were obtained by making microconidia from positive transformants and spore PCR analysis as well as race tube experiments were performed with pure microconidia derived transformant strains. Only one PCR derived band was observed from some transformants with *his-3* locus specific primers (Appendix XXII - A). The PCR results confirmed that the strains are pure homokaryotic transformants with the correct genotype.

Race tube experiments with or without 100μM choline containing media were performed with pure microconidia derived transformant strains. Clear banding patterns were observed on the race tube experiment (Appendix XXII – B & Appendix XXII - C). The rescue of the rhythmic phenotype on race tubes in an NCU05950 deletion strain indicated that the cloned gene produces a functional fusion protein that can restore the wild type phenotype.

Furthermore, as with the GFP tagged strains, the strains expressing NCU05950::3xFLAG fusion protein from both the native NCU05950 promoter and the high expression *cgc-1* promoter can rescue the rhythmic phenotype in

the NCU05950 deletion mutant background (Appendix XXII – B & Appendix XXII - C). This also indicates that the native promoter is not required for the clock function of NCU05950.

All the above-mentioned strains (FN-strains expressing 3xFLAG fusion protein from the native NCU05950 promoter & FX- strains expressing 3xFLAG fusion protein from the high-expression *cgg-1* promoter) contain a functional FRQ-WCC oscillator. In order to create a strain expressing NCU05950::3xFLAG fusion protein in *frq¹⁰* background with the FRQ-less oscillator, a genetic cross was made between a strain expressing 3xFLAG fusion protein from the native NCU05950 promoter (Male parent- SG#444 - *csp-1 his-3⁺*:: Native promoter -NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*) and a strain with *frq¹⁰* background (Female parent-SG#59 *ras-1^{bd}*; *frq¹⁰* *mat A*). This genetic cross produced two different types of strains (NCU05950 wild type gene at its own locus and NCU05950^{KO} at its own locus – Table 6.1 and Appendix XXIII).

6.3.1.2: Preliminary assay of NCU05950-FLAG fusion protein expression

In order to check the expression of tagged protein Western blotting was conducted. Samples grown under constant dark (DD) and constant light (LL) were tested and also NCU05950-FLAG fusion protein expressed from high expression *cgg-1* promoter as well as native NCU05950 promoter were tested. No difference was observed in expression levels of the fusion protein detected from samples grown under constant dark (DD) and constant light (LL) (Figure 6.1). This observation indicates NCU05950 promoter as well as the NCU05950 protein is not responding to light. However, the upstream regulatory sequence of NCU05950 gene contains several GATN repeats (Figure 6.2), which are identified as light response elements (LREs) (He and Liu 2005).

As expected NCU05950-FLAG fusion protein expressed from the high-expression *cgc-1* promoter exhibited higher expression level and NCU05950-FLAG fusion protein expressed from NCU05950 native promoter also offered satisfactory signals for immuno-detection of Western blots with Mouse monoclonal M2-Anti-FLAG (DYKDDDDK) antibodies. Therefore, it is more appropriate to use a strain expressing the FLAG-tagged protein driven by its native promoter as it displays physiologically relevant expression.

As a control, FRQ protein levels were analyzed using the same protein extracts prepared from NCU05950-FLAG fusion protein expressing strains. When analyzing FRQ protein levels, 50 µg of total protein were needed to give sufficient signal with anti-FRQ monoclonal antibodies (Appendix XXIV) and this amount was used in subsequent experiments.

6.3.1.3: NCU05950 protein levels are not rhythmic

To assess time dependent changes in the protein level of NCU05950 gene product time course experiments were performed by Western blotting of cell extracts from cultures harvested at different clock times. Several experiments were performed to optimize the time course experimental conditions and following variables were tested : two different strains (a strain expressing 3xFLAG fusion protein from the high-expression *cgc-1* promoter and a strain expressing 3xFLAG fusion protein from the native NCU05950 promoter), two different primary antibodies (Mouse monoclonal M2-Anti-FLAG (1:10000) antibodies and Rabbit polyclonal – Anti-FLAG (1:10000) antibodies), different dilutions of Mouse monoclonal M2-Anti-FLAG antibody (1:10000 and 1:20000), different amounts of total protein (10 µg, 20 µg and 40 µg), protein extraction with and without DTT, different culture conditions (with and without 2 hours heat pulse at 35°C) and different culture methods (low

glucose liquid media and on top of cellophane overlaid on solid agar medium where the cultures are expressing the rhythm of conidiation). 20 µg of total protein and 1:10000 dilution of Mouse monoclonal M2-Anti-FLAG antibodies were recognized as the best conditions that give clear results on Western blot analysis.

For initial experiments, a strain expressing 3xFLAG fusion protein from the high-expression *cgc-1* promoter with the genotype; *csp-1 his-3⁺:: pccg-1::NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a* (SG#439 = FX-5-b) and a strain expressing 3xFLAG fusion protein from the native NCU05950 promoter with the genotype; *csp-1 his-3⁺:: Native promoter - NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a* (SG#444 = FN-16-a) were used and latter experiments were conducted only with the strains expressing 3xFLAG fusion protein from the native NCU05950 promoter.

Samples for immunoblotting were grown either in conditions of slow growth in low-glucose liquid media in flask on shaker or growing rapidly on top of cellophane overlaid on solid agar. Liquid media cultures were grown using methods modified from Edgar, *et al.* 2012 and details are mentioned in section 2.3.2. Briefly, cultures were grown in liquid medium in constant light and to synchronize the biological clocks or to reset the circadian phase (hours in dark), 2 hours of heat pulse at 35°C were given under constant light (LL) before transfer to constant dark (DD) at 25°C. Samples were collected at various times and the level of NCU05950 protein was assayed by Western blotting followed by immunodetection. Protein loading was determined by Coomassie staining of the membrane. Three independent experiments were carried out at each time point. As shown in Figure 6.3, NCU05950 protein levels in SG#444, which expresses the FLAG tagged NCU05950 protein under its own promoter were rhythmic. NCU05950 protein levels reach a peak after approximately 16

hours and protein levels drop to a minimum after 24 hours. Furthermore, it gives a second peak between 32-hours and 36-hours time points.

To test whether the heat pulses influence the protein levels, time course experiments were performed again without the heat pulse. Cultures were grown in liquid medium in constant darkness and constant 25 °C after synchronizing their clocks with a light-to-dark transition, and samples were collected at various times. The level of NCU05950 protein was assayed by immunoblotting and the protein was found at nearly constant levels across two circadian cycles (Figure 6.4). As a control, levels of FRQ protein were assayed in the same samples and the expected rhythm of phosphorylation state was found, demonstrating that the clock in these cultures was functional (Figure 6.4).

The experiments above used samples harvested from cultures grown in low-glucose liquid medium in which growth is inhibited. Using culture methods previously described in Li *et al.*, 2011, similar results (Figure 6.5) were obtained from cultures growing rapidly on top of cellophane overlaid on solid agar medium and expressing the rhythm of conidiation. Therefore, the function of NCU05950 gene product does not require rhythmicity of the protein.

Strains expressing GFP- and FLAG-tagged proteins from both the native NCU05950 promoter and the constitutive / high expression *cgc-1* promoter were constructed and both types of constructs can rescue the rhythmic phenotype in the NCU05950 deletion mutant background (Appendix XX - B and Appendix XXII - B). This indicates that the native promoter is not required for the clock function of NCU05950, in agreement with our finding that rhythmicity of the protein levels is not required for clock function.

All of these experiments were carried out in strains with a functional FRQ-WCC oscillator and a strain was created with *frq¹⁰* background expressing NCU05950::3xFLAG fusion protein by crossing SG#444 and SG#59. This

genetic cross produced two different types of strains (NCU05950 wild type gene at its own locus and NCU05950^{KO} at its own locus). In order to check the expression of NCU05950::3xFLAG fusion proteins in *frq*¹⁰ background, Western blotting followed by immunodetection was carried out and as shown in Figure 6.6. It produced the expected size band (NCU05950::3xFLAG - 20.65 kDa) and the absence of FRQ does not have a large effect on NCU05950 expression.

Since NCU05950 protein is not rhythmic in the wild-type background, I do not expect it to be rhythmic in the *frq*¹⁰ background. Therefore, I did not assay a time-course of NCU05950 protein levels in strains with the *frq*¹⁰ background. These strains will be used to identify the NCU05950 binding partners when the FRQ-WCC oscillator is not functioning.

6.3.2: Rhythmicity of NCU05950 mRNA

6.3.2.1: Preliminary tests to optimize Northern-blotting technique for NCU05950 mRNA

Northern blotting was used to assay the levels of NCU05950 mRNA at regular intervals across two circadian cycles. Pilot experiments were conducted with two strains (Wild type = SG#26: *csp-1; chol-1 ras-1^{bd} mat a* & Negative control = SG#361: *csp-1; chol-1 ras^{bd}; NCU05950^{KO} mat a*) to optimize the Northern blotting protocols. 24 well plates containing 1 mL (on each well) Vogel's minimum liquid medium with 2% glucose & 100 µM choline were inoculated with SG#26 & SG#361 strains and allowed to grow for 2 days at room temperature (22°C) in constant light (LL). RNA was extracted and Northern blotting, hybridization with DIG- labelled probe and immunodetection

was carried out. As predicted, signals were detected only from SG#26, but not from SG#361, which is the NCU05950 knockout strain (Figure 6.7).

6.3.2.2: NCU05950 mRNA is not rhythmic

Time course experiments were performed using cultures (SG#26) grown in low-glucose liquid medium in flasks on shaker in constant darkness and constant 25 °C after synchronizing their clocks with a light-to-dark transition. Mycelia were collected at 4 hours intervals and samples were collected from 0 hour to 48 hours in DD (13 samples).

RNA was extracted using Trizol reagent protocol. From each sample 20 µg of RNA was run on 1.2% formaldehyde-agarose gel and Northern blotting was performed. RNA loading was determined by viewing the gel on a UV transilluminator and staining the blotted membrane with methylene blue. Three independent experiments were carried out at each time point and quantitated by densitometry. For each experiment, RNA extracted from SG#361 was used as the negative control. As shown in Figure 6.8, NCU05950 transcripts in the SG#26 strain with clock wild-type background are not rhythmically expressed.

In order to check the clock functionality and validity of the culture conditions and the method, Northern analysis was done to examine *frq* mRNA at 4 hours intervals over 48 hours under constant conditions using the same experimental samples of SG#26. As shown in Appendix XXV, the *frq* transcript showed a rhythmic accumulation that peaked at 12 to 16 and 32 to 36 hours in dark as reported by Aronson 1994.

Table 6.1: Genotypes of progeny strains from cross between SG#59 (*ras-1^{bd}; frq¹⁰ mat A*) and SG#444 (*csp-1 his-3⁺:: Native promoter - NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*).

A. *csp+* and *csp-1* progeny from cross between SG#59 (*ras-1^{bd}; frq¹⁰ mat A*) and SG#444 (*csp-1 his-3⁺:: Native promoter - NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*) strains.

Total spores picked	<i>csp-1</i> progeny	<i>csp+</i> progeny*	Non-germinated spores*
188	50 (26.60 %)	64 (34.04 %)	74 (39.36 %)

**csp+* and non-germinating progeny were excluded from further testing.

B. Summary of genotyping experiments.

Total progeny strains analyzed on race tubes	32
Controls used for the race tube analysis	- SG#26 (<i>csp-1; chol-1 ras-1^{bd} mat a</i>) - SG#361 (<i>csp-1; chol-1 ras-1^{bd}; NCU05950^{KO} mat a</i>) - SG#80 (<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i>) - SG#444 (<i>csp-1 his-3⁺:: Native promoter - NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>)

<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly::3xFLAG; chol-1 ras^{bd}; frq¹⁰</i>	7 pg#10, pg#17, pg#24, pg#28, pg#40, pg#46, pg#51 (Has two copies of NCU05950 gene.)
<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} frq¹⁰</i>	4 pg#27, pg#29, pg#50, pg#76
<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly::3xFLAG; chol-1 ras^{bd}</i>	8 pg#19, pg#21, pg#31, pg#34, pg#44,pg#60, pg#68, pg#97
<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly::3xFLAG; ras^{bd}; frq¹⁰</i>	1 pg#72
<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly::3xFLAG; ras^{bd}</i>	6 pg#2, pg#33, pg#43, pg#75, pg#100, pg#105
<i>csp-1; chol-1 ras^{bd}; frq¹⁰</i>	3 pg#42, pg#84, pg#92
<i>csp-1; chol-1 ras^{bd}</i>	0
<i>csp-1; ras^{bd}; frq¹⁰</i>	1 pg#88
<i>csp-1; ras^{bd}</i>	0

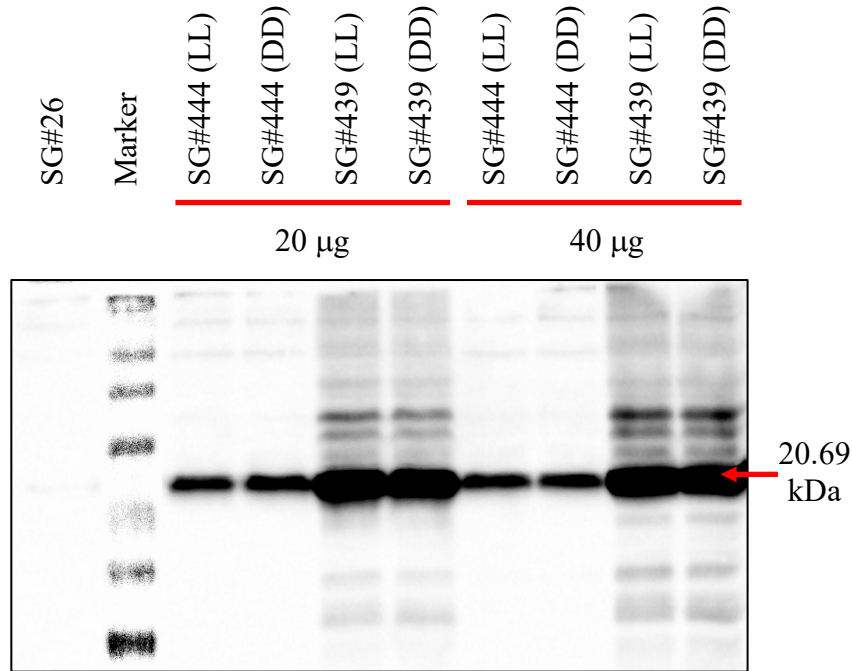


Figure 6.1: Expression of NCU05950::3xFLAG fusion protein from the native NCU05950 promoter (SG#444 - *csp-1 his-3⁺*:: Native promoter - NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; NCU05950^{KO} mat a) and the high-expression *ccg-1* promoter (SG#439 - *csp-1 his-3⁺*:: *pccg-1*::NCU05950 ::Gly::3xFLAG; *chol-1 ras^{bd}*; NCU05950^{KO} mat a) under constant light and constant dark conditions. Total protein from each sample was loaded on to 15 % gel and for immunodetection mouse monoclonal M2-Anti-FLAG (DYKDDDDK) antibodies were used as the primary antibody.

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1      TCGTGGTCCGTTCTCTGATGATTCAATCCTTGTAGTGTACATATACCTAGG
101    TACATAGGTAGTTGAAGGTGTTTTTGGTTGGGTGATCAACCACTGATCGA 100
      TAGCGACCAACAAGGTTAAATGGCGTATAAGTGCGCATTGACCAACTG
201    TTTCTACCACCCACCAGGACAGCAGCGCCTTGGTAGGCTGATGAGGCTAG
      AGGCTAGAGGTAGTCTGGATAGCCGACGTGCACTCGCGCCTGCTGTTCA 300
301    GCCCCAGGGCCACACAGCACACAGGGTTCGCTAGAGTTCGTTGGGCTC
      TGGCGCCTGTACACTAGCAAAAGTTTGGCCGAGGCGGTGAATAAGGCA 400
401    CGAGCAAGGATGAACCAACCAATGATGACCGCTCCAATCCTTGCCTCGGTGG
      ATTTTTAGCCGCACATGGCAGGCGCATTGAACCGTGACACATTTGTTGCT 500
501    GCAATCACCAATTCAATTCAATACGCGACCGAGAAGACCTTGTGCTGGGC
      TCCAGTTGCGGGAAGGAACGCTTGGAAATGAGTCGGGGCGCGTTGAAATT 600
601    TGGAAACAGCGACGACGACGATCGATATCGATAAGGACAACTTTCCCTAG
      GTTCCTTGGGTTCCCTTATCCGTCATTATCCCTACCTAGGTCCTGCCT 700
701    CACCTCGCGCAATCATCGCCGATTCCAAGCCGACGGCCGACCAATTTC
      GGGACACTGCAGCAAGTCCATCGCGACACATTCCTTCCTTGCCTGATGA 800
801    ACCGACCTCGCTAGCAGTCCCTCCCCCGGGGACCCGACAGAAGCCGA
      AGCAACTCGACTAAACCAGCACCACAAGCCATTTTCACGACGGGACCCAA 900
901    CTTTTTTTTTTTTTTTTTTTTTGGTTTTTTTTTGTGCGTCTGATCTCATT
      TCGTATCGATCGAGTTTATCTCTTCGCGACGGTCTTTCTCCCAACTT 1000
1001   TACTTCGACGACGTCTGGCTTCAAGACGAGACTTTTTCTTGTGCTTCGC
      GCGAATTCCAATTGACAAACGTATCGACGACTTGATACCGACCGACCG 1100
1101   ACCGACCACATCCGACTGGCGACACGGCACATAAAGAAGGGAGGACAAG
      ATAACAAGATGGGCAACTTTTGCTCAACCTGCTTCGGCGGTAGGAGGAGC 1200
1201   GATGACTACGATGAGGTAGGTTTCGGCATAGCGGCCACAGTCCGATGACA
      TGGCCATCAAAAGAGGCATTTCGACGGAACAAGAGAACTGACGTGGACTGT 1300
1301   TCGTGACACAGGAGGACGAGGCCAGCATCTCTTCGACGAGAACAACAT
      GCACTACGGGAGCTTTGATCAGCAACACATGATGAACCAGGAAGATCCCC 1400
1401   AAGAGACAGAAAGGGAATTGCGGCTCTGCAGGGCGTGGTGAACGCACA
      TCAAAAGTATGCCCTCCACCTGAAATGTCTCCCATCCAAGATCTAGTCCG 1500
1501   AGACCCAAGTCCACGGAGCCGGTAGAGCGAATAACTGACTTGGAACCGCG
      CAGTAACATGGTCGACATCTATGATATGGTCCCGCATGATAAACCCATGC 1600
1601   AGGACGCTCCTGCTCCCTACGGCTTTGCCAACCAGCGCTACAATGCCCTG
      CTTTCCAAGCTCTCAACCCACGATGACATGGCCGCCGTTGCACGGGTGA 1700
1701   CTGGGGCACCCCTGAGGACGACAGCATGGAGATGCTGCGCAAAGCTTCCT
      TGCCAACCATCCCATCAAGGCTGAAGGAGGCGAGGCCCTCGTCGGTAAC 1800
1801   TTTACCGATGCCGCTGCCGCCATGCGCTGT 1830

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Figure 6.2: NCU05950 sequence with GATN repeats (LREs).

Key:

Upstream region of NCU05950 gene – Yellow

GATN repeats – Orange

Start codon of NCU05950 gene – Purple

Exon 1,2, & 3 of NCU05950 gene – Blue

Stop codon of NCU05950 gene – Red

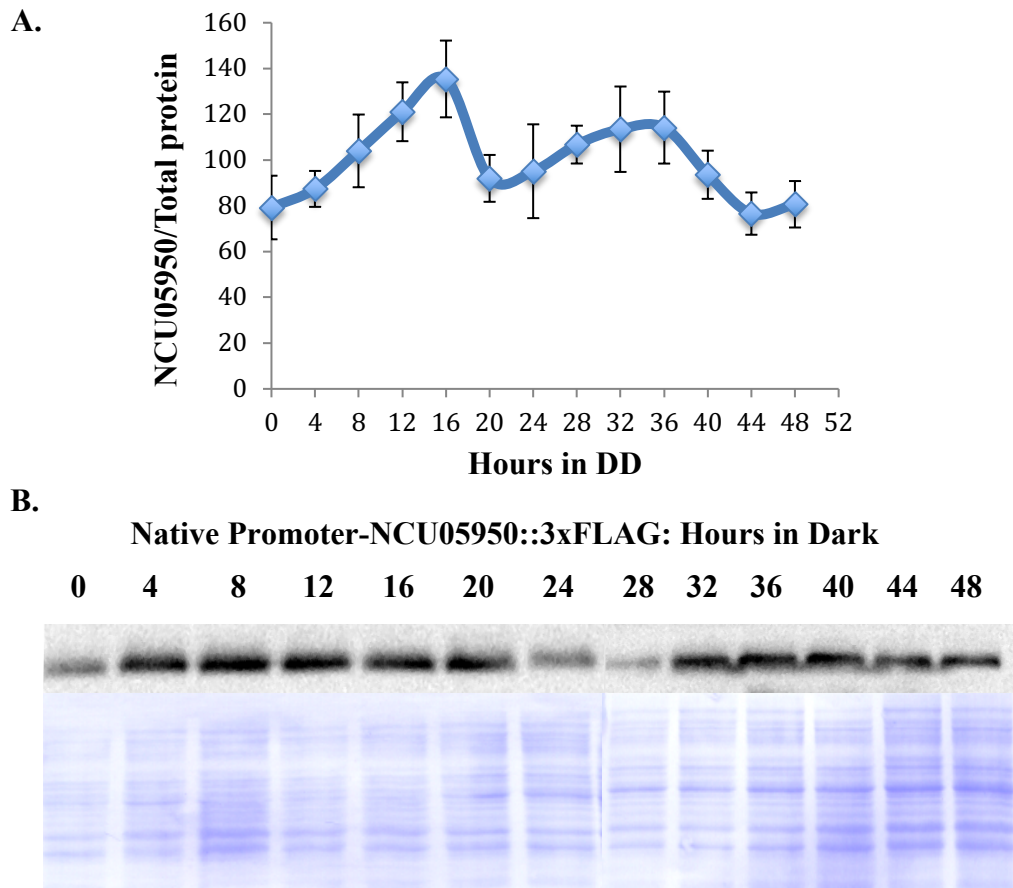


Figure 6.3: Expression of NCU05950 protein. SG#444 (*csp-1 his3+::NCU05950::FLAG; ras^{bd}chol-1; NCU05950^{KO}*) was grown on liquid media with high choline to repair the *chol-1* defect. Cultures were grown in DD for varying lengths of time after synchronizing the clock with 2 hours of heat pulse at 35°C under constant light and protein extracts were assayed for NCU05950-FLAG protein by immunoblotting. A. Relative level of NCU05950-FLAG normalized to total protein by Coomassie staining. Points are mean normalized ratios from three independent experiments. B. Upper panel: Immunoblotting of NCU05950-FLAG protein at different time points (0–48 h in DD) corresponding to the time-series in A. Lower panel: Coomassie stained membrane of samples in upper panel.

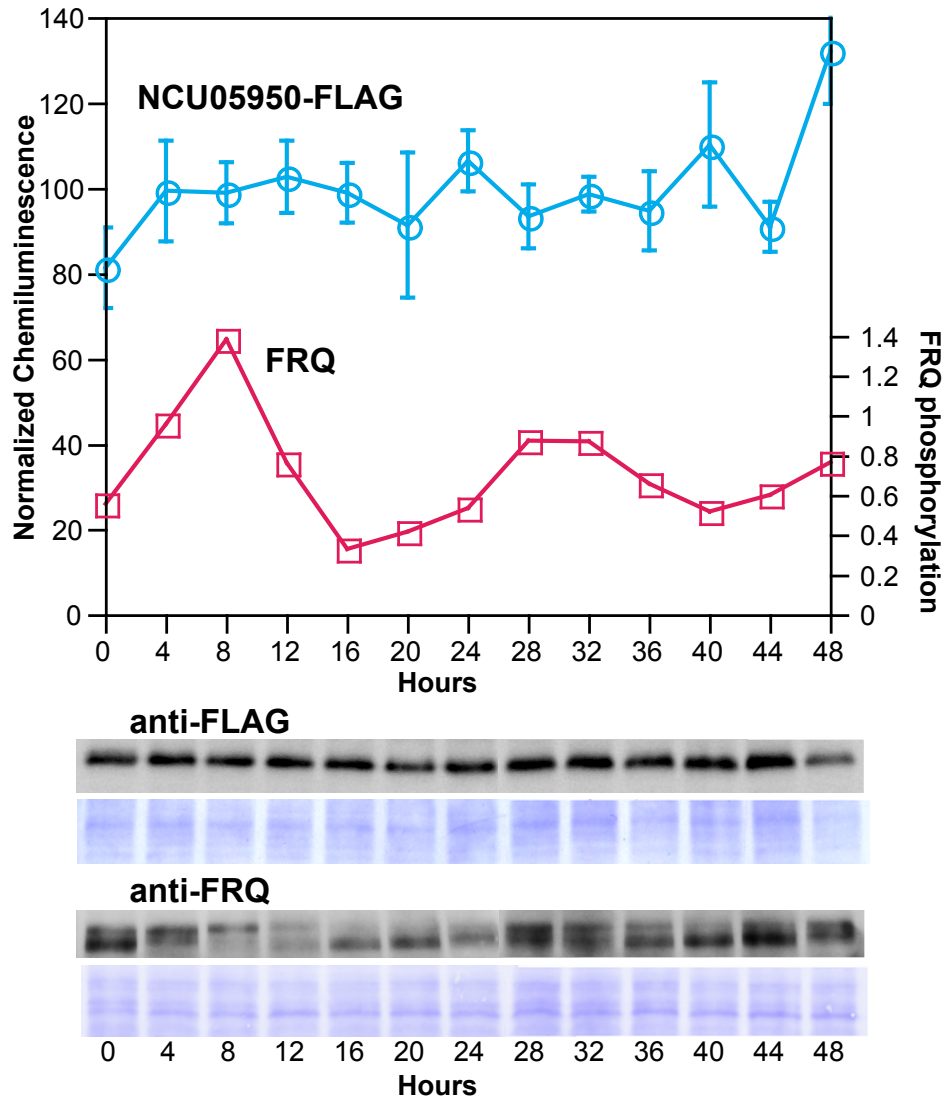


Figure 6.4: Absence of rhythmicity in NCU05950 protein levels. SG#444 (*csp-1 his3+::NCU05950::FLAG; ras^{bd}chol-1; NCU05950^{KO}*) was grown on liquid media with high choline to repair the *chol-1* defect. Cultures were grown in DD for varying lengths of time after synchronizing the clock with light to dark transition. Samples were collected every 4 hours across two circadian cycles from cultures expressing the NCU05950-FLAG fusion protein from the

native promoter. The genotype was *csp-1 his-3+::NCU05950::FLAG; ras^{bd} chol-1; NCU05950^{KO}*. Western blotting was carried out using anti-FLAG antibody. Relative densities of chemiluminescence images within each replicate time-course were normalized to mean = 100 (left Y-axis). Data points are mean $\pm$ S.E.M. of three independent experiments. A representative blot with anti-FLAG antibody and Coomassie-stained protein is shown below. The same samples were run on a second gel and the blot is shown with anti-FRQ antibody and Coomassie stained protein. The FRQ phosphorylation state was calculated as the ratio of the upper FRQ band (phosphorylated) to the lower FRQ band (unphosphorylated) and the ratio is plotted against the right Y-axis. Data for FRQ are from one experiment (Ratnayake *et al.*, 2018).

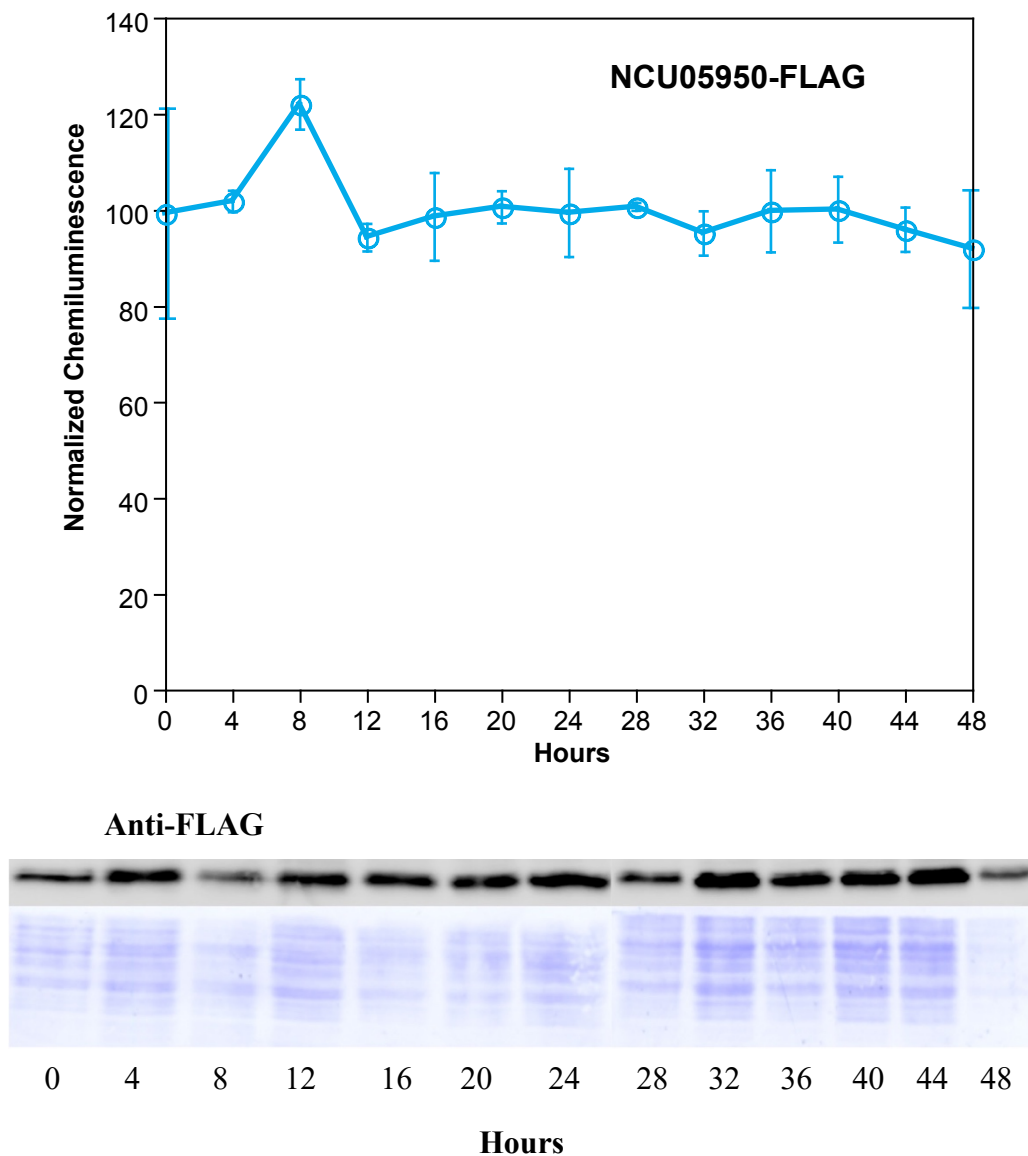


Figure 6.5: NCU05950 protein levels on agar medium. Cultures were grown on top of cellophane overlaid on solid agar medium. Samples were collected every 4 hours, processed for immunoblotting and analysed. Data points are mean $\pm$ S.E.M. of three independent experiments. A representative blot with anti-FLAG antibody and Coomassie-stained protein is shown below.

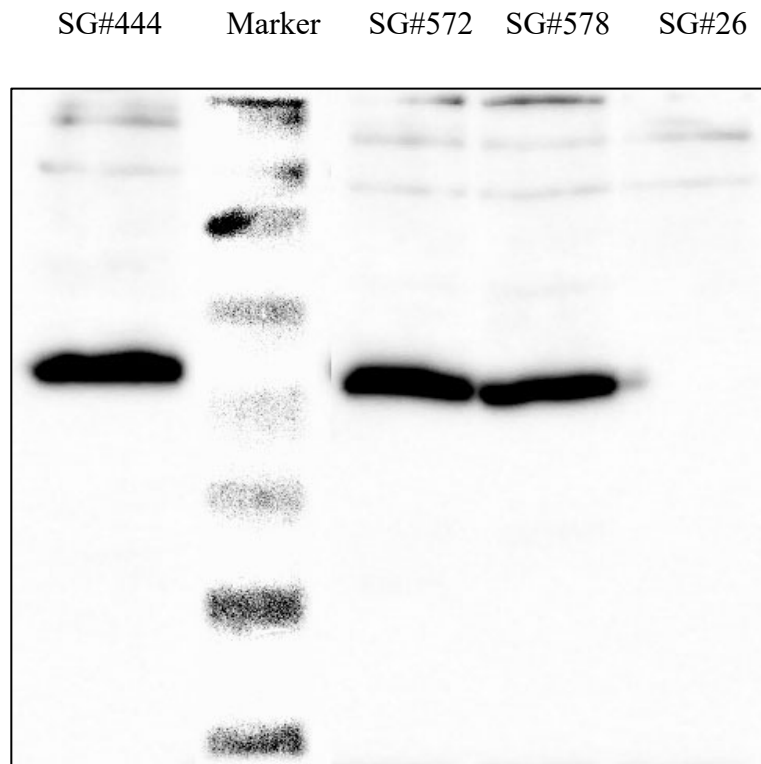


Figure 6.6: Analysis of expression of NCU05950::3xFLAG fusion protein in *frq*¹⁰ background.

SG#444 – positive control with the genotype *csp-1 his-3*⁺:: Native promoter - NCU05950::Gly::3xFLAG; *chol-1 ras*^{bd}; NCU05950^{KO}.

SG#26 – Negative control with the genotype *csp-1*; *chol-1 ras*^{bd}.

SG#572 - *csp-1 his-3*⁺:: Native promoter - NCU05950::Gly::3xFLAG; *chol-1 ras*^{bd}; *frq*¹⁰; NCU05950^{KO}

SG#578 - *csp-1 his-3*⁺:: Native promoter - NCU05950::Gly::3xFLAG; *chol-1 ras*^{bd}; *frq*¹⁰

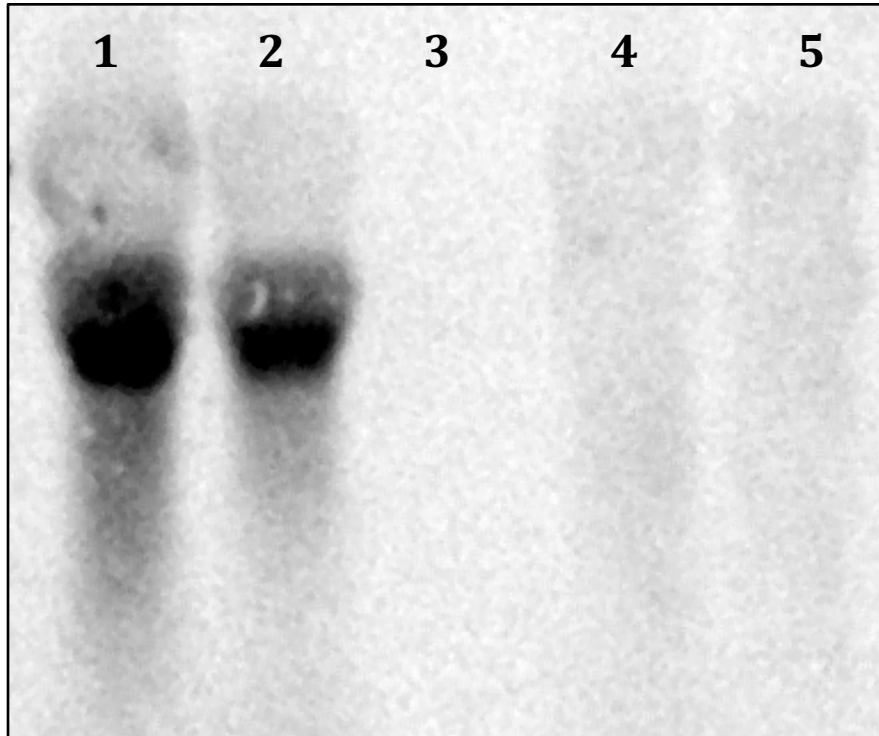
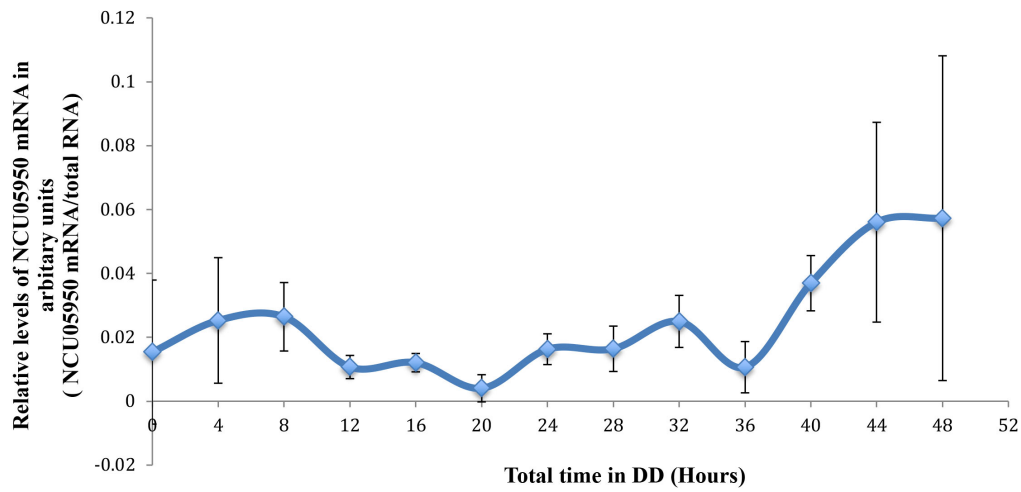


Figure 6.7: Pilot experiment to optimize Northern blot protocol.

Lane No. 1: 40 µg of RNA from SG#26: *csp-1*; *ras^{bd} chol-1* ; Lane No. 2: 20 µg of RNA from SG#26: *csp-1*; *ras^{bd} chol-1* ; Lane No. 3: empty ; Lane No. 4: 20 µg of RNA from SG#361: *csp-1*; *ras^{bd} chol-1*; *NCU05950^{KO}* ; Lane No. 5: 40 µg of RNA from SG#361: *csp-1*; *ras^{bd} chol-1*; *NCU05950^{KO}*

A.



B.

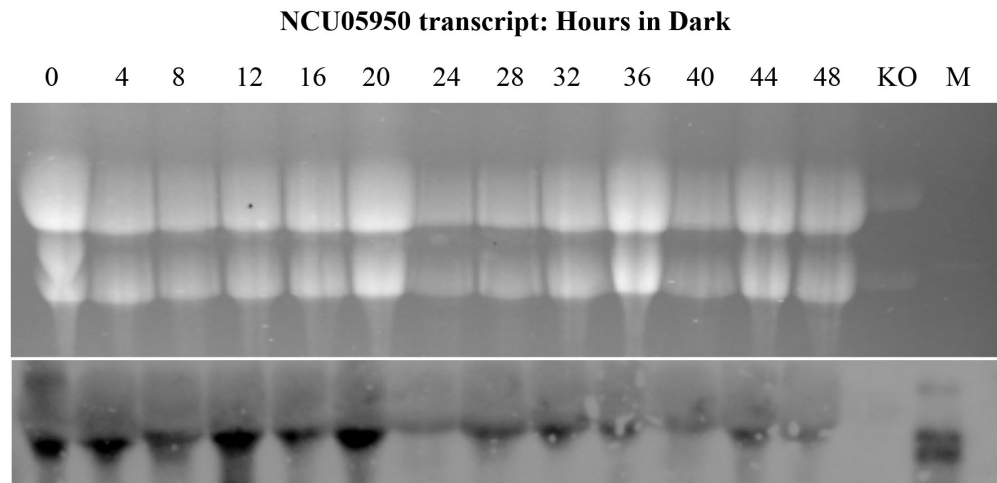


Figure 6.8: Analysis of NCU05950 transcript by Northern blotting.

SG#26 (*csp-1*; *ras^{bd} chol-1*) was grown on high choline to repair the *chol-1* defect. Cultures were grown in DD for varying lengths of time. SG#361 (*csp-1*; *ras^{bd} chol-1*; *NCU05950^{KO}*) was used as the control strain. Digoxigenin-labeled probe (251bp long fragment from the 3rd exon of the NCU05950 gene) was used for hybridization.

A. Relative level of NCU05950 transcript levels normalized to total RNA by methylene blue staining of membrane and ethidium bromide staining of the gel. Points are mean normalized ratios from three independent experiments. B. Upper panel: Ethidium bromide staining of total RNA at different time points (0–48 h in DD) corresponding to the time-series in A and total RNA sample (KO) of SG#361 (*csp-1*; *ras^{bd}* *chol-1*; *NCU05950^{KO}*). M – Molecular weight marker. Lower panel: Immunodetected Northern blot of samples in upper panel. (RNA extraction, Northern blotting, immunodetection and data analysis were done by Alexandra Pana and Aryan Lajevardi).

CHAPTER 07: Importance of N-terminus of NCU05950 gene product for its localization and clock related function.

7.1: Introduction

The proteins homologous to NCU05950 protein, human p18 and yeast EGO1/Meh1p/Gse2p, have amino acid sequence signals near the amino terminus for lipid modification such as myristoylation and palmitoylation (Kogan *et al.*, 2010; Dubouloz *et al.*, 2005; Nada *et al.*, 2009; Nada *et al.*, 2014). These lipid modifications are essential for membrane anchoring of yeast EGO complex and human “Regulator” complex (Kogan *et al.*, 2010).

The putative acylation sites at the N-terminus of the NCU05950 protein (Figure 4.1) may be responsible for localizing the protein to membranes. To test this hypothesis and determine whether it is important for the function of NCU05950, a deletion mutant lacking the terminal glycine (amino acid #2 - $\Delta 1$) and also a deletion mutant lacking 7 amino acids (#2–8 – $\Delta 7$), which would delete both putative acylation sites, were constructed. However, the 7 amino acid deletion ($\Delta 7$) leaves two glycines near the N-terminus, which could potentially serve as new acylation sites. Therefore, a deletion of 10 amino acids (#2–11- $\Delta 10$) was constructed to eliminate that possibility. Strains expressing defective NCU05950::GFP fusion proteins were subjected to live –cell imaging to determine the importance of lipid modifications for localization of the NCU05950 protein. Strains expressing defective NCU05950::3xFLAG fusion proteins were used for race tube experiments to determine the importance of the N-terminus of the NCU05950 protein for the clock related function.

Furthermore, strains expressing defective NCU05959::3xFLAG fusion proteins can be used to identify whether other proteins interact with NCU05950 protein using the N-terminus of NCU05950 protein.

7.2: Methods

7.2.1: Construction of strains expressing defective NCU05950::GFP and NCU05959::3xFLAG fusion proteins from the high-expression *cgc-1* promoter.

Defective coding sequence of the candidate gene NCU05950 without regulatory sequences was isolated from previously constructed pCCG::NCU05950::C-Gly::GFP (GX10) and pCCG::NCU05950::C-Gly::3XFLAG (FX8) plasmids by PCR and inserted into the XbaI-PacI site of the *his-3* locus targeting pCCG::C-Gly::GFP plasmid and pCCG::C-Gly::3xFLAG between the highly expressed *cgc-1* promoter and the 10X Gly linker upstream of the GFP or 3XFLAG sequence. The following forward primers were used: for 1-deletion ($\Delta 1$) – UV90 $\Delta 1$ XbaIF; for 7-deletion ($\Delta 7$) – UV90 $\Delta 7$ XbaIF and for 10-deletion ($\Delta 10$) – UV90 $\Delta 10$ XbaIF (Appendix IV). The reverse primer for all six constructs was UV90PacIR (Appendix IV).

The plasmid and isolated gene fragments were digested with XbaI-PacI (to obtain highly expressed fusion protein of NCU05950 and GFP or 3XFLAG from the *cgc-1* promoter) and were gel purified. Ligation mixtures were transformed to chemically competent *E.coli*. Several colonies were picked and colony PCR was carried out to analyze the transformed bacterial cells using UV90AFor and R2 primers (Appendix III). Several colonies gave the desired bands for six constructs and those colonies were selected for further testing. Plasmid DNA isolated from PCR positive colonies was digested with different

restriction enzymes to confirm the presence of the inserted candidate gene on the recombinant plasmids (Appendix XXVII - A). Recombinant plasmids with *ccg-1* promoter followed by defective NCU05950 gene coding sequence and GFP tag were named as pΔ1GX, pΔ7GX, and pΔ10GX. Recombinant plasmids with *ccg-1* promoter followed by defective NCU05950 gene coding sequence and 3XFLAG tag were named as pΔ1FX, pΔ7FX, and pΔ10FX.

After confirming the cloning by sequencing, pΔ1GX-7, pΔ7GX-4, pΔ10GX-2, pΔ1FX-3, pΔ7FX-1, and pΔ10FX1 were selected for transformation. Plasmid DNA was transformed to the spores of NCU05950 knockout strain (carrying the deletion of NCU05950) SG #359 (*csp-1 his-3; chol-1 ras^{bd}; NCU05950^{KO} mat a*) as the . Fungal strain SG #359 grown on histidine and choline containing media was used to harvest conidiospores for transformation. Cloned plasmid (10-15 µg/ 15 µL) was linearized with NdeI and added to 40 µL of washed conidia. Conidiospores were transformed with the plasmid DNA by electroporation and plated on Top agar media without histidine supplement to select *his-3⁺* transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline and the transformants that showed healthy growth with bright orange spores were further analyzed.

Spore PCR was carried out with different combinations of *his-3* locus specific (HIS-3F1 & HIS-3R - Appendix VI) and NCU05950 gene specific (UV90AFor & UV90BRev - Appendix III) primers. Some transformants gave expected bands and it indicates successful transformation as well as integration at the *his-3* locus. Silica stocks were prepared from positive heterokaryotic transformants of Δ1GX strains (SG#598-600), Δ7GX strains (SG# 521-527), Δ10GX strains (SG#528-530), Δ1FX strains (SG#531-535), Δ7FX strains (SG#536-539) and Δ10FX strains (SG#540-541). Details of strain identification

names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

Heterokaryotic transformants of only $\Delta 1GX$, $\Delta 7GX$ and $\Delta 10GX$ strains were purified by making microconidia. $\Delta 1FX$, $\Delta 7FX$ and $\Delta 10FX$ strains were not subject to purification by making microconidia as no experiments for the near future have been planned. In order to make microconidia, the method described in section 2.7 was used. Spore PCR was performed with pure microconidia derived transformant strains with *his-3* locus specific (HIS-3F1 & HIS-3R) primers (Appendix VI). Only the strains that gave one PCR derived band from the *his-3* locus specific primers, which are pure homokaryotic transformants with correct genotype, were used for race tube experiments and for microscopy analysis. Several attempts were made to purify $\Delta 1GX$ strains. Unfortunately, no homokaryon was obtained for $\Delta 1GX$ strains. Therefore, heterokaryons of $\Delta 1GX$ strains were used for microscopy experiments. Silica stocks were prepared from positive homokaryotic transformants of $\Delta 7GX$ (SG#547, 551-553) and $\Delta 10GX$ strains (SG# 544 – 546, SG#548-550, SG#554,555). Details of strain identification names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

7.2.2: Construction of strains expressing defective NCU05950::GFP and NCU05959::3xFLAG fusion proteins from NCU05950 promoter

Gene fragments (Nat-Del1-NCU05950 = 611bp, Nat-Del7-NCU05950= 593bp and Nat-Del10- NCU05950= 584bp) with *Sal*I and *Bgl*II restriction sites on either sides of the synthetic gene were obtained from Integrated DNA Technologies, Inc., Coralville, IA, USA. These gene fragments

were received on ampicillin resistant pUCIDT vector. 4 µg of each plasmid (pUCIDT- Nat-Del1-NCU05950, pUCIDT- Nat-Del7-NCU05950 and pUCIDT- Nat-Del10-NCU05950) were received and each was dissolved in 20 µL of ddH₂O. 1 µL from each plasmid was transformed to chemically competent *E.coli*. Two colonies were picked from each construct and plasmid DNA was isolated and digested with EcoRI-BglII restriction enzymes to confirm the presence of the synthetic gene on the plasmids.

These synthetic gene fragments were sub cloned to SanDI and BglII restriction sites on previously constructed pCCG::C-Gly::3xFLAG and pCCG::C-Gly::GFP vectors harboring the NCU05950 wild type gene with native promoter (GN10 plasmid and FN33). Ligation mixtures were transformed to chemically competent *E.coli*. Several colonies were picked and colony PCR was carried out to analyze the transformed bacterial cells using F2 and UV90BRev primers. Several colonies gave the desired bands for six constructs and those colonies were selected for further testing. Plasmid DNA isolated from PCR positive colonies was digested with different restriction enzymes to confirm the presence of the inserted candidate gene on the recombinant plasmids (Appendix XXIX - A). Recombinant plasmids with coding sequence of defective NCU05950 gene along with upstream regulatory sequences and GFP tag were named as pΔ1GN, pΔ7GN, and pΔ10GN. Recombinant plasmids with coding sequence of defective NCU05950 gene along with upstream regulatory sequences 3XFLAG tag were named as pΔ1FN, pΔ7FN, and pΔ10FN.

After confirming the cloning by sequencing, pΔ1GN-E, pΔ7GN-5, pΔ10GN-a, pΔ1FN-A, pΔ7FN-1, and pΔ10FN-g were selected for transformation. Plasmid DNA was transformed to the spores of NCU05950 knockout strain (carrying the deletion of NCU05950) SG #359 (*csp-1 his-3*;

chol-1 ras^{bd}; NCU05950^{KO} mat a). Fungal strain SG #359 grown on histidine and choline containing media was used to harvest conidiospores for transformation. Cloned plasmid (10-15 µg/ 15 µL) was linearized with NdeI and added to 40 µL of washed conidia. Conidiospores were transformed with the plasmid DNA by electroporation and plated on Top agar media without histidine supplement to select *his-3⁺* transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline and the transformants that showed healthy growth with bright orange spores were further analyzed.

Spore PCR was carried out with different combinations of *his-3* locus specific (HIS-3F1 & HIS-3R - Appendix VI) and NCU05950 gene specific (UV90AFor & UV90BRev - Appendix III) primers. Some transformants gave expected bands and it indicates successful transformation as well as integration at the *his-3* locus. Silica stocks were prepared from positive heterokaryotic transformants of Δ 1GN strains (SG#601-603), Δ 7GN strains (SG# 604-606) and Δ 10GN strains (SG#607-609). Heterokaryotic transformants were purified by microconidia preparation and the presence of the cloned gene in the homokaryons was confirmed by spore PCR analysis with *his-3* locus specific (HIS-3F1 & HIS-3R) primers (Appendix VI). Silica stocks were prepared from positive homokaryotic transformants of Δ 1FN (SG#556- 558), Δ 7FN (SG#559-565) and Δ 10FN strains (SG# 582-585). Details of strain identification names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

7.3: Results & Discussion

7.3.1: Construction of strains expressing defective NCU05950::GFP and NCU05959::3xFLAG fusion proteins from the high-expression *cpg-1* promoter.

Strains carrying deletions of 1 (G), 7 (GNFCSTC) or 10 (GNFCSTCFGG) amino acids of NCU05950::GFP and NCU05959::3xFLAG expressed from the *cpg-1* promoter were constructed by replacing either the first 2, first 8 or first 11 amino acid codons (Appendix XXVI) with an initiator methionine codon to determine if these amino acids facilitate interaction with the vacuolar membrane and are therefore important for the function of NCU05950 gene product.

Restriction enzyme digestion and sequencing of recombinant plasmids with *cpg-1* promoter followed by defective NCU05950 gene coding sequence and GFP tag or 3xFLAG tag gave the expected results and confirmed the presence of the insert on the recombinant plasmid (Appendix XXVII – A and Appendix XXVII – B). Linearized pΔ1GX-7, pΔ7GX-4, pΔ10GX-2, pΔ1FX-3, pΔ7FX-1, and pΔ10FX1 plasmids were transformed into macrospores of NCU05950 knockout strain SG#359 and successful integration at the *his-3* locus was confirmed by carrying out PCR with different combinations of *his-3* locus specific (HIS-3F1 – HIS-3R) and NCU05950 gene specific (UV90AFor – UV90BRev) primers. Transformants that gave expected bands indicate successful transformation as well as integration at the *his-3* locus.

Homokaryons were obtained by making microconidia from positive transformants. Spore PCR analysis was performed with pure microconidia derived transformants and only pure homokaryotic transformants with correct genotype were used for race tube experiments and for microscopy analysis.

7.3.2: Construction of strains expressing defective NCU05950::GFP and NCU05959::3xFLAG fusion proteins from NCU05950 promoter

To construct the strains expressing NCU05950 defective protein under its native promoter, a different strategy was used and utilized a *SanDI* restriction site (GGGACCC) 261–267 nt upstream of the start codon, and a *BglII* restriction site (AGATCT) 328–333 nt downstream (Appendix XXVIII).

Gene sequences between these two sites, but missing 1, 7 or 10 amino acid codons as described above were commercially synthesized. These synthetic gene fragments were sub cloned to *SanDI* and *BglII* restriction sites on previously constructed pCCG::C-Gly::3xFLAG and pCCG::C-Gly::GFP vectors harboring the NCU05950 wild type gene with native promoter (GN10 plasmid and FN33). Restriction enzyme digestion and sequencing of recombinant plasmids with coding sequence of defective NCU05950 gene along with upstream regulatory sequences and GFP tag or 3xFLAG tag gave the expected results and confirmed the presence of the correct insert on the recombinant plasmid (Appendix XXIX – A and Appendix XXIX - B). Linearized pΔ1GN-E, pΔ7GN-5, pΔ10GN-a, pΔ1FN-A, pΔ7FN-1, and pΔ10FN-g plasmids were transformed into macrospores of NCU05950 knockout strain SG#359 and successful integration at the *his-3* locus was confirmed by carrying out PCR with different combinations of *his-3* locus specific (HIS-3F1 – HIS-3R) and NCU05950 gene specific (UV90AFor – UV90BRev) primers. Transformants that gave expected bands indicate successful transformation as well as integration at the *his-3* locus. Homokaryons were obtain by making microconidia from positive transformants. Pure homokaryotic transformants with correct genotype were

used for race tube experiments, for microscopy analysis and protein expression analysis by Western blotting.

7.3.3: Subcellular localization of NCU05950 N-terminal deletion mutants.

GFP tagged deletion mutant strains were observed using confocal microscopy to determine any changes in subcellular localization of the protein. GFP-fusion proteins with these deletions ($\Delta 7GX$ & $\Delta 10GX$) expressed from the *cpg-1* promoter were found to be diffusely localized in the cytosol (Figure 7.1), in contrast to the vacuolar localization of the wild-type protein and deletion mutant lacking the terminal glycine (amino acid #2 - $\Delta 1GX$). Similar results were found with deletion mutants $\Delta 1GN$, $\Delta 7GN$ & $\Delta 10GN$ expressed from the native NCU05950 promoter (Figure 7.1).

In order to check the expression of defective NCU05950 proteins (*pccg-1:: $\Delta 7$ -NCU05950::10xGly::GFP* & *pccg-1:: $\Delta 10$ -NCU05950::10xGly::GFP*) Western blotting was carried out using anti-GFP antibodies. Figure 7.2 demonstrates that the proteins with N-terminal 7- and 10-amino acid deletions were stably expressed and produced the expected size bands (NCU05950::GFP - 44.6 kDa, $\Delta 7$ -NCU05950::GFP - 43.91 kDa & $\Delta 10$ -NCU05950::GFP - 43.65 kDa).

Quantitation of pixel brightness from confocal images (Figure 7.3) confirmed that GFP-tagged proteins were concentrated at the vacuolar membranes in the wild type control and $\Delta 1$ strains but not in the $\Delta 7$ and $\Delta 10$ strains, for both the *cpg-1* and native promoters. All these results indicate that the NCU05950 gene product is a LAMTOR-like protein localized to the vacuolar membrane, probably by acyl group anchors.

7.3.4: Vacuolar localization is required for the rhythm-related function of NCU05950.

After purifying homokaryons by making microconidia, the strains expressing defective NCU05950 gene product ($\Delta 7GX$, $\Delta 10GX$, $\Delta 1GN$, $\Delta 7GN$, $\Delta 10GN$, $\Delta 1FN$, $\Delta 7FN$ and $\Delta 10FN$) were grown on race tubes to check their ability to produce rhythms. FLAG-tagged as well as GFP tagged N-terminal deletion constructs expressed from the native NCU05950 promoter ($\Delta 7GN$, $\Delta 10GN$, $\Delta 7FN$ and $\Delta 10FN$) as well as *ccg-1* promoter ($\Delta 7GX$ and $\Delta 10GX$), which are missing 7 or 10 amino acids, failed to rescue the defective phenotype of the NCU05950 gene deletion strain (Figure 7.4 & Figure 7.5). In contrast, a deletion of only the N-terminal glycine at position #2, the putative site for myristoylation, did not disrupt vacuolar localization of the protein in $\Delta 1GN$ and $\Delta 1GX$ strains (Figure 7.1) and did not disrupt the rhythm-related function of the protein on $\Delta 1GN$ and $\Delta 1FN$, as this deletion was able to rescue the phenotype of the gene deletion mutant (Figure 7.4 & Figure 7.5). Acylation at the N-terminal glycine is therefore not required for correct vacuolar localization and function.

In order to check the expression of defective NCU05950 proteins (Native promoter:: $\Delta 1$ - NCU05950 / $\Delta 7$ - NCU05950 / $\Delta 10$ - NCU05950 ::3xFLAG) Western blotting was carried out using anti-FLAG antibody and it produced the expected size bands (NCU05950::3xFLAG - 20.71 kDa , $\Delta 1$ - NCU05950::3xFLAG - 20.65 kDa, $\Delta 7$ - NCU05950::3xFLAG - 20.0 kDa & $\Delta 10$ - NCU05950::3xFLAG -19.73 kDa as shown in the Figure 7.6.

Table 7.1: Periods and growth rates of GFP-tagged N-terminal deletion strains.

A.

Genotype	+ Choline		- Choline	
	Period (h)	Growth Rate (mm/h)	Period (h)	Growth Rate (mm/h)
Control	21.69 ± 0.06	1.41 ± 0.01	50.34 ± 1.66	0.53 ± 0.01
NCU05950 ^{KO}	22.30 ± 0.09	1.28 ± 0.01	N.R.	0.47 ± 0.014
pccg-1:: NCU05950:: 3xFLAG	21.56 ± 0.05	1.37 ± 0.02	41.22 ± 1.66	0.54 ± 0.01
pccg-1:: Δ7- NCU05950:: GFP	22.02 ± 0.07	1.40 ± 0.01	N.R.	0.50 ± 0.01
pccg-1:: Δ10- NCU05950:: GFP	22.52 ± 0.07	1.33 ± 0.02	N.R.	0.46 ± 0.006

Genotype abbreviations as for Figure 7.4 - A. All strains are also *csp-1*; *ras^{bd}* *chol-1*.

Strains were grown with (+) or without (-) 100 μM choline in the medium.

Data are from one isolate of each genotype and five replicate race tubes per condition.

Data are reported as mean ± S.E.M. (N=5).

N.R.: Not rhythmic.

B.

Genotype	+ Choline		- Choline	
	Period (h)	Growth Rate (mm/h)	Period (h)	Growth Rate (mm/h)
Control	21.3 ± 0.08	1.55 ± 0.01	46.3 ± 4.5	0.53 ± 0.03
NCU05950 ^{KO}	22.4 ± 0.01	1.36 ± 0.01	N.R.	0.53 ± 0.03
NCU05950:: GFP	21.2 ± 0.04	1.46 ± 0.003	38.8 ± 1.8	0.54 ± 0.01
Δ1- NCU05950:: GFP	21.6 ± 0.14	1.47 ± 0.01	43.5 ± 2.0	0.52 ± 0.003
Δ7- NCU05950:: GFP	22.0 ± 0.16	1.41 ± 0.003	N.R.	0.56 ± 0.01
Δ10- NCU05950:: GFP	22.1 ± 0.13	1.48 ± 0.01	N.R.	0.56 ± 0.01

Genotype abbreviations as for Figure 7.4 - B. All strains are also *csp-I*; *ras*^{bd} *chol-I*.

Strains were grown with (+) or without (-) 100 μM choline in the medium.

Data are from one isolate of each genotype and three replicate race tubes per condition.

Data are reported as mean ± S.E.M. (N=3).

N.R.: Not rhythmic.

Table 7.2: Periods and growth rates of 3xFLAG-tagged N-terminal deletion strains.

Genotype	Number of Isolates	+ Choline		-Choline	
		Period (h)	Growth Rate (mm/h)	Period (h)	Growth Rate (mm/h)
Control	1	21.3 ± 0.1 (10)	1.34 ± 0.02 (10)	51.8 ± 1.7 (10)	0.49 ± 0.01 (10)
NCU05950 ^{KO}	1	22.3 ± 0.3 (10)	1.16 ± 0.04 (10)	N.R.	0.49 ± 0.01 (10)
NCU05950::3xFLAG	4	22.0 ± 0.1 (16)	1.28 ± 0.01 (16)	45.6 ± 1.5 (14)	0.51 ± 0.01 (14)
Δ1-NCU05950::3xFLAG	2	21.9 ± 0.2 (8)	1.27 ± 0.003 (8)	47.7 ± 2.8 (8)	0.49 ± 0.01 (8)
Δ7-NCU05950::3xFLAG	2	23.4 ± 0.3 (8)	1.17 ± 0.06 (8)	N.R.	0.50 ± 0.01 (8)
Δ10-NCU05950::3xFLAG	2	24.2 ± 0.3 (8)	1.04 ± 0.01 (8)	N.R.	0.50 ± 0.01 (8)

Genotype abbreviations as for Figure 7.5. All strains are also *csp-1*; *ras^{bd} chol-1*.

Data from transformants pooled from several independent isolates with the indicated genotype. Strains were grown with (+) or without (-) 100 μM choline in the medium.

Data reported as mean ± S.E.M. (N)

N.R.: Not rhythmic.

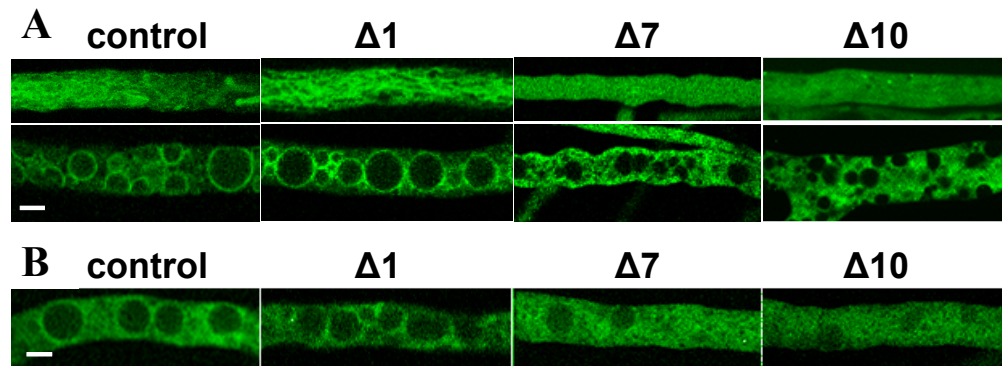


Figure 7.1: Subcellular localization of NCU05950 N-terminal deletion mutants. (A) The GFP fusion protein of NCU05950 expressed from the *cgc-1* promoter was modified by deleting either 1, 7 or 10 amino acids after the initial methionine at the N-terminus (indicated as Δ1, Δ7 and Δ10). GFP localization was observed in living hyphae by confocal microscopy. Upper panels show young hyphae. Lower panels show older hyphae with mature round vacuoles. Control = full-length NCU05950. Scale bar = 5 μ m. (B) Subcellular localization of GFP fusion proteins driven by the native promoter. The GFP fusion protein of NCU05950 was expressed from the NCU05950 promoter. N-terminal deletions and microscopy as for (A). Older hyphae are shown. Control = full-length NCU05950. Scale bar = 5 μ m.

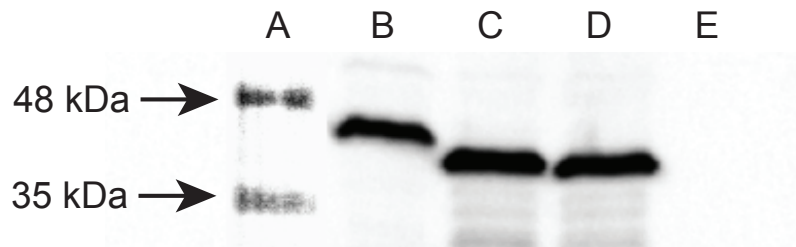


Figure 7.2: Expression of GFP tagged N-terminal deletion constructs. A Western blot demonstrating that the GFP tagged proteins with N-terminal 7- and 10-amino acid deletions expressed from the *ccg-1* promoter were stably expressed and produced the expected size bands (NCU05950::GFP - 44.6 kDa , $\Delta 7$ -NCU05950::GFP - 43.91 kDa & $\Delta 10$ -NCU05950::GFP - 43.65 kDa). A. Molecular weight marker; B. NCU05950::GFP ; C. $\Delta 7$ -NCU05950::GFP ; D. $\Delta 10$ -NCU05950::GFP ; E. Negative control.

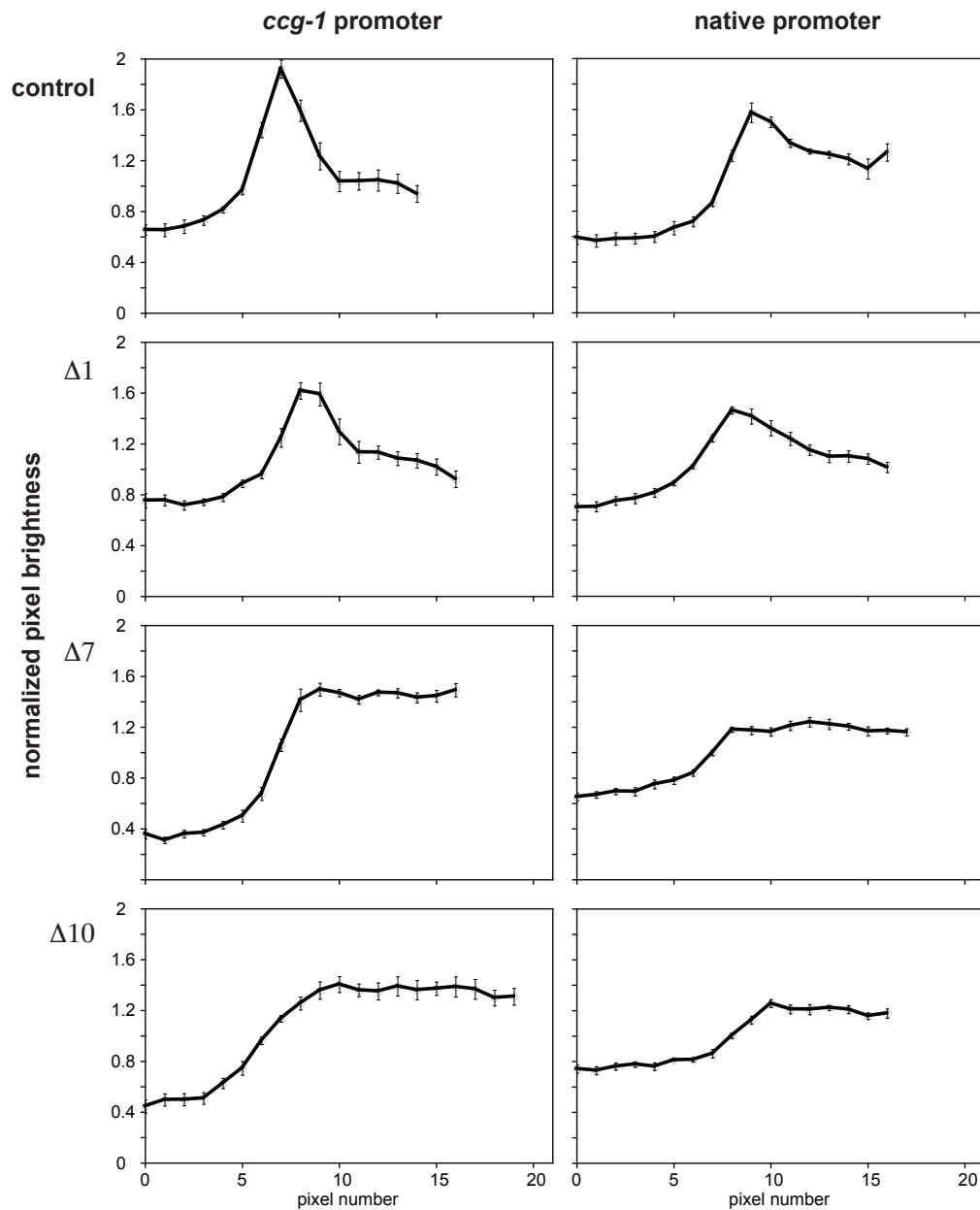


Figure 7.3: Localization of NCU05950-GFP near vacuolar membranes.

Images related to Figure 7.1 were analyzed by quantitating pixel brightness across transects drawn from the interior of vacuoles across the vacuolar

membrane into the cytosol. Pixel number zero is in the interior of the vacuole. Scale: 10 pixels = 4 μ m. Six vacuoles per strain were quantitated. Error bars are mean $\pm$ SEM (N=6). Left column: NCU05950-GFP driven by the *ccg-1* promoter. Right column: NCU05950-GFP driven by the native promoter. Top row: full-length NCU05950 gene fused to GFP (control). Rows 2, 3 and 4: N-terminal deletions of NCU05950 fused to GFP, deleting 1, 7 or 10 amino acids. (Data collection and analysis were done by Dr. Patricia Lakin-Thomas.)

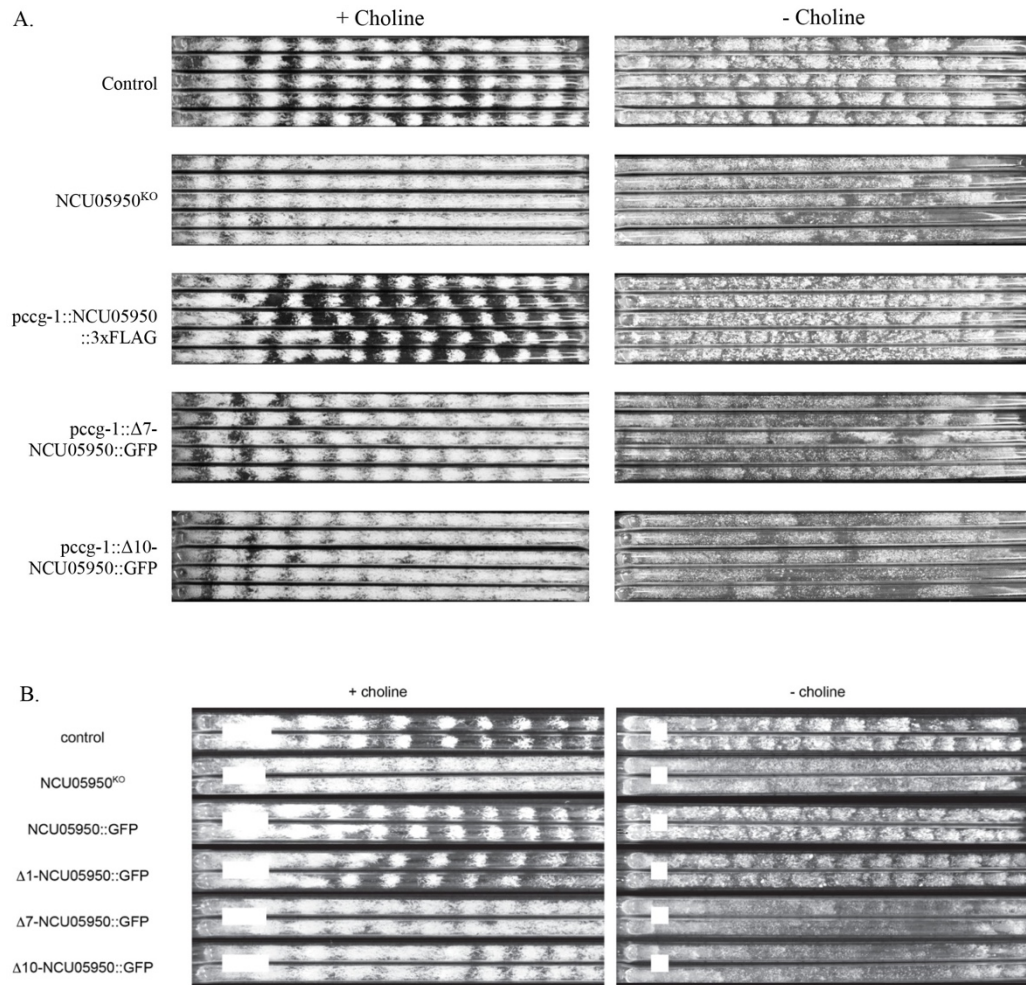


Figure 7.4: Rescue of the phenotype of the NCU05950 deletion mutant by the NCU05950-GFP.

All strains, including control, have the *csp-1*; *ras^{bd}* *chol-1* genetic background. All strains except the control also carry the NCU05950 deletion allele (NCU05950^{KO}). GFP-tagged fusion genes were inserted at the *his-3* locus of a NCU05950^{KO} strain. Strains were grown with (+, left panel) or without (-, right panel) 100 μM choline in the medium. Growth is from left to right. Periods and growth rates are reported in Table 7.1. (A) The fusion genes were expressed

from the high expression *ccg-I* promoter. $\Delta 7$ and $\Delta 10$: The GFP fusion protein of NCU05950 expressed from the *ccg-I* promoter was modified by deleting either 7 or 10 amino acids after the initial methionine at the N-terminus. Five representative replicate tubes for each condition are shown. As a control a strain expressing 3xFLAG fusion protein of NCU5950 from the *ccg-I* promoter was used. (B) The fusion genes were expressed from the native NCU05950 promoter. $\Delta 1$, $\Delta 7$ and $\Delta 10$: The GFP fusion protein of NCU05950 expressed from the native NCU05950 promoter was modified by deleting either 1, 7 or 10 amino acids after the initial methionine at the N-terminus. Two representative replicate tubes for each condition are shown. White bars indicate average growth in 24 hours.

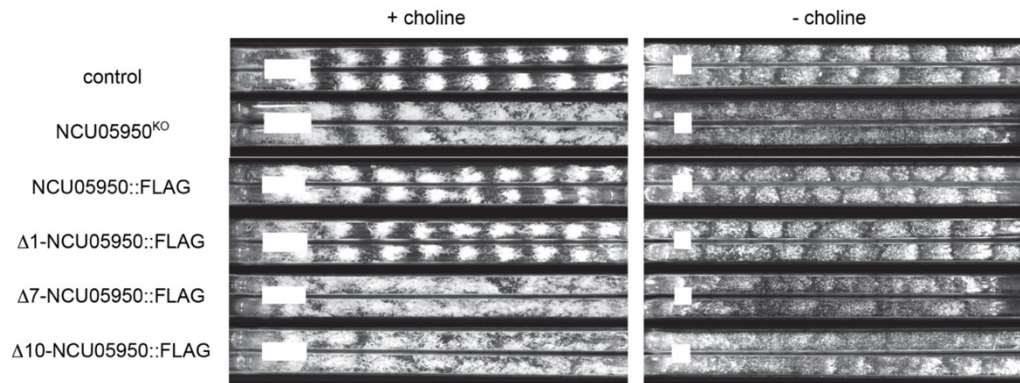


Figure 7.5: Rescue of the phenotype of the NCU05950 deletion mutant by the NCU05950-3xFLAG. All strains, including control, have the *csp-1*; *ras^{bd}* *chol-1* genetic background. All strains except the control also carry the NCU05950 deletion allele (NCU05950^{KO}). 3xFLAG-tagged fusion genes were inserted at the *his-3* locus of a NCU05950^{KO} strain. The fusion genes were expressed from the native NCU05950 promoter. Δ1, Δ7 and Δ10: The 3xFLAG fusion protein of NCU05950 expressed from the native NCU05950 promoter was modified by deleting either 1, 7 or 10 amino acids after the initial methionine at the N-terminus. Strains were grown with (+, left panel) or without (-, right panel) 100 μM choline in the medium. Two representative replicate tubes for each condition are shown. Growth is from left to right. White bars indicate average growth in 24 hours. Periods and growth rates are reported in Table 7.2.

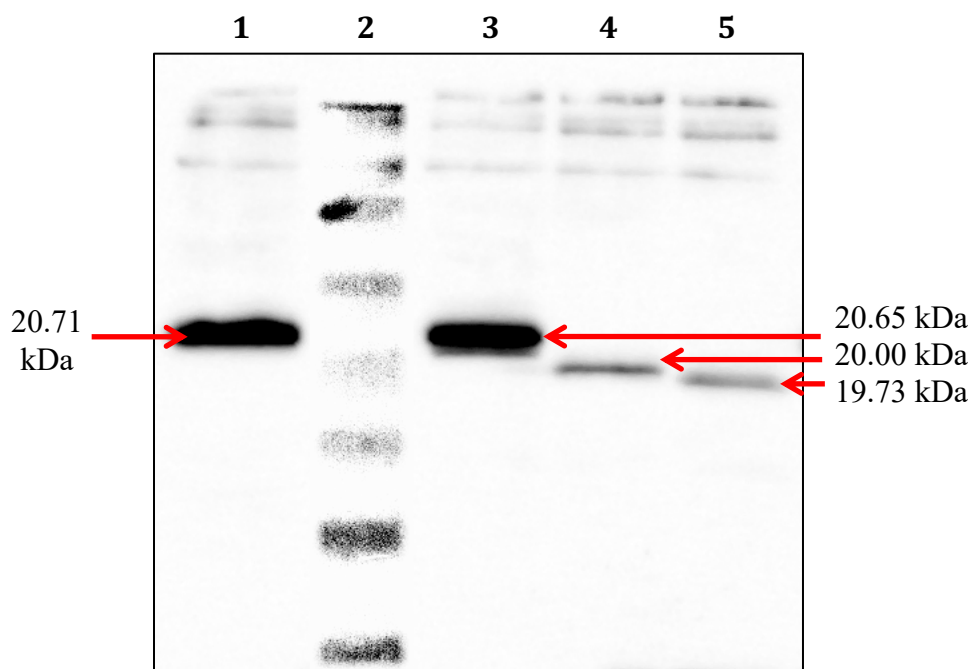


Figure 7.6: Expression of FLAG tagged defective NCU05950 protein.

SG#444, defective NCU05950::3xFLAG (Native promoter:: $\Delta 1$ NCU05950::3xFLAG, Native promoter:: $\Delta 7$ NCU05950::3xFLAG & Native promoter:: $\Delta 10$ NCU05950::3xFLAG) strains were grown on high choline to repair the *chol-1* defect. Cultures were grown in LL for 2 days at 25 °C and protein extracts were assayed for NCU05950-FLAG protein by immunoblotting.

Lane No. 1: SG#444 (NCU05950::3xFLAG $\rightarrow$ 20.71kDa)

Lane No. 2: Colored protein marker

Lane No. 3: Native promoter:: $\Delta 1$ NCU05950::3xFLAG (20.65 kDa)

Lane No. 4: Native promoter:: $\Delta 7$ NCU05950::3xFLAG (20.00 kDa)

Lane No. 5: Native promoter:: $\Delta 10$ NCU05950::3xFLAG (19.73 kDa)

CHAPTER 08: Proteins interacting with VTA.

8.1: Introduction

Studying the proteins interacting with the VTA will contribute to understanding the function of the VTA. Co-Immunoprecipitation (Co-IP) is a powerful technique used to investigate physiologically relevant protein–protein interactions and is one of the most widely used techniques in modern cell biology. This technique uses an antibody for a specific target protein bound to beads, to indirectly identify interacting proteins within a protein complex.

NCU05950 protein is homologous to human p18/LAMTOR 1 and yeast EGO1/Meh1p/Gse2p. Therefore, we can predict the interacting partners as well as the function of NCU05950 protein in the context of the TOR signaling pathway. The TOR signaling pathway has not been studied extensively in *Neurospora crassa* and only very little is known about this pathway in other filamentous fungi, which includes the identification of rapamycin binding proteins (Solscheid and Tropschug 2000; Pinto *et al.*, 2008), growth defects in knockouts of TOR and its downstream targets in *Neurospora crassa* (Park *et al.*, 2011) and TOR involvement in development as well as virulence in *Fusarium graminearum* (Yu *et al.*, 2014). Therefore, identification of NCU05950 protein binding partners may lead to predicting the components of TOR signaling pathway and discovering the targets as well as role of TOR signaling pathway in *Neurospora crassa*. Hence, these results can be used to define a new signaling pathway in *Neurospora crassa*. NCU05950 protein may also interact with some other proteins which are involved in maintaining circadian rhythms in *Neurospora crassa*. Pulling down the protein complexes in which NCU05950 protein participates by co-immunoprecipitation and identifying the proteins that are interacting with NCU05950 protein by mass

spectrometry (MS) is important to understand the function of NCU05950 protein.

Since no antibody for NCU05950 gene product is available, *Neurospora crassa* strains with NCU05950 gene product tagged with FLAG epitope were used for Co-IP. The *Neurospora crassa* expression vector constructed by Honda and Selker (2009) containing the FLAG epitope (pCCG::C-Gly::3XFLAG) was used to make the strains expressing NCU05950 gene product tagged with FLAG epitope as described in Chapter 6. The strains expressing FLAG tagged NCU05950 protein from its native promoter as well as the strains expressing FLAG tagged NCU05950 protein from high expression promoter *pccg-1* were selected for co-IP and MS analysis.

8.2: Methods

8.2.1: *Neurospora crassa* vacuolar membrane extraction

Vacuolar membrane extraction was performed as described by Bowman and Bowman 1988. *Neurospora crassa* cultures for vacuolar membrane extraction were initiated by inoculating spores of SG#444 (*csp-1 his-3⁺:: Native promoter* -NCU05950::Gly::3xFLAG; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*) into a stock tube containing 3 mL of 1xVogel's salts, 2% glucose, 100 μ M choline and 2% agar and allowed to grow for 10 days at room temperature. Spores and mycelia of the entire tube were transferred to 500 mL flask containing 100 mL of Vogel's liquid media with 2% glucose and 100 μ M choline. These cultures were incubated at 30°C under LL for 48 hours with shaking at 150 r.p.m.

Mycelia were harvested by transferring the contents of the culture flask onto vacuum filtration system with Whatman No.1 filter papers. Mycelia were washed 3 times with distilled water and washed with acetone to remove water.

Mycelial samples were then quickly transferred to pre-weighed 50 mL falcon tubes and the tube with mycelia was weighed and immediately frozen in liquid nitrogen. Frozen samples were stored at -80°C until vacuolar membrane extraction was performed.

70 mL of vacuolar preparation medium (VPM) was mixed with 0.7 mL of 100 mM Na₂ATP and 7 µL of 20mg/mL Chymostatin and chilled on ice. The mortar and pestle were rinsed with VPM and chilled on ice. Pre-chilled 250 mL centrifuge bottles were filled 3/4 with VPM (allowing room for cells) and placed in ice. Just prior to grinding the cultures 2mL of 100 mM Na₂ATP, 2mL of 100 mM PMSF and 20 µL Chymostatin were added to VPM in 250 mL centrifuge bottle.

Liquid nitrogen was poured into pre-rinsed mortar to cool the mortar and pestle. The frozen fungus sample was transferred into the mortar and ground into powder. Powdered fungus was transferred into the 250 mL centrifuge bottle containing VPM, Na₂ATP, PMSF, Chymostatin and vortexed for 20 seconds. Mortar and pestle were rinsed with VPM, Na₂ATP, Chymostatin solution and the rinsate was added to the 250 mL centrifuge bottle.

250 mL centrifuge bottles were centrifuged at 4°C at 2500 rpm (approx. 1000x g in the JA 14 rotor in Beckman centrifuge) for 10 minutes. The pellet (P1) contains cell wall, nuclei and cell debris. The supernatant (S1) was poured off into 2 pre-chilled 250 mL centrifuge bottles. Samples were centrifuged at 4°C at 10000 rpm (approx. 15000x g) for 30 minutes. The supernatant (S2) can be used to isolate plasma membrane and other light membranes. The soft and runny pellet (P2) contains mitochondria and vacuoles. The 250 mL centrifuge bottles were placed on ice and any extra supernatant and loose mitochondrial slurry was suctioned off. The pellet was gently re-suspended with 0.5 mL of VPM - Na₂ATP - Chymostatin. The re-suspended pellets were pooled into a

chilled glass Dounce homogenizing vessel. Each bottle was rinsed with 0.25 mL of VPM - Na₂ATP - Chymostatin and the total volume of the re-suspended pellets was brought to 4 mL. The re-suspended pellets were homogenized by hand with 3-4 strokes of the pestle. The homogenate was layered onto two sucrose step gradients (Appendix II) to separate mitochondria and vacuoles and centrifuged at 4°C at 19000rpm (approx. 46000x g) for 60 minutes.

The supernatant (S3) was removed from the vacuolar pellet (P3). The vacuoles were lysed by re-suspending each pellet in 0.5 mL ECA (Appendix II) - PMSF (13 mL ECA & 0.13mL PMSF).

The re-suspended pellets were pooled into a chilled glass-homogenizing vessel. Each bottle was rinsed with 0.5 mL of ECA and the total volume of the re-suspended pellets was brought to 6.0mL. The Teflon homogenizer pestle (3-4 smooth passes) was used to blend the mixture and it was distributed into 2 pre-chilled TLA 100.3 rotor compatible 5 mL centrifuge tubes (3mL per tube). Tubes were centrifuged at 4°C using TLA 100.3 rotor in Beckman TLA 100 ultracentrifuge, for 5 min at 20000rpm (approx. 20000x g). The supernatant (S4) was transferred to a new pre-chilled TLA 100.3 rotor compatible 5 mL centrifuge tube (The pellets P4 contain organelles with Woronin bodies and any remaining mitochondria and cell wall). The supernatant (S4) was centrifuged at 4°C using TLA 100.3 rotor in Beckman TLA 100 ultracentrifuge for 10 min at 60000rpm (approx. 195000x g).

The supernatant (S5), which contains vacuolar contents, was removed and the pellet (P5), which contains vacuolar membranes, were re-suspended in 3mL ECA total. Tubes were centrifuged at 4°C using TLA 100.3 rotor in Beckman TLA 100 ultracentrifuge for 10 min at 55000rpm (approx. 164000x g). The supernatant (S6) was removed and the vacuolar membrane pellet (P6) was re-suspended in 150-300 µL ECA. Re-suspended P6 pellet was transferred

to a small homogenizer, mixed and distributed into aliquots of about 50 µL per tube plus a 30 µL aliquot for initial assays for protein. All the samples were frozen in liquid nitrogen and stored at -80°C.

8.2.2: Immuno-precipitation (IP) & Mass-spectrometry analysis

Neurospora crassa cultures for immuno-precipitation (IP) were initiated by inoculating spores of SG#444 (*csp-1 his-3⁺:: Native promoter - NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*), the positive control strain SG # 588 (*csp-1 his-3⁺:: Native promoter - NCU07839::Gly::3xFLAG; chol-1 ras^{bd}*) and the negative control (un-tagged wild type strain) SG# 26 (*csp-1; chol-1 ras^{bd}; mat a*) into a stock tube containing 3 mL of 1xVogel's salts, 2% glucose, 100 µM choline and 2% agar and allowed to grow for 7 days at room temperature. Spores and mycelia of the entire tube were transferred to 500 mL flask containing 100 mL of Vogel's liquid media with 2% glucose and 100 µM choline. These cultures were incubated at 25°C under LL for 24 hours with shaking at 150 r.p.m.

Mycelia were harvested by transferring the contents of the culture flask into a vacuum filtration system with Whatman No.1 filter papers. Mycelia were washed 3 times with distilled water and in PBS (Appendix II). Mycelial samples were then quickly transferred to pre-weighed 50 mL conical tubes. The tube with mycelia was weighed and immediately frozen in liquid nitrogen. Frozen samples were stored at -80°C until protein extraction was performed.

The mortar and pestle were rinsed with ice-cold IP lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.02% (v/v) NP40, 1 mM EDTA, 1 mg/mL leupeptin, 1 mg/mL pepstatin A and 1 mM PMSF – Appendix II) and placed on ice. Liquid nitrogen was poured into pre-rinsed mortar to cool the mortar and pestle. The frozen fungus sample was transferred into the mortar

and ground into powder. Powdered fungus was transferred into several pre-cooled 1.5 mL screw capped tubes containing 500 μ L of IP lysis buffer. Tubes were inverted for 20 seconds to mix and incubated for 5 minutes at 4°C. Cell debris was pelleted by centrifuging at 4°C for 10 minutes at 12000xg. Supernatants were collected into a 5 mL tube and stored at -80°C as IP extracts.

The cell debris pellets were re-suspended in an equal volume of SDS protein extraction buffer (Appendix II) and boiled for 5 minutes, vortexed for 20 seconds and immediately put on ice for 5 minutes. Tubes were centrifuged at 12000xg for 10 minutes at 25 °C. The supernatants were kept as insoluble protein content, which was used to assess the extraction efficiency. Concentrations of the samples were measured using BioRad DC protein assay method.

Sepharose 4B beads (Sigma-Aldrich) were used to pre-clear the cell lysates as this significantly reduces non-specific interactions. 50 μ L of Sepharose 4B beads in a 5 mL tube were washed 5 times with 1 mL of IP lysis buffer by centrifuging at 500xg at 4°C for 1 minute each time. IP extract with 10 mg of total protein in 5 mL total volume was added to 5 mL tube with 50 μ L of pre-washed Sepharose 4B beads and incubated for 1 hour at 4°C with end-over-end agitation. Centrifuging at 500xg at 4°C for 1 minute pelleted the Sepharose 4B beads and the cleared supernatant was recovered.

10 μ L of Anti-FLAG M2 Affinity Gel beads (Sigma – Aldrich) in a 5 mL tube were washed two times with 1 mL of IP lysis buffer by centrifuging at 500xg at 4°C for 1 minute each time. Pre-washed Anti-FLAG M2 Affinity Gel beads were then incubated with 1 mL of 0.1 M glycine-HCl at pH 3.5 for 10 minutes at room temperature and washed 3 more times with 1 mL of IP lysis buffer by centrifuging at 500xg at 4°C for 1 minute each time.

Pre-cleared cell lysate was added to the 5 mL tube containing pre-washed Anti-FLAG M2 Affinity Gel beads and incubated overnight at 4°C with gentle end-over-end agitation at about 12 rpm. Anti-FLAG M2 Affinity Gel beads were pelleted by centrifuging at 750xg at 4°C for 1 minute and supernatant was removed. An aliquot of supernatant of post immuno-precipitation was kept to examine capture efficiency. Pelleted Anti-FLAG M2 Affinity Gel beads were washed 3 times with IP lysis buffer, and then 2 times with FLAG-rinsing buffer (Appendix II) by centrifuging at 500xg at 4°C for 1 minute each time. FLAG rinsing buffer helps to remove trace amounts of NP-40 detergent that would interfere with Mass-spectrometry analysis.

Washed beads were stored at -80°C and frozen beads were sent to SPARC BioCentre (SickKids Proteomics, Analytics, Robotics & Chemical Biology Centre, Hospital for Sick Children, Totonto, Canada) for sample preparation and Mass-spectrometry analysis. The results of Mass-spectrometry analysis were received as a Scaffold file (Scaffold 4.8.1 - Proteome Software Inc).

8.2.2.1: Immuno-precipitation of the vacuolar membrane fraction

Immuno-precipitation was performed with 1 mg of protein of the vacuolar membrane fraction from SG#444 (*csp-1 his-3⁺*:: Native promoter - NCU05950::Gly::3x FLAG ; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*) and total cell lysate of SG#444 as well as SG#26 prepared using IP lysis buffer and 30 µL Anti-FLAG M2 Affinity Gel beads (Sigma – Aldrich). The beads were washed with IP lysis buffer and proteins were eluted from beads using SDS protein extraction buffer. Eluted proteins were separated by SDS-PAGE using a 15 % polyacrylamide gel.

8.2.2.2: Optimizing the co-immunoprecipitation protocol

Cultures of SG#444 (*csp-1 his-3⁺*:: Native promoter -NCU05950:: Gly:: 3xFLAG ; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*) and SG#26 (*csp-1*; *chol-1 ras^{bd}*; *mat a*) were grown in 100 mL Vogel's minimal media with 2% glucose at 25°C. After 24 hours tissues were harvested by filtration, washed with PBS (10 mM phosphate pH 7.4, 137 mM NaCl and 2.7 mM KCl), dried on filter paper, frozen in liquid N₂ and stored at -80°C.

Protein lysates were prepared by grinding the frozen fungus in liquid N₂ and suspending the powdered fungus in ice cold IP lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.02% NP40, 1mM EDTA, 1 µg/mL leupeptin, 1 µg/mL pepstatin A and 1 mM PMSF). After centrifugation at 12000 rpm for 10 minutes at 4 °C, supernatants aliquots were transferred to microfuge tubes and stored at -80°C.

Cell debris containing pellets were re-dissolved in ice-cold SDS protein extraction buffer (50 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA-Na₂, 0.1 M DTT and 1mM PMSF) at a ratio of 1:1 (buffer: mycelia pellet). Vortexed samples were immediately heated to 100°C for 5 min and then cooled on ice. Samples were then centrifuged at 25°C at 12000xg for 5 minutes and supernatants were stored -80°C.

BioRad protein assay was conducted on both cell extract and pellet samples to calculate the protein concentration. For Western blot analysis, 20 µg and 40 µg of protein from each sample was loaded on 15% polyacrylamide gel. Bromophenol blue (final concentration 0.1%) was added and the samples were boiled again for 5 minutes before loading on the gel. After electrophoresis, Western blotting and immunodetection for FLAG tag was conducted with primary antibody (monoclonal Anti-FLAG M2 - diluted 1:10000 in freshly diluted 1xblocking buffer) and secondary antibody (goat anti-mouse IgG-

horseradish peroxidase conjugate - diluted 1:50000 in freshly diluted 1xblocking buffer).

After washing the membrane with TBST, chemiluminescent detection reagent (1:1 - Luminol reagent: Peroxide solution) was added and incubated for 5 minutes. The light produced by chemiluminescent substrate was recorded with MicroChemi imager. Protein loading was determined by gel electrophoresis and Coomassie staining of gels loaded with equal volumes of lysate from each sample.

8.2.2.3: Preliminary experiment for the immuno-precipitation assay

As a preliminary experiment for the immuno-precipitation (IP) assay, pre-clearing of the cell extracts (to eliminate non-specific binding of proteins) was done by incubating 1 mg of total cell extract with 50 μ L pre-washed, packed Sepharose beads for 1 hour at 4°C with end-over-end agitation. Supernatants were recovered by centrifugation at 750g for 1 minute at 4°C. 10 μ L of pre-washed Anti-FLAG M2 beads (A2220, Sigma) was added to pre-cleared cell extracts and incubated overnight at 4°C with end-over-end agitation. (20 μ L aliquot from the pre-cleared lysate was kept as Input of IP to use for SDS-PAGE).

This experiment was performed with the following variations: No pre-clearing with Sepharose beads and 3 hours with Anti-FLAG M2, No pre-clearing with Sepharose beads and overnight with Anti-FLAG M2 beads, 1 hour with Sepharose beads and 3 hours with Anti-FLAG M2 beads, 1 hour with Sepharose beads and overnight with Anti-FLAG M2 beads. The beads were washed three times with lysis buffer and two times with FLAG-rinsing buffer (50 mM NH_4HCO_3 pH 8, 75 mM KCl, 2mM EDTA) and elution was done with 20 μ L Leammli buffer at 95 °C for 5 minutes. The Anti-FLAG M2 affinity

beads were pelleted down by centrifugation at 750g for 1 minute at 25°C and the supernatants were kept as IP-pull down. Samples were loaded on 12.5% SDS-PAGE and western blotting was performed.

8.2.3: Construction of GTR2::GFP expressing strains

The candidate Gtr2 gene (NCU00376) was cloned to the *his-3* locus targeting *Neurospora crassa* expression vector pCCG::C-Gly::GFP→8509bp (Figure 2.2) , which can be used to detect the protein under the control of high-expression *ccg-1* promoter. To construct a highly expressed fusion protein of Gtr2 and GFP, the candidate gene without regulatory sequences (2632bp) was isolated from *Neurospora crassa* genomic DNA by PCR using GTR2XbaIF and GTR2PacIR primers (Appendix IV) and inserted into the XbaI-PacI site of the *his-3* locus targeting pCCG::C-Gly::GFP plasmid between the highly expressed *ccg-1* promoter and the 10X Gly linker upstream of the GFP sequence.

The plasmid and isolated gene fragments were digested with XbaI-PacI restriction enzymes and were gel purified. Ligation mixtures were transformed to chemically competent *E.coli*. Several colonies were picked and colony PCR was carried out to analyze the transformed bacterial cells using the primers used to isolate the gene. Several colonies gave the desired bands and those colonies were selected for further testing. Plasmid DNA isolated from PCR positive colonies was digested with different restriction enzymes (XbaI-PacI and BamHI-HindIII) to confirm the presence of the inserted candidate gene on the recombinant plasmids. Recombinant plasmids with *ccg-1* promoter, NCU00376 gene and GFP tag were named as pGTR-GFP (pGG 1-10).

After confirming the cloning by sequencing, pGG18 recombinant plasmids was selected for transformation. pGG18 plasmid DNA was

transformed to the spores of NCU05950 knockout strain (carrying the deletion of NCU05950) SG #359 (*csp-1 his-3; chol-1 ras^{bd}; NCU05950^{KO} mat a*) and NCU05950 wild type strain SG#274 (*csp-1 his-3; chol-1 ras^{bd} mat a*) by electroporation. Fungal strains (SG #359 and SG#274) grown on histidine and choline containing media was used to harvest conidiospores for transformation. Cloned plasmid (10-15 µg/ 15 µL) was linearized with NdeI and added to 40 µL of washed conidia. Conidiospores were transformed with the plasmid DNA by electroporation and plated on Top agar media without histidine supplement to select *his-3⁺* transformants. Colonies observed after 3-5 days were transferred to tubes containing minimal medium with choline and the transformants that showed healthy growth with bright orange spores were further analyzed.

Spore PCR was carried out with different combinations of *his-3* locus specific, NCU00376 gene specific and GFP tag specific primers. Some transformants gave expected bands and it indicated successful transformation as well as integration at the *his-3* locus.

Strains expressing GTR2::GFP fusion protein in NCU05950 knockout background were named as NCU05950^{KO} GTR2-GFP (KOGG) strains and had the genotype; *csp-1 his-3⁺:: pccg-1::NCU00376::C-Gly::GFP; chol-1 ras^{bd} :: NCU05950^{KO} (mat a)*. Silica stocks were prepared from positive heterokaryotic transformants of NCU05950^W GTR2-GFP (WGG) strains (SG# 610-612). Strains expressing GTR2::GFP fusion protein in NCU05950 wild type background were named as NCU05950^W GTR2-GFP strains and had the genotype; *csp-1 his-3⁺:: pccg-1::NCU00376::C-Gly::GFP; chol-1 ras^{bd} (mat a)*. Silica stocks were prepared from positive heterokaryotic transformants of NCU05950^W GTR2-GFP strains (SG# 613-615). Details of strain identification names used during the experiments and corresponding silica stock numbers were mentioned in Appendix I.

8.3: Results & Discussion

8.3.1: Vacuolar membrane extraction is not necessary

As mentioned in Chapter 5 and Chapter 7, NCU05950 gene product is a LAMTOR-like protein that localizes to the vacuolar membrane, probably by acyl group anchors. High stringency buffers that contain anionic detergents such as SDS are not used for co-IP experiments, as they dissociate protein complexes. Low stringency buffers that contain non-ionic detergent NP-40 are used, as it allows protein association to remain intact. However, this detergent may not remove the protein from vacuolar membrane. Therefore, the abundance of this protein may be low in the cell lysate compared to total protein amount. To eliminate this problem, vacuolar membrane extraction was performed.

NCU05950-FLAG fusion protein expressed from NCU05950 native promoter offered satisfactory signals for immuno-detection of Western blots with anti-FLAG antibodies compare to the NCU05950-FLAG fusion protein expressed from *csg-1* promoter (Chapter 6) and also it is more physiologically relevant to use a strain expressing the FLAG-tagged protein driven by its native promoter. Therefore, only SG#444 (*csp-1 his-3⁺::* Native promoter - NCU05950::*Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*) was used for co-IP experiments and as the negative control an untagged wild type strain (SG#26 - *csp-1: chol-1 ras-1^{bd} mat a*). As the positive FLAG expressing control SG#588 (*csp-1 his-3⁺::* Native promoter - NCU07839::*Gly::3xFLAG; chol-1 ras^{bd}; mat a*) was used.

Vacuolar membrane extraction was performed as described in section 8.2.1 with SG#444 (*csp-1 his-3⁺::* Native promoter -NCU05950::*Gly:: 3xFLAG ; chol-1 ras^{bd}; NCU05950^{KO} mat a*) and Western blotting was done with every fraction obtained at each step along with total protein extracted from cell

lysate using IP lysis buffer. As shown in figure 8.1, IP lysis buffer can extract a sufficient amount of the target fusion protein (Native promoter - NCU05950::Gly::3x FLAG).

Immuno-precipitation (Section 8.2.2 & 8.2.2.1) was performed with 1 mg of protein of the vacuolar membrane fraction from SG#444 (*csp-1 his-3⁺::* Native promoter -NCU05950::Gly::3x FLAG ; *chol-1 ras^{bd}*; NCU05950^{KO} *mat a*) and total cell lysate of SG#444 as well as SG#26 prepared using IP lysis buffer and 30 µL Anti-FLAG M2 Affinity Gel beads. Western blotting and immunodetection using anti-FLAG antibodies showed that there was not a significant difference between vacuolar membrane fraction and total cell extract (Figure 8.2). Therefore, all the experiments were performed with total cell extracts prepared using IP lysis buffer.

8.3.2: Optimizing the co-immunoprecipitation protocol

In order to discover the effectiveness of IP lysis buffer, protein lysates were prepared from cultures of SG#444 and SG#26 using IP lysis buffer and cell debris containing pellets were re-dissolved in SDS protein extraction buffer. For Western blot analysis, 20 µg and 40 µg of protein from each sample were used.

As shown in figure 8.3, only SG#444, which express the FLAG tagged NCU05950 protein under its own promoter gave the band corresponding to NCU05950::3xFLAG protein which is 20.71kDa in size. Therefore, comparison of protein extraction with IP lysis buffer and SDS protein extraction buffer shows that IP lysis buffer is effective at extracting the FLAG - tagged protein.

As a preliminary experiment for the immuno-precipitation (IP) assay, pre-clearing of the cell extracts (to eliminate non-specific binding of proteins)

was done by incubating 1 mg of total cell extract with 50 μ L pre-washed, packed Sepharose beads. This experiment was performed with the following variations (Appendix XXX); No pre-clearing with Sepharose beads and 3 hours with Anti-FLAG M2, No pre-clearing with Sepharose beads and overnight with Anti-FLAG M2 beads, 1 hour with Sepharose beads and 3 hours with Anti-FLAG M2 beads, 1 hour with Sepharose beads and overnight with Anti-FLAG M2 beads). As shown in Appendix XXX, unconjugated sepharose beads did not reduce the abundance of NCU05950::3xFLAG protein in the IP-input. However, coomassie staining of the membrane did not display the band corresponding to NCU05950::3xFLAG.

Therefore, co-IP assay was performed with 10 mg of total cell extracts and 30 μ L of pre-washed Anti-FLAG M2 beads (A2220, Sigma) followed by Colloidal Coomassie G-250 staining (see section 2.14.7) of the gel. The stained gel showed the amount of proteins eluted from Anti-FLAG M2 affinity beads were noticeably less than the co-IP-input and several clear bands were observed. After confirming the presence of the band corresponding to NCU05950::3xFLAG by Colloidal Coomassie G-250 staining, co-IP samples were prepared with 10 mg of total cell extracts and 30 μ L of pre-washed Anti-FLAG M2 beads (A2220, Sigma) and sent for mass spectrometry analysis at the Hospital for Sick Children, Toronto.

Based on the results obtained at each round of experiment, Co-IP assay and MS analysis were carried out with variations described in Appendix XXXI to optimize the conditions. A sample from SG#26 (*csp-1*; *chol-1 ras^{bd}*; *mat a*) was used as the negative control (un-tagged wild type strain) and a sample from SG#588 (*csp-1 his-3⁺*:: *Native promoter-NCU07839*:: *Gly*::3xFLAG; *chol-1 ras^{bd}*) was used as the FLAG tagged positive control. Fewer bound proteins, higher spectral counts, enriched in test sample SG#444 are the criteria for

improving the method. The amount of Anti-FLAG M2 beads was reduced to 10 μ L to reduce non-specific binding of proteins and also to reduce the background. Incubation time with Anti-FLAG M2 beads was reduced to 2 hours. But the spectral count given by SG#444 (NCU05950::3XFLAG) decreased compared to the spectral count given when incubated overnight with Anti-FLAG M2 beads. Therefore, incubation overnight with Anti-FLAG M2 beads was used in later experiments. Furthermore, to remove any unbound antibodies, Anti-FLAG M2 beads were washed with glycine. All the incubations for Co-IP were performed in 5 mL tubes and total reaction was kept at 5 mL.

8.3.3: Proteins interacting with NCU05950 gene product

The proteins which were co-immunoprecipitated with NCU05950::3xFLAG protein on Anti-FLAG M2 beads were sent for Mass-spectrometry analysis. SG# 26 was used as the negative control and SG#588 was used as the FLAG tagged positive control. The results of Mass-spectrometry analysis were received as a Scaffold file (Scaffold 4.8.1 - Proteome Software Inc) and the proteins with 95% or more existence probability were chosen for analysis. Results of experiment trial number 9, 10 and 11 were compiled and proteins that appeared 2 or 3 times only in the SG#444 sample and proteins that had higher spectral counts were selected as candidate interacting partners for NCU05950 protein (Table 8.1).

As predicted, proteins homologous to yeast GTR1 and GTR2 were identified as candidate binding proteins of NCU05950 protein (Table 8.1). Furthermore, there were some potential NCU05950 binding partners that were ribosomal proteins and some were hypothetical or unnamed protein products.

8.3.4: NCU05950 recruits the small GTPase Gtr2 to the vacuoles

Co-immunoprecipitation analyses identified highly conserved proteins, the small Ras-related GTP binding proteins Gtr1(NCU01099) and Gtr2 (NCU00376) as proteins that interact with NCU05950 protein. The homologous yeast proteins (GTR1 and GTR2) form the heterotetrameric EGO complex which acts as an upstream activator of the TORC1 and the EGO complex is located at the vacuolar membrane (Evans *et al.*, 2014). The EGO complex is the potential functional homolog of the mammalian Ragulator – Rag complex (Ragulator and RagA/ B, RagC/D) which is localized to the surface of late endosomes/lysosomes.

To determine if GTR2 is localized to the vacuole in *Neurospora crassa* and if the vacuolar association of GTR2 is dependent on NCU05950, localization of GTR2::GFP was compared in a wild type and NCU05950 deletion mutant strain. In order to achieve this, candidate Gtr2 gene (NCU00376) was cloned to the *his-3* locus targeting *Neurospora crassa* expression vector pCCG::C-Gly::GFP→8509bp (Figure 2.2) , which can be used to detect the protein under the control of high-expression *cgc-1* promoter.

Restriction enzyme digestion and sequencing of recombinant plasmid (pGG18) with *cgc-1* promoter followed by NCU00376 gene coding sequence gave the expected results and confirmed the presence of the insert on the recombinant plasmid. Linearized plasmid was transformed into macrospores of NCU05950 knockout strain SG#359 as well as NCU05950 wild type strain SG#274. Successful integration at the *his-3* locus was confirmed by carrying out spore PCR with different combinations of *his-3* locus specific, NCU00376 gene specific and GFP tag specific primers (Appendix XXXII – A and Appendix XXXII – B). Transformants that gave expected bands indicate successful transformation as well as integration at the *his-3* locus.

Expression of fluorescence was monitored from live cell cultures grown on 3% agar media on slides, under fluorescence microscopy and confocal laser scanning microscope with the GFP filter (See section 2.17). Organelle GFP and/or RFP marker strains (Vacuoles: *vma-5* with GFP tag & *vam-3* with RFP tag, Mitochondria: *arg-4* with GFP tag, Golgi: *vps-52* with GFP tag, Endoplasmic reticulum: *nca-1* with GFP tag, nuclei: *hH1* with RFP tag) which were constructed by Bowman *et al.*, 2009 were compared to the GTR2-GFP strains. Strains expressing NCU05950-GFP fusion protein from both *ccg-1* as well as the endogenous promoter and untagged strains (SG#26 & SG#259) as negative controls were also used to compare to the test specimens (Details about all the strains are mentioned in Appendix I). Fluorescence expression was detected from strains expressing NCU00376-GFP fusion protein in both NCU05950 wild type background as well as NCU05950 knockout background. In NCU05950 wild type background, NCU00376::GFP fusion protein was detected on small vesicles that could be endosomes and inside the large old vacuoles (Figure 8.4) and in NCU05950 knockout background, NCU00376::GFP fusion protein was found to be diffusely localized in the cytosol, less on small vesicles and none in the large vacuoles (Figure 8.4). These results demonstrate that localization of Gtr2 (NCU00376) is dependent on NCU05950 and it suggest that NCU05950 is required to recruit Gtr2 to the vacuoles.

Table 8.1: List of proteins binding to NCU05950 protein.

NCU Number	Identified Proteins in <i>Neurospora crassa</i> OR74A	Accession Number	Molecular Weight	Total Spectrum counts	Name and Homologue
NCU00376	GTR/RAG family GTPase Gtr2	ESA43353.1 ESA43352.1 XP_011393826.1 KHE87845.1 (+1)	048 kDa or 044kDa	38	GTR2
NCU04811	Hypothetical protein NCU04811	EAA31201.1 (+2) KHE89013.1	033 kDa	22	
NCU01099	GTP-binding protein GTR1	EAA32475.1 CAD21343.1 (+3)	045 kDa	11	GTR1
NCU02075	Unnamed protein product	CBC46356.1 (+1) CAW36942.1 (+6)	065 kDa	9	
NCU02509	60S ribosomal protein L11	EAA36404.2 (+3) Q7RVN0.2	020 kDa	6	
NCU07830	40S ribosomal protein S14	CBD28188.1 (+3) EAA33715.1 CAA37766.2	016 kDa	8	

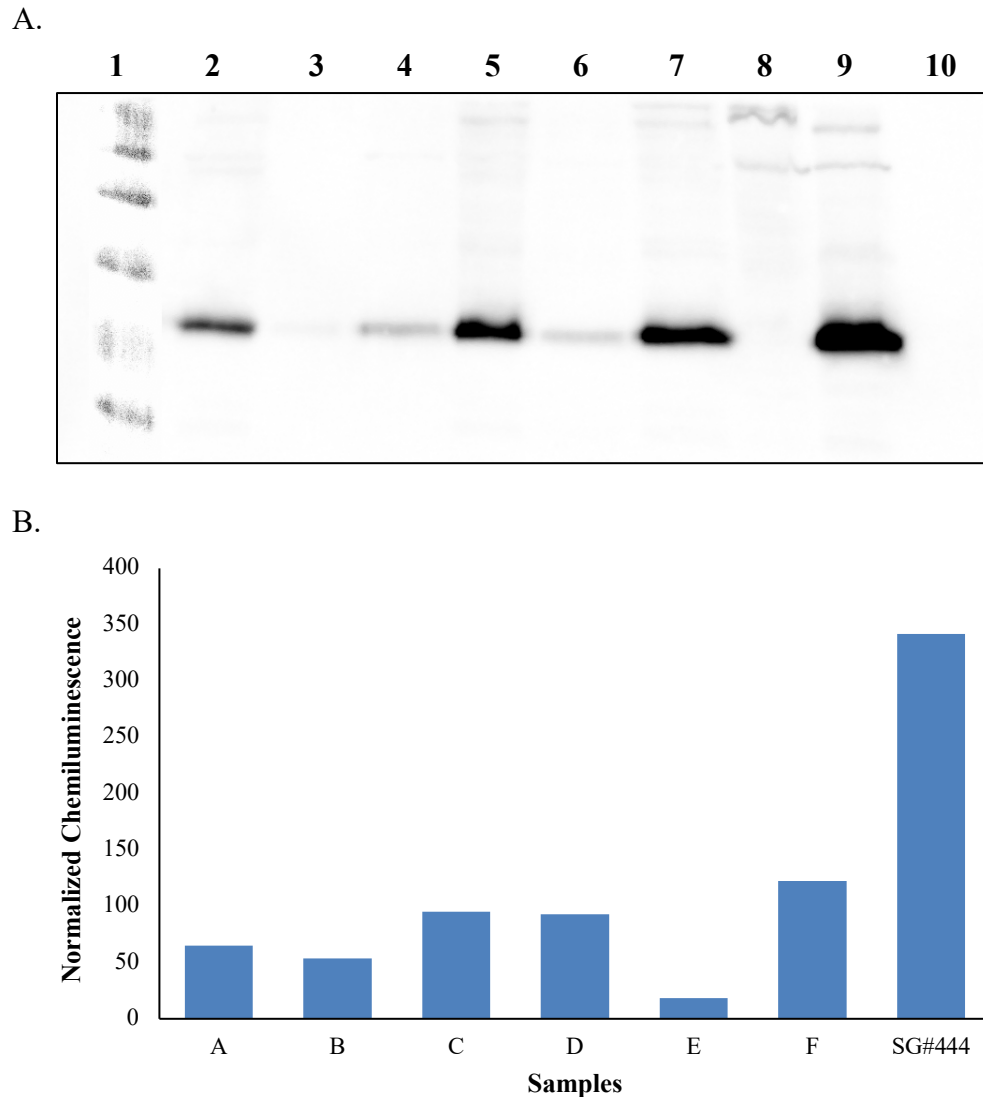


Figure 8.1: Western blot analysis of fractions collected during the vacuolar membrane preparation. (A) Western blot. (B) Quantification of Western blot on (A). Samples were collected during the vacuolar membrane extraction procedure from a culture (SG#444) expressing the NCU05950-3xFLAG protein from the native promoter. The genotype was *csp-1 his-3⁺:: Native promoter - NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950KO* mat *a*. 20 µg of

protein loaded on to 15% gel and Western blotting was carried out using anti-FLAG antibody.

Lane No. 1: Colored protein marker

Lane No. 2: Cell wall, nuclei pellet - 20 µg (A)

Lane No. 3: Plasma membrane and other membranes supernatant - 20 µg (B)

Lane No. 4: Supernatant from sucrose gradient - 20 µg (C)

Lane No. 5: Pellet containing organelles with Woronin bodies and any remaining mitochondria and cell wall - 20 µg (D)

Lane No. 6: Supernatant containing vacuolar contents - 20 µg (E)

Lane No. 7: Vacuolar membrane pellet - 20 µg (F)

Lane No. 8: SG#26 - total protein extracted with boiling SDS method - 20 µg

Lane No. 9: SG#444 - total protein extracted with boiling SDS method - 20 µg

Lane No. 10: Empty.

A. Cell wall, nuclei containing pellet of vacuolar membrane extraction procedure dissolved in IP buffer.

B. Plasma membrane and other light membranes containing supernatant of vacuolar membrane extraction procedure.

C. Supernatant from sucrose gradient used for vacuolar membrane extraction procedure.

D. Pellet containing organelles with Woronin bodies and any remaining mitochondria and cell wall of vacuolar membrane extraction procedure dissolved in IP buffer.

E. Supernatant containing vacuolar contents during vacuolar membrane extraction procedure.

F. Vacuolar membrane pellet of vacuolar membrane extraction procedure dissolved in ECA buffer. SG#444 - Total protein extracted using boiling SDS method.

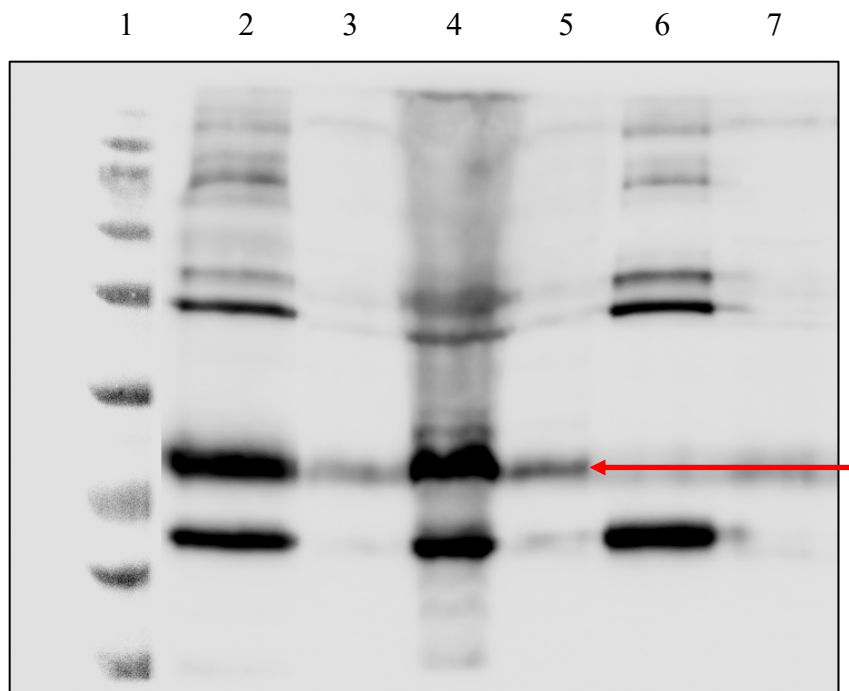


Figure 8.2: Western blot analysis of immunoprecipitates prepared from vacuolar membrane fractions. The arrow indicates the 20.71 kDa size VTA::3xFLAG protein.

Lane No. 1: Colored protein marker

Lane No. 2: SG#444 - Immunoprecipitate with 1 mg of total protein

Lane No. 3: SG#444 – Supernatant after incubating with Anti-FLAG M2 beads - 20 µg

Lane No. 4: SG#444 vacuolar membrane fraction - Immunoprecipitate with 1 mg of total protein

Lane No. 5: SG#444 vacuolar membrane fraction - Supernatant after incubating with Anti-FLAG M2 beads - 20 µg

Lane No. 6: SG#26 - Immunoprecipitate with 1 mg of total protein

Lane No. 7: SG#26 – Supernatant after incubating with Anti-FLAG M2 beads - 20 µg.

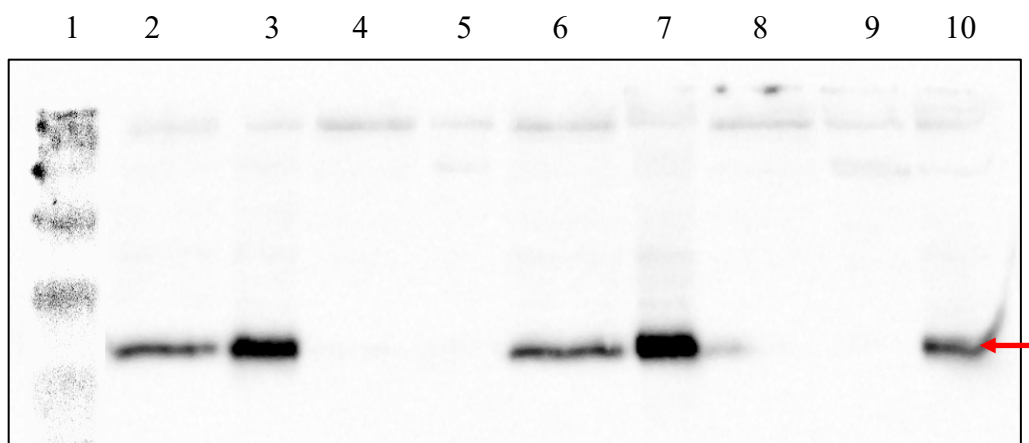


Figure 8.3: Analysis of cell extracts prepared for immunoprecipitation.

SG#444 (*csp-1 his-3⁺:: Native promoter -NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*) and SG#26 (*csp-1 ; chol-1 ras^{bd}*) strains were grown on high choline to repair the *chol-1* defect. The arrow indicates the 20.71 kDa size VTA::3xFLAG protein.

Lane No. 1: Colored protein marker

Lane No. 2: SG#444 - total cell extract 20 µg

Lane No. 3: SG#444 - extract from cell debris pellet 20 µg

Lane No. 4: SG#26 - total cell extract 20 µg

Lane No. 5: SG#26 - extract from cell debris pellet 20 µg

Lane No. 6: SG#444 - total cell extract 40 µg

Lane No. 7: SG#444 - extract from cell debris pellet 40 µg

Lane No. 8: SG#26 - total cell extract 40 µg

Lane No. 9: SG#26 - extract from cell debris pellet 40 µg

Lane No. 10: SG#444 – total protein extracted with boiling SDS method - 20 µg.

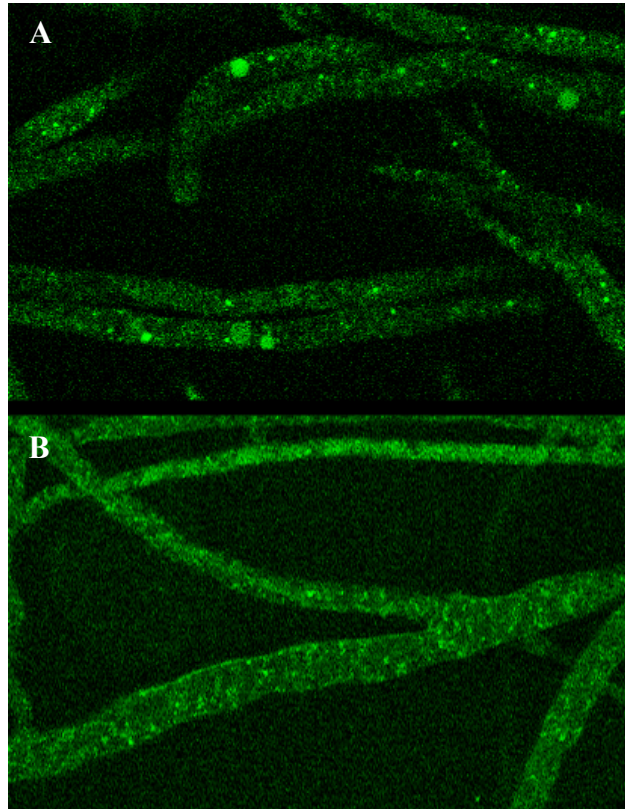


Figure 8.4: Subcellular localization of NUC00376 (GTR2) protein.

Neurospora crassa expressing NCU00376::GFP under the control of high expressing promoter *ccg-1* was analyzed by fluorescence microscopy. (A) In NCU05950 wild type background, NCU00376::GFP fusion protein was seen on small vesicles and inside the large old vacuoles. (B) In NCU05950 knockout background, NCU00376::GFP fusion protein diffusely localized in the cytosol.

CHAPTER 09: Contribution of the TOR signaling pathway to clock function

9.1: Introduction

Upstream regulators of yeast TORC1, which includes EGO complex (EGO1, EGO2, EGO3, GTR1 and GTR2) as well as FPR2, TORC1 (TOR1 or TOR2, KOG1, LST8 and TCO89), TORC2 (TOR2, AVO1, AVO2, AVO3 or TSC11, LST8 and BIT61) and TORC1 targets, such as Sch9 and Tap42 are considered as the main components of the yeast TOR signaling network (Loewith and Hall 2011; Powis K *et al.*, 2015). Apart from the above-mentioned components, Vam6, which has been identified as a guanine nucleotide exchange factor for Gtr1, is considered as an important element.

To determine whether the other proteins in the TOR pathway also influence rhythmicity in *Neurospora crassa*, homologous genes of yeast TOR pathway components were identified using the available literature and bioinformatics searches and their rhythmic phenotype was assayed.

9.2: Methods

9.2.1: Lab stock culture initiation and preparation of silica stocks

The strains received from FGSC were inoculated into stock tubes containing Vogel's minimum media with hygromycin (200 µg/mL) and incubated at room temperature for 10 days. Silica stocks were made for all the strains (FKR5^{KO} = FGSC#13000 – SG#508, TOR2^{KO} = FGSC#13432 - SG#509, GTR2^{KO} = FGSC#16041 - SG#510, VAM6^{KO} = FGSC#18870 - SG#511, PIK1a^{KO} = FGSC#19786 - SG#512, TAP42^{KO} = FGSC#21741 - SG#513, STK-10^{KO} = NCU03200 – SG#571).

9.2.2: Setting up genetic crosses

Usually *csp*⁺ gene knockout strains were used as the female parent. These strains were grown on slants of synthetic crossing medium (SCM) (Appendix II) with 100 µM choline as well as 200 µg/mL hygromycin in glass tubes at room temperature for 7-10 days or until protoperithecia were visible. SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) were used as the male parents and those were grown on slants of 1xVogel's salts with 2% glucose along with 100 µM choline for 7-10 days at room temperature. Fertilization, and spore picking were performed as described in section 2.4.2.

9.2.3: Analysis of progeny

Spores were picked as described in section 2.4.2 and allowed to germinate in baby tubes containing 1xVogel's salts with 2% glucose along with 100 µM choline for 7-10 days at room temperature.

9.2.3.1: Tube “tap test”

The “tap test” was performed on the baby tubes to visually check for *csp-1* and *csp*⁺ progeny as described in section 2.4.2. *csp*⁺ and non-germinating progeny were excluded from further testing.

9.2.3.2: Spore PCR to test the genotype of progeny strains

Spore PCR was conducted to test the genotype of progeny strains as described in section 2.12.2. Primers and the sizes of expected products are given in Table 9.1.

9.2.3.3: Race tube analysis

Progeny strains in the *csp-1* background were inoculated onto maltose-arginine medium both with and without 100 μ M choline in race tubes as described in section 2.3.1.1. along with some control strains (Table 9.2). After completing growth in the race tube, the linear growth rate and period (for the strains carrying *ras-1^{bd}* mutation) were calculated as previously described in (Lakin-Thomas, 1998) using the scanned race tube pictures with daily growth marks and Microsoft Excel program “MacTau” developed by Dr. Patricia Lakin-Thomas. Then the progeny strains were visually separated into *ras-1^{bd}* or *ras-1^{bd+}* phenotypes based on conidiation banding phenotype as well as slower growth determined by comparing growth fronts marked daily on the tubes. The *chol-1* mutation bearing strains were identified by comparing the growth rate on choline supplemented and choline deficient media. The *chol-1* progeny have slow growth on choline deficient media and rapid growth on choline supplemented media. The race tubes were further separated into groups based on visual comparisons to the controls, considering growth rate and PCR results.

9.3: Results & Discussion

In order to find homologous genes to yeast TOR pathway components in *Neurospora crassa*, amino acid sequences of proteins as well as nucleotide sequences of the genes involved in the yeast TOR signaling pathway were obtained from Saccharomyces Genome Database (SGD - <http://www.yeastgenome.org/>) (Appendix XXXIII). Using the information available in the *Neurospora crassa* genome database (FungiDB, <http://fungidb.org/fungidb/> ; Stajich, *et al.* 2012) and BLAST searches using the

yeast sequences, candidate homologous proteins in *Neurospora crassa* TOR signaling pathway were discovered (Table 9.3).

Very little is known about the TOR signaling pathway in *Neurospora crassa* and rapamycin binding proteins (FKBPs) have been identified in the fungus (Solscheid and Tropschug 2000; Pinto *et al.*, 2008). A screen of Ser/Thr kinase gene deletions in *Neurospora crassa* (Park *et al.*, 2011) identified a homolog of yeast TOR1/TOR2 by sequence similarity. The same screen identified a homolog of the yeast Sch9 gene, which is homologous to the TOR kinase substrate S6K in mammals (Park *et al.*, 2011).

The gene knockout strains for those homologous proteins were obtained from FGSC (McCluskey *et al.*, 2010), which contains gene knockout strains created by knocking out individual genes using a cassette containing Hygromycin B resistance gene (Colot *et al.*, 2006). Lab stock culture were initiated for the strains received from FGSC and silica stocks were made for all the strains.

In order to check the contribution of a candidate gene to clock function, race tube experiments should be performed to assess the rhythmic phenotype in gene knockout strains. Therefore, these deletion mutants were crossed to standard lab strains (Table 9.4) to get the same genetic background with other strains that contain the *csp-1*, *chol-1*, *ras^{bd}* and *frq¹⁰* mutations.

Genotypes of progeny were determined by tube tap test (for *csp-1*), spore PCR (knocked-out gene and *Frq* gene presence/absence) and race tube experiment (*ras^{bd}* and *chol-1*) (Figure 9.1). Progeny strains obtained from five different crosses (GTR2, TAP42, STK10, FKR5 and PIK1a) were analyzed.

9.3.1: Genetic cross with GTR2^{KO} (FGSC#16041)

A genetic cross was performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and GTR2^{KO} strains. 191 spores were picked and the “tap test” was performed on the baby tubes to visually check for *csp-1* and *csp+* progeny. Out of 191 spores, 85 spores were germinated, 43 (22.51%) were found to be *csp-1*, 42 (21.98%) were *csp+* and 106 (55.49%) did not germinate (Table 9.4).

Spore PCR analysis was conducted with *csp-1* progeny (1 to 43) to uncover presence or absence of knocked-out gene using GTR2F & GTR2R primers. Out of 43 tested progeny strains, 18 were GTR2^{KO} strains (Appendix XXXIV - A). GTR2^{KO} strains were tested for the presence or absence of *Frq* gene and out of 18 tested progeny strains, 6 were *Frq* wild type strains (Appendix XXXIV - B).

18 progeny strains with GTR2^{KO} allele in the *csp-1* background were inoculated onto maltose-arginine medium both with and without 100 μ M choline in race tubes as described in section 2.3.1.1. The progeny strains were separated into *ras-1^{bd}* or *ras-1^{bd+}* phenotypes and 9 were found to be *ras-1^{bd+}* and 9 were *ras-1^{bd}* (Figure 9.2 and Appendix XXXV - A). The race tubes were further separated into groups based on visual comparisons to the controls, considering growth rate and PCR results (Appendix XXXV - B). Progeny groups and number of progenies belonging to each group are given in Table 9.5. Periods and growth rates of all the tested strains are listed in Table 9.5 & Appendix XXXV - B, and conidiation phenotypes are presented in Figure 9.2 and Appendix XXXV - A.

GTR2 wild type progeny strains were also tested for the presence or absence of *Frq* gene and out of 25 tested progeny strains, 15 were *Frq* type strains (Appendix XXXIV - C). A race tube experiment was again performed with *csp-1* and *gtr2* wild type progeny to check the segregation pattern. Apart

from *gtr2* wild type progeny, the strains identified as *csp-1; gtr2^{KO}; chol-1 ras-I^{bd}* (pg#3, pg#4 & pg#26) and *csp-1; gtr2^{KO}; chol-1 ras-I^{bd}; frq¹⁰* (pg#14, pg#20, pg#30, pg#34 & pg#37) during the previous race tube experiment, were subjected to race tube analysis. The race tubes were separated into the groups based on visual comparisons to the controls, considering growth rate and PCR results (Table 9.6, Appendix XXXV – C & Appendix XXXV - D). Silica stocks of the progeny strains with the required genotypes were made (*csp-1; gtr2^{KO}; chol-1 ras-I^{bd}* – SG#590 -592 & *csp-1; gtr2^{KO}; chol-1 ras-I^{bd}; frq¹⁰* – SG#593-597).

Progeny from the cross performed between SG#300 (*csp-1: chol-1 ras-I^{bd}; frq¹⁰ mat A*) and GTR2^{KO} strain (FGSC#16041 = SG#510) were grown on race tubes to determine whether the GTR2 knockout resembled *uvr90* mutant or NCU05950 knockout or whether the GTR2 knockout showed any other phenotype such as different growth rate, changes to the period of conidiation rhythm or morphological changes in conidial banding pattern. Clear and rhythmic conidiation patterns were observed in progeny strains with the genotype *csp-1; gtr2⁺; ras-I^{bd}* grown on MA media containing choline, as the control strain SG# 26 with the genotype *csp-1: chol-1 ras-I^{bd}* (Figure 9.2 & Appendix XXXV - A). Progeny strains with the genotype *csp-1; gtr2⁺; ras-I^{bd}; frq¹⁰* exhibited fluffy and arrhythmic phenotype as the control strain SG#80 with the genotype *csp-1: chol-1 ras-I^{bd}; frq¹⁰* (Figure 9.2 & Appendix XXXV - A).

The progeny strains with the genotype *csp-1; gtr2^{KO}; ras-I^{bd}* appeared to have low amplitude rhythms at the beginning of the race tubes that tapered off to an arrhythmic pattern as the control strain SG# 361 with the genotype *csp-1; chol-1 ras-I^{bd}; NCU05950^{KO}* (Figure 9.2 & Appendix XXXV - A) and the progeny strains with the genotype *csp-1; gtr2^{KO}; ras-I^{bd}; frq¹⁰* exhibited

arrhythmic conidiation as the control strain SG# 213 with the genotype *csp-1*; *chol-1 ras-1^{bd}*; *uv90*; *frq¹⁰* (Figure 9.2 & Appendix XXXV - A).

On media without choline, strains with genotype *csp-1*; *gtr2⁺*; *chol-1 ras-1^{bd}* exhibited rhythmic conidiation banding patterns, but this banding pattern was not as distinct as the banding pattern produced on media containing choline. This is the result of the *chol-1* mutation, which exhibits slower growth rate and longer period when choline levels are depleted (Lakin-Thomas, 1996). The progeny strains with the genotype *csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}* appeared arrhythmic on media without choline as the control strain SG# 361 (Figure 9.2 & Appendix XXXV - A). Similar to the control strain SG#80, the progeny strains with the genotype *csp-1*; *gtr2⁺*; *chol-1 ras-1^{bd}*; *frq¹⁰* exhibited long period, low amplitude conidiation rhythm, as expected (Figure 9.2 & Appendix XXXV - A). *csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}*; *frq¹⁰* strains abolish the conidiation rhythm and appeared completely arrhythmic as the control strain SG# 213 (Figure 9.2 & Appendix XXXV - A).

The phenotypes and growth rates of *gtr2* deletion mutant strains are identical to the *uv90* mutant as well as NCU05950 deletion mutant strains. These results may indicate that *gtr2* and NCU05950 are participants in a single pathway that is responsible for conidiation rhythm generation in *Neurospora crassa*. As with the NCU05950 deletion mutation and *uv90* mutation, the *gtr2* deletion mutation phenotype appears to be completely arrhythmic in the *frq¹⁰* background on high choline and it also abolishes the rhythm found in strains with the genotype *csp-1*; *chol-1 ras-1^{bd}*; *frq¹⁰* on choline deficient media. These observations give strong evidence that the *gtr2* deletion mutant affects the free running conidiation rhythms under choline deficient conditions, which is known to be a FRQ-less rhythm (Li *et al.*, 2011). Therefore, similar to

NCU05950, GTR2 could be a component of the FRQ-less oscillator (FLO) and may also be required to maintain the amplitude of the FRQ-WCC TTFL.

9.3.1.1: Backcrosses with selected *gtr2^{KO}* strains

Backcrosses were performed with SG#180 (*ras-1^{bd} mat a*) as well as SG#4 (*ras-1^{bd} mat A*), to confirm the genotype of the *gtr2^{KO}* strains and to find the mating type of the selected *gtr2^{KO}* strains (pg#4 - *csp-1; gtr2^{KO}; chol-1 ras-1^{bd}* and pg#20 - *csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰*). Both pg#4 and pg#20 had mating type “A”.

9.3.1.1.1: Genetic cross with pg#4 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}*)

192 spores were picked from the genetic cross performed between SG#180 (*ras-1^{bd} mat a*) and pg#4 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}*) strains. Out of 192 spores, 166 spores were germinated, 77 (40.10 %) were found to be *csp-1*, 89 (46.35 %) were *csp+* and 26 (13.54 %) did not germinate.

Spore PCR analysis was conducted with *csp-1* progeny (1 - 77) to uncover presence or absence of knocked-out gene using GTR2F & GTR2R primers. Out of 74 tested progeny strains, 35 were GTR2^{KO} strains (pg#15, pg#16 & pg#17 were excluded from further testing as the baby tubes were contaminated).

Race tube analysis was performed with all the *csp-1* progeny strains. Both the parental strains were *ras-1^{bd}*. Therefore, all the progeny strains were *ras-1^{bd}* (Appendix XXXVI - A). Out of 74 tested progeny strains, 31 were *chol-1* strain. The race tubes were further separated into groups based on visual comparisons to the controls, considering growth rate and PCR results (Table 9.7 & Appendix XXXVI - B). Genotypes, periods and growth rates of all the tested

strains are listed in Table 9.7 & Appendix XXXVI - B, and conidiation phenotypes are presented in Appendix XXXVI - A.

The *gtr2^{KO}* phenotype was found to segregate in this backcross with expected 1:1 ratio for segregation of a single gene mutation. In the backcross of SG#180 (*ras-1^{bd} mat a*) and pg#4 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd} mat A*), out of 74 *chol-1 ras-1^{bd}* progeny assayed, 35 were classified as *gtr2^{KO}* and 39 as *gtr2⁺* based on spore PCR results as well as banding phenotype on choline supplemented and choline deficient media. The results confirm the genotype of the parental *gtr2^{KO}* strain, which is *csp-1; gtr2^{KO}; chol-1 ras-1^{bd} mat A*.

9.3.1.1.2: Genetic cross with pg#20 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰*)

192 spores were picked from the genetic cross performed between SG#180 (*ras-1^{bd} mat a*) and pg#20 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰ mat A*) strains. Out of 192 spores, 163 spores were germinated, 83 (43.23%) were found to be *csp-1*, 80 (41.67 %) were *csp+* and 29 (15.10 %) did not germinate.

Spore PCR analysis was conducted with *csp-1* progeny (1 - 83) to uncover presence or absence of knocked-out gene using GTR2F & GTR2R primers. Out of 83 tested progeny strains, 38 were *GTR2^{KO}* strains. All the progeny strains were tested for the presence or absence of *Frq* gene with FrqF & FrqR primers and out of 83 tested progeny strains, 39 were *Frq* wild type strains.

Race tube analysis was performed with all the *csp-1* progeny strains. Both the parental strains were *ras-1^{bd}*. Therefore, all the progeny strains were *ras-1^{bd}* (Appendix XXXVI - C). Out of 83 tested progeny strains, 35 were *chol-1* strain.

The race tubes were further separated into groups based on visual comparisons to the controls, considering growth rate and PCR results

(Appendix XXXVI - D). Genotypes, periods and growth rates of all the tested strains are listed in Table 9.8 & Appendix XXXVI - D, and conidiation phenotypes are presented in Appendix XXXVI - C. Silica stocks of the progeny strains with the required genotypes were made (*csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}*; *frq¹⁰* – SG#616-618, *csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}* – SG#619 -621, *csp-1*; *gtr2^{KO}*; *chol⁺ ras-1^{bd}*; *frq¹⁰* – SG#622-624 & *csp-1*; *gtr2^{KO}*; *chol⁺ ras-1^{bd}* – SG#625-627).

The *gtr2^{KO}* phenotype was found to segregate in this backcross with expected 1:1 ratio for segregation of a single gene mutation. In the backcross of SG#180 (*ras-1^{bd} mat a*) and pg#20 (*csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}*; *frq¹⁰ mat A*), out of 35 *chol-1 ras-1^{bd}* progeny assayed, 16 were classified as *gtr2^{KO}* and 19 as *gtr2⁺* based on spore PCR results as well as banding phenotype on choline supplemented and choline deficient media. These results confirm the genotype of parental *gtr2^{KO}* strain as *csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}*; *frq¹⁰ mat A*.

We can conclude that the *gtr2* deletion mutation severely compromises the function of the FRQ-less oscillator by completely disrupting conidiation rhythms in the *chol-1*; *frq¹⁰* background under choline deficient conditions. *Gtr2* deletion mutant also damps the amplitude of the conidiation rhythms in *frq* wild type strains that contain a functional FRQ-WCC TTFL.

9.3.2: Effects of GTR2 deletion mutation on entrainment to heat pulses

To assay the presence of a functional heat-entrainable oscillator in FRQ-less strains, the repeated heat-pulse entrainment protocol was used as described in section 2.3.1.2. The heat-entrainable rhythm is the second example of a FRQ-less rhythm. Cultures were grown on 100 μ M choline supplemented MA medium in constant darkness at 22°C and subjected to repeated 2-hour pulses of 32°C at regular intervals, using three different (16 hours, 20 hours and 24

hours) T- cycles (where T=total number of hours in the cycle) to entrain the conidiation rhythms. Density traces were collected and analyzed as described in Lakin-Thomas, 2006c & Li and Lakin-Thomas, 2010. Changes in peak timing and peak shape in different T-cycles were examined as they are indicative of entrainment of an underlying oscillator (Li *et al.*, 2011). In the FRQ-less background, Li *et al.*, 2011 found that the NCU05950 deletion mutant (SG#365) had a dramatic effect on the peak timing and the major peak shifted to a much earlier time, compared to the control strain SG#80. Therefore, to confirm whether the behavior of *gtr2* deletion mutant is similar to NCU05950 deletion mutant or *uvr90* mutant, heat entrained rhythms were assayed. If the *gtr2* gene product and the NCU05950 gene product are components of a single pathway or if they are interacting with each other to make a complex, progeny with genotype *csp-1*; *gtr2*^{KO}; *chol-1 ras-1*^{bd}; *frq*¹⁰ should give a similar result for the heat pulse experiment as the NCU05950 deletion mutant or *uvr90* mutant strain.

Three FRQ-less strains (Pg#14 = SG#593, Pg#20 = SG#594 & Pg#30 = SG#595) with the genotype *csp-1*; *gtr2*^{KO}; *chol-1 ras-1*^{bd}; *frq*¹⁰ from the cross between SG#300 (*csp-1*; *chol-1 ras-1*^{bd}; *frq*¹⁰ *mat A*) and GTR2^{KO} strains were used as test strains, SG#80 (Genotype: *csp-1*; *chol-1 ras*^{bd}; *frq*¹⁰) was used as the wild-type control and SG#365 (Genotype: *csp-1*; *chol-1 ras*^{bd}; *NCU05950*^{KO}; *frq*¹⁰) was used as the NCU05950 deletion mutant control. Changes in peak timing and peak shape in different T-cycles were monitored. All three test strains showed similar peak timing and peak shape as the NCU05950 deletion mutant control strain SG#365. The control strain SG#365 and all three test strains (Pg#14 = SG#593, Pg#20 = SG#594 & Pg#30 = SG#595), showed a dramatic effect on the peak timing and shifted the major peak to a much earlier time compare to the wild type control strain SG#80 (Figure 9.3).

The phenotype of the *gtr2* knockout progeny are displayed in Figure 9.3 - D, where we have re-plotted heat pulse profiles to compare the effects of different T-cycles within each strain. The conidiation peaks in wild type strain occur at varying times after the heat pulse depending on the T-cycle, as expected from an entrained oscillator (Lakin-Thomas, 2006c; Merrow, *et al.* 1999). In contrast, the main peaks in the NCU05950 deletion mutant and *gtr2* knockout progeny strains occur earlier in the cycle than the wild type and at nearly the same time. This observation demonstrates, that the loss of NCU05950 or GTR2 function disables the oscillator that is revealed by the heat pulse entrainment. Therefore, like NCU05950, the GTR2 gene produces an effect on entrainment behavior in the absence of functional FRQ (in the *frq¹⁰* strain) and it therefore, affects the FLO.

9.3.3: Genetic cross with Stk-10^{KO} (FGSC#17939)

A genetic cross was performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and Stk-10^{KO} strain. 191 spores were picked and out of 191 spores, 175 spores were germinated, 95 (49.74%) were found to be *csp-1*, 80 (41.88 %) were *csp+* and 16 (8.37 %) did not germinate.

csp-1 progeny strains (1 to 95) were subjected to spore PCR analysis to find the presence or absence of knocked-out gene using Stk10F & Stk10R primers. Out of 95 tested *csp-1* progeny strains, 8 were Stk10^{KO} strains, which was expected due to the fact that the *Stk10* and *csp* genes are on the same chromosome i.e. on linkage group “I”. Since those two genes are linked, there were only a few recombinations due to the close physical distance. All the *csp-1* progeny strains were tested for the presence or absence of *Frq* gene and out of 95 tested progeny strains, 43 were *Frq* wild type strains.

8 progeny strains with *Stk-10^{KO}* allele in the *csp-1* background were subjected to race tube analysis. The progeny strains were visually separated into *ras-1^{bd}* or *ras-1^{bd+}* phenotypes based on conidiation banding phenotype as well as slower growth determined by comparing growth fronts marked daily on the tubes. All the tested progeny strains were found to be *ras-1^{bd}* and *chol-1* (Appendix XXXVII - A).

The race tubes were further separated into groups based on visual comparisons to the controls, considering growth rate and PCR results (Table 9.9 and Appendix XXXVII - B). Genotype, periods and growth rates of all the tested strains are listed in Table 9.9 & Appendix XXXVII - B, and conidiation phenotypes are presented in Figure 9.4 & Appendix XXXVII - A. Silica stocks of the progeny strains with the required genotypes were made (*csp-1 stk-10^{KO}*; *chol-1 ras-1^{bd}*; *frq¹⁰* - SG#628-630 & *csp-1 stk-10^{KO}*; *chol-1 ras-1^{bd}* – SG#631-633).

A second race tube experiment was performed with some of the *csp-1* and *stk-10* wild type gene containing progeny strains to check the segregation pattern. The race tubes were separated into the groups based on visual comparisons to the controls, considering growth rate and PCR results (Table 9.10, Appendix XXXVII - C & Appendix XXXVII - D).

Progeny from the cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}*; *frq¹⁰ mat A*) and STK-10^{KO} strain (FGSC#17939 = SG#571) were grown on race tubes to determine whether the STK-10 knockout resembled *uv90* mutant or NCU05950 knockout or whether the STK-10 knockout shows any other phenotype such as a difference in growth rate, changes to the period of conidiation rhythm or morphological changes in conidial banding pattern. Clear and rhythmic conidiation patterns were observed in progeny strains with the genotype *csp-1 stk-10⁺*; *ras-1^{bd}* grown on MA media containing choline, similar

to the control strain SG# 26 with the genotype *csp-1; chol-1 ras-1^{bd}* (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C). Progeny strains with the genotype *csp-1 stk-10⁺; ras-1^{bd}; frq¹⁰* exhibited fluffy and arrhythmic phenotype similar to the control strain SG#80 with the genotype *csp-1; chol-1 ras-1^{bd}; frq¹⁰* (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C).

The progeny strains with the genotype *csp-1 stk-10^{KO}; ras-1^{bd}* appeared to have low amplitude conidiation rhythms and conidiation development was not intense as the control strain SG# 361 with the genotype *csp-1; chol-1 ras-1^{bd}; NCU05950^{KO}* (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C). On the other hand, the progeny strains with the genotype *csp-1 stk-10^{KO}; ras-1^{bd}; frq¹⁰* exhibited arrhythmic conidiation under constant conditions similar to the control strain SG# 213 with the genotype *csp-1; chol-1 ras-1^{bd}; uv90; frq¹⁰* (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C). All of these strains had very thin hyphae and the conidiation morphology was not similar to control strains SG#361 and SG#213. However, all of these strains had nearly equivalent growth rates and periods of conidiation rhythms (Appendix XXXVII - B & Appendix XXXVII - D).

On media without choline, strains with genotype *csp-1 stk-10⁺; chol-1 ras-1^{bd}* exhibited rhythmic conidiation banding patterns. The progeny strains with the genotype *csp-1 stk-10^{KO}; chol-1 ras-1^{bd}* appeared arrhythmic on media without choline similar to the control strain SG# 361 (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C). However, the progeny strains had fluffy mycelia, which was different from the control strain. Similar to the control strain SG#80, the progeny strains with the genotype *csp-1 stk-10⁺; chol-1 ras-1^{bd}; frq¹⁰* exhibited long period, low amplitude conidiation rhythms, as expected (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C). *csp-1 stk-10^{KO}; chol-1 ras-1^{bd}; frq¹⁰* strains abolish the conidiation rhythm and

appeared completely arrhythmic similar to the control strain SG# 213 (Figure 9.4, Appendix XXXVII - A & Appendix XXXVII - C).

The period of conidiation rhythms and growth rates of *stk-10* deletion mutant strains are identical to *uvr90* mutant as well as NCU05950 deletion mutant strains. However, the phenotype or morphology of conidial banding and hyphae were not similar to *uvr90* mutant or NCU05950 deletion mutant strains or to wild type strains. These results indicate that *stk-10* and NCU05950 may be participants of a single pathway that is responsible for conidiation rhythm generation in *Neurospora crassa* and *stk-10* may also have some function in conidial and hyphae morphogenesis. Similar to the NCU05950 deletion mutation and *uvr90* mutation, the *stk-10* deletion mutation phenotype appears to be completely arrhythmic in the *frq¹⁰* background and it also abolishes the rhythm found in strains with the genotype *csp-1; chol-1 ras-1^{bd}; frq¹⁰* on choline deficient media. These observations give strong evidence that the *stk-10* deletion mutant affects the free running conidiation rhythms under choline deficient conditions, which is known to be a FRQ-less rhythm (Li *et al.*, 2011). Therefore, similar to the NCU05950, STK-10 could be a component of the FRQ-less oscillator (FLO) and it may also be required to maintain the amplitude of the FRQ-WCC TTFL.

Backcrosses were performed with SG#180 (*ras-1^{bd} mat a*) as well as SG#4 (*ras-1^{bd} mat A*), to confirm the genotype of the *stk-10^{KO}* strains and to find the mating type of the selected *stk-10^{KO}* strains (pg#11 - *csp-1 stk-10^{KO}*; *chol-1 ras-1^{bd}* and pg#34, pg#46, pg#48 - *csp-1 stk-10^{KO}; chol-1 ras-1^{bd}; frq¹⁰*). All the tested strains had mating type “a”. Progeny strains need to be analyzed to confirm the genotypes.

9.3.4: Effects of STK-10 deletion mutation on entrainment to heat pulses

To assay the presence of a functional heat-entrainable oscillator in FRQ-less *Stk-10* deletion mutant strains, the repeated heat-pulse entrainment protocol was used as described in section 2.3.1.2. If the *stk-10* gene product and the NCU05950 gene product are components of a single pathway or if they are interacting with each other to make a complex, progeny with genotype *csp-1 stk-10^{KO}; chol-1 ras-1^{bd}; frq¹⁰* should give a similar result for the heat pulse experiment as the NCU05950 deletion mutant or *uvr90* mutant strain.

Three FRQ-less strains (Pg#34 = SG#628, Pg#46 = SG#629 & Pg#48 = SG#630) with the genotype *csp-1 stk-10^{KO}; chol-1 ras-1^{bd}; frq¹⁰* from the cross between SG#300 (*csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat A*) and STK-10^{KO} strains were used as test strains, SG#80 (Genotype: *csp-1; chol-1 ras^{bd}; frq¹⁰*) was used as the wild-type control and SG#365 (Genotype: *csp-1; chol-1 ras^{bd}; NCU05950^{KO}; frq¹⁰*) was used as the NCU05950 deletion mutant control. Changes in peak timing and peak shape in different T-cycles were monitored. Two of the test strains (Pg#46 = SG#629 & Pg#48 = SG#630) showed similar peak timing and peak shape and Pg#34 (SG#628) had completely different peak timing as well as peak shape. None of the test strains were similar to NCU05950 deletion mutant or wild-type control SG#80. All three test strains showed a dramatic effect on the peak timing and peak shape compared to the wild type control strains (Figure 9.5).

The phenotype of the *Stk-10* knockout progeny strains are displayed in Figure 9.5 - D, where we have re-plotted heat pulse profiles to compare the effects of different T-cycles within each strain. The conidiation peaks in wild type strain occurs at varying times after the heat pulse depending on the T-cycle, as expected from an entrained oscillator (Lakin-Thomas, 2006c; Merrow, *et al.* 1999). In contrast, the main peaks in the NCU05950 deletion mutant and

two of the *stk-10* knockout progeny strains occur earlier in the cycle than the wild type and at nearly the same time. This observation demonstrates that the loss of NCU05950 or STK-10 function disables the oscillator that is revealed by the heat pulse entrainment. Therefore, like the NCU05950, STK-10 gene product has an effect on entrainment behavior in the absence of functional FRQ (in the *frq¹⁰* strain) and it affects the FLO.

9.3.5: Genetic cross with Tap42^{KO} (FGSC#21741)

A genetic cross was performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and Tap42^{KO} strain. 192 spores were picked and out of 192 spores, 133 spores were germinated, 82 (42.71 %) were found to be *csp-1*, 51 (26.56 %) were *csp+* and 16 (30.73 %) did not germinate.

csp-1 progeny strains (1 to 82) were subjected to spore PCR analysis to uncover presence or absence of the Tap42 knocked-out gene using hphF & hphR primers as well as Tap42F2 & Tap42R2 primers. All the *csp-1* progeny strains were also tested for the presence or absence of *Frq* gene with FrqF & FrqR primers. The results obtained from spore PCR are were unclear. Therefore, spore PCR analysis should be performed again to find the correct genotypes.

Race tube analysis was conducted for all 82 progeny strains with *csp-1* background. Periods and growth rates of all the tested strains are listed in Appendix XXXVIII - B and conidiation phenotypes are presented in Appendix XXXVIII - A.

The race tube experiment conducted with progeny strains obtained from the cross between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and Tap42^{KO} strain, did not show any strain similar to *uv90* mutant or NCU05950 deletion

mutant strains. There was a set of progeny strains that exhibited different phenotype (morphologically different conidiation) from the control strains.

We received the Tap42 knock out strain (FGSC#21741) as microconidia from the FGSC. It will be beneficial to check the purity of these microconidia and confirm it is not a heterokaryon with nuclei of different genotypes. If Tap42 is an essential gene for *Neurospora crassa*, deletion of it may be lethal for the fungus.

9.3.5: Genetic cross with FGSC#19786 (pik1)

BLAST search with yeast TOR1 amino acid sequence identified NCU10397 in *Neurospora crassa* as a homolog. NCU10397 is phosphatidylinositol 4-kinase PIK1a and it is from linkage group III. A genetic cross was performed between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and PIK1a^{KO} strain. 146 spores were picked and out of 146 spores, 58 spores were germinated, 29 (19.86 %) were found to be *csp-1*, 29 (19.86 %) were *csp+* and 88 (60.27 %) did not germinate. Out the 29 *csp-1* progeny strains 7 progeny strains had thin, white mycelium.

Healthy looking *csp-1* progeny were numbered from 1b to 22b and poorly grown progeny were numbered from 1c to 7c. Spore PCR analysis was conducted to uncover presence or absence of knocked-out gene using PIK1aF & PIK1aR primers. Out of 29 tested progeny strains, 15 were PIK1a^{KO} strains (Appendix XXXIX - A).

Progeny strains with PIK1a^{KO} allele in the *csp-1* background were analyzed on race tubes and the progeny were visually separated into *ras-1^{bd}* or *ras-1^{bd+}* phenotypes based on conidiation banding phenotype as well as slower growth determined by comparing growth fronts marked daily on the tubes (Appendix XXXIX - B).

The race tubes were further separated into groups based on visual comparisons to the controls, considering growth rate and PCR results (Table 9.11 & Appendix XXXIX - C). Periods and growth rates of all the tested strains are listed in Table 9.11 & Appendix XXXIX – C and conidiation phenotypes are presented in Appendix XXXIX - B.

A second race tube experiment was performed with selected progeny strains. Three replicates for each strain were inoculated onto maltose-arginine medium both with and without 100 µM in race tubes. SG#26 (*csp-1; chol-1 ras-1^{bd} mat a*), SG#224 (*csp-1; chol-1 ras-1^{bd}; uv90 mat a*), SG#80 (*csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a*) and SG#213 (*csp-1; chol-1 ras-1^{bd}; uv90 mat a*) were used as the control strains. Periods and growth rates of all the tested strains are listed in Appendix XXXIX – E and conidiation phenotypes are presented in Appendix XXXIX - D.

PIK1a^{KO} strains did not show any interesting conidiation rhythm related phenotype. Therefore, I did not proceed further with *PIK1a^{KO}* progeny strains. Stock tubes were made for following strains and stored in the -20°C freezer; 5b, 7b, 10b, 11b, 18b, 19b, 22b & 2c.

9.3.6: Genetic cross with FGSC#13000 (*fkr5*)

BLAST search with yeast FPR2 amino acid sequence identified NCU02455 in *Neurospora crassa* as a homolog. NCU02455 is FKBP (FK506 binding protein) -type peptidyl-prolyl cis-trans isomerase (*fkr5* = FK506-resistant-5) and it is on linkage group VII. The *Frq* gene is also located in the same chromosome as *fkr5* and a genetic cross was performed between SG#80 (*csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a*) and FKR5^{KO} strains, assuming *fkr5* and *frq* genes will segregate independently. Spores were picked and the “tap test” was performed on the baby tubes to visually check for *csp-1* and *csp+* progeny.

There were 40 *csp-1* progeny strains and spore PCR analysis was conducted to uncover the presence or absence of the knocked-out gene using FKR5F & FKR5R primers. Out of 40 tested progeny strains, 20 were FKR5^{KO} strains.

Selected progeny strains with *csp-1* background were inoculated onto maltose-arginine medium both with and without 100 µM choline in race tubes. The following control strains were used for the experiment: SG#26 (*csp-1*; *chol-1 ras-1^{bd}* mat a), SG#224 (*csp-1*; *chol-1 ras-1^{bd}*; *uv90* mat a), SG#80 (*csp-1*; *chol-1 ras-1^{bd}*; *frq¹⁰* mat a) and SG#213 (*csp-1*; *chol-1 ras-1^{bd}*; *uv90* mat a). One tube for each strain were inoculated. The progeny strains were visually separated into *ras-1^{bd}* or *ras-1^{bd+}* phenotypes. Genotypes, periods and growth rates of all the tested strains are listed in Appendix XL - C. Most of the baby tubes as well as race tubes were contaminated with grey color fungus and those tubes were discarded. Stock tubes were made for following strains and stored in the -20°C freezer; pg#5, pg#6, pg#9, pg#10, pg#12, pg#14 & pg#28.

218 spores were picked again and out of 218 spores, 93 spores were germinated, 50 were found to be FKR5^{KO} and 3 were *frq¹⁰* (Appendix XL - A and Appendix XL - B). I did not proceed with race tube experiment as there were only three strains with *frq¹⁰* and priority was given to other experiments.

Table 9.1: Analysis of progeny strains by Spore PCR.

Gene	Primers	Product size	Comments
<i>Frq</i>	FrqF & FrqR	484 bp	484 bp long PCR product from the <i>Frq</i> wild type strains. No PCR products from the <i>frq</i> ¹⁰ strains.
<i>GTR2</i>	GTR2F & GTR2R	1935 bp Or ~1500 bp	1935 bp long PCR product from the wild type <i>GTR2</i> gene containing progeny strains. ~ 1500 bp PCR product from <i>GTR2</i> gene knocked out strains that has the Hygromycin B resistance gene at the <i>GTR2</i> genomic locus.
<i>Stk10</i>	Stk10F & Stk10R	3268 bp Or ~1500 bp	3268 bp long PCR product from the wild type <i>Stk10</i> gene containing progeny strains. ~ 1500 bp PCR product from <i>Stk10</i> gene knocked out strains that has the Hygromycin B resistance gene at the <i>Stk10</i> genomic locus.
<i>Tap42</i>	hphF & hphR Tap42F2 & Tap42R2	~1500 bp or 1077 bp	No PCR product from the wild type <i>Tap42</i> gene containing progeny strains with hphF & hphR primers. ~ 1500 bp PCR product from <i>Tap42</i> gene knocked out strains that has the Hygromycin B resistance gene at the

			<i>Tap42</i> genomic locus with hph cassette specific primers. 1077 bp long PCR products from the <i>Tap42</i> wild type strains and no PCR products from the <i>Tap42</i> gene knocked out strains with <i>Tap42</i> gene specific primers Tap42F2 & Tap42R2.
<i>PIK1a</i>	PIK1aF & PIK1aR	3562 bp Or ~1500 bp	3562 bp long PCR product from the wild type <i>PIK1a</i> gene containing progeny strains. ~ 1500 bp PCR product from <i>PIK1a</i> gene knocked out strains that has the Hygromycin B resistance gene at the <i>PIK1a</i> genomic locus.
<i>FKR5</i>	FKR5F & FKR5R	1161 bp Or ~1500 bp	1161 bp long PCR product from the wild type <i>fkr5</i> gene containing progeny strains. ~ 1500 bp PCR product from <i>fkr5</i> gene knocked out strains that has the Hygromycin B resistance gene at the <i>fkr5</i> genomic locus

Table 9.2: Race tube analysis of progeny strains obtained from different crosses.

Background of the progeny strains	Control strains used for the Race tube experiment	Comments
Progeny strains with <i>GTR2</i> ^{KO} allele in the <i>csp-1</i> background	SG#26: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} <i>mat a</i> SG#361: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>NCU05950</i> ^{KO} <i>mat a</i> SG#80: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>frq</i> ¹⁰ <i>mat a</i> SG#213: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>uv90</i> <i>mat a</i> SG#300: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>frq</i> ¹⁰ <i>mat A</i>	Two replicates for each strain were inoculated.
Progeny strains with <i>GTR2</i> wild type allele in the <i>csp-1</i> background	SG#26: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} <i>mat a</i> SG#361: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>NCU05950</i> ^{KO} <i>mat a</i> SG#80: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>frq</i> ¹⁰ <i>mat a</i> SG#213: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>uv90</i> <i>mat a</i> SG#300: <i>csp-1</i> ; <i>chol-1 ras-1</i> ^{bd} ; <i>frq</i> ¹⁰ <i>mat A</i> pg#3, pg#4 & pg#26: <i>csp-1</i> ; <i>gtr2</i> ^{KO} ; <i>chol-1 ras-1</i> ^{bd} pg#14, pg#20, pg#30, pg#34 &	Race tube experiment was performed with <i>csp-1</i> and <i>gtr2</i> wild type progeny to check the segregation pattern. Two replicates for each strain were inoculated.

	pg#37: <i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i>	
Progeny strains obtained from genetic backcross performed with pg#4 (<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i>)	SG#26: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> mat a SG#361: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>NCU05950^{KO}</i> mat a SG#80: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i> mat a SG#213: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>uv90</i> mat a SG#300: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i> mat A SG#180: <i>ras-1^{bd}</i> mat a Pg#4: <i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> mat A	One tube for each strain were inoculated.
Progeny strains obtained from genetic backcross performed with pg#20 (<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i>)	SG#26: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> mat a SG#361: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>NCU05950^{KO}</i> mat a SG#80: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i> mat a SG#213: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>uv90</i> mat a SG#300: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i> mat A SG#180: <i>ras-1^{bd}</i> mat a Pg#20: <i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> <i>frq¹⁰</i> mat A	One tube for each strain were inoculated.
Progeny strains	SG#26: <i>csp-1</i> ; <i>chol-1 ras-1^{bd}</i> mat a	8 progeny strains

with <i>Stk-10^{KO}</i> allele in the <i>csp-1</i> background	SG#361: <i>csp-1; chol-1 ras-1^{bd}; NCU05950^{KO} mat a</i> SG#80: <i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i> SG#213: <i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i> SG#571: <i>stk-10^{KO} mat a</i> SG#300: <i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat A</i>	with <i>Stk-10^{KO}</i> allele in the <i>csp-1</i> background were subjected to race tube experiment. Two replicates for each strain were inoculated.
Progeny strains with <i>Stk-10</i> wild type allele in the <i>csp-1</i> background.	SG#26: <i>csp-1; chol-1 ras-1^{bd} mat a</i> SG#361: <i>csp-1; chol-1 ras-1^{bd}; NCU05950^{KO} mat a</i> SG#80: <i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i> SG#213: <i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i> SG#571: <i>stk-10^{KO} mat a</i> SG#300: <i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat A</i> pg#34, pg#46 & pg#48: <i>csp-1 stk-10^{KO}; chol-1 ras-1^{bd}; frq¹⁰</i> pg#11, pg#23 & pg#44: <i>csp-1 stk-10^{KO}; chol-1 ras-1^{bd}; frq⁺</i>	Race tube experiment was performed with some of the <i>csp-1</i> and <i>stk-10</i> wild type gene containing progeny strains to check the segregation pattern. 10 progeny strains with <i>csp-1, frq¹⁰, stk-10⁺</i> genetic background (pg#4, pg#10, pg#12, pg#19, pg#26, pg#29, pg#33, pg#36, pg#40 & pg#47) and 10 progeny strains with

		<i>csp-1</i>, <i>frq</i>⁺, <i>stk-10</i>⁺ genetic background (pg#1, pg#7, pg#16, pg#18, pg#20, pg#28, pg#37, pg#39, pg#42 & pg#45) were selected. Three replicates for each strain were inoculated.
Progeny strains with <i>Tap42</i> ^{KO} allele or <i>Tap42</i> wildtype allele in the <i>csp-1</i> background	SG#26: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd} mat a SG#361: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd}; <i>NCU05950</i>^{KO} mat a SG#80: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd}; <i>frq</i>¹⁰ mat a SG#213: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd}; <i>uv90</i> mat a SG#513: <i>Tap42</i>^{KO} mat a SG#300: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd}; <i>frq</i>¹⁰ mat A	All the <i>csp-1</i> progeny strains obtained from cross between SG#300 (<i>csp-1</i>; <i>chol-1 ras-1</i>^{bd}; <i>frq</i>¹⁰ mat A) and <i>Tap42</i>^{KO} strains were subjected to race tube experiment. One tube for each strain were inoculated.
Progeny strains with <i>PIK1a</i> ^{KO} allele in the <i>csp-1</i> background	SG#26: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd} mat a SG#80: <i>csp-1</i>; <i>chol-1 ras-1</i>^{bd}; <i>frq</i>¹⁰ mat a.	One tube for each strain was inoculated.

Progeny strains with <i>FKR5^{KO}</i> allele in the <i>csp-1</i> background	SG#26: <i>csp-1; chol-1 ras-1^{bd} mat a</i> SG#224: <i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i> SG#80: <i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i> SG#213: <i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i>	One tube for each strain were inoculated.
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Table 9.3: TOR signaling pathway associated genes in Yeast and possible equivalent genes in *Neurospora crassa*.

Heterokaryon: Knockouts are probably lethal. We cannot use heterokaryons for the experiment.

Yeast	<i>Neurospora crassa</i>	<i>Neurospora crassa</i> Knock Out strains
TOR1	NCU10397.7: Phosphatidylinositol 4-kinase PIK1a	FGSC #19786 Mating Types: A Genotype: NCU10397.2
TOR2	NCU05608.7: Phosphatidylinositol 3-kinase tor2	FGSC #13432 Genotype: NCU05608.2 heterokaryon
KOG1	NCU00621: TORC1 growth control complex subunit Kog1	FGSC #16121 Mating Types: a Genotype: NCU00621.2 heterokaryon
Vam6	NCU01539 - vacuolar morphogenesis protein AvaB	FGSC #18870 Mating Types: a Genotype: NCU01539.2 heterokaryon
Tap42	NCU08268 - TOR signaling pathway regulator	FGSC #21741 Mating Types: a Genotype: NCU08268.2 (Available as microconidia)
FPR2	NCU02455 - FKBP-type	FGSC #13000

	peptidyl-prolyl cis-trans isomerase	Mating Types: A Genotype: NCU02455.2 (fkr-5)
GTR2	NCU00376.7: GTR/RAG family GTPase Gtr2 (GTP-28 Guanine triphosphate binding-28)	FGSC #16041 Mating Type: a Genotype: NCU00376.2
LST8	NCU04281: WD-repeat protein pop3	FGSC #20964 Mating Type: a Genotype: NCU04281.2 heterokaryon
AVO1	NCU00324: Hypothetical protein (CRIM and PH_Avo1 domain-containing protein)	FGSC #16038 Mating Type: a Genotype: NCU00324.2 heterokaryon
SCH9	NCU03200: Serine/threonine-protein kinase sck1 (<i>stk-10</i> - Serine / Threonine - protein kinase – 10)	FGSC #17939 Mating Type: a Genotype: NCU03200.2 (<i>stk-10</i>)

Table 9.4: Crosses made with TOR signaling pathway associated gene

knockout strains.

TOR related gene name	Knockout strain details	Linkage group	Remarks
GTR2	FGSC#16041 SG#510 <i>mat a</i>	III	Crossed with SG#300: <i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A</i> - 191 spores were picked. - 85 were germinated. - <i>csp-1</i> - 43 (22.51 %) - <i>csp+</i> - 42 (21.98 %) - Non-germinated – 106 (55.49%)
Tap42	FGSC#21741 SG#513 <i>mat a</i>	IV	Crossed with SG#300: <i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A</i> - 192 spores were picked. - 133 were germinated. - <i>csp-1</i> - 82 - <i>csp+</i> - 51
Stk-10	FGSC#1793 SG#571 <i>mat a</i>	I	Crossed with SG#300: <i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A</i> - 191 spores were picked. - 175 were germinated. - <i>csp-1</i> - 95 - <i>csp+</i> - 80
FKR5	FGSC#13000 SG#508 <i>mat A</i>	VII	Crossed with SG#80: <i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a</i> 1 st Round - - 119 spores were picked. - 40 were germinated. 2 nd Round - - 218 spores were picked. - 93 were germinated.
PIK1a	FGSC#19786 SG#512 <i>mat A</i>	III	Crossed with SG#80: <i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a</i> - 146 spores were picked. - 58 were germinated.

Table 9.5: *csp-1* & GTR2^{KO} progeny from cross between SG#300 (*csp-1*: *chol-1 ras-1^{bd}*; *frq¹⁰* mat A) and GTR2^{KO} strains tested on MA medium as well as 100 μ M choline containing MA medium.

Total <i>csp-1</i> & GTR2 ^{KO} progeny analyzed on race tubes	18
Controls used for the race tube analysis	- SG#26 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i> mat a) - SG#361 - (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>NCU05950^{KO}</i> mat a) - SG#80 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰</i> mat a) - SG#213 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>uv90</i> mat a) - SG#300 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰</i> mat A).
<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq⁺</i>	3 pg#3, pg#4 & pg#26
<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd+}</i> ; <i>frq⁺</i>	3 pg#28, pg#29 & pg#38
<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i>	5 pg#14, pg#20, pg#30, pg#34 & pg#37
<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd+}</i> ; <i>frq¹⁰</i>	1 pg#27
<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol+ ras-1^{bd}</i> ; <i>frq¹⁰</i>	1 pg#43
<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol+ ras-1^{bd+}</i> ; <i>frq¹⁰</i>	5 pg#7, pg#22, pg#33, pg#36 & pg#40

Table 9.6: *csp-1* & GTR2^{wild type} progeny from cross between SG#300 (*csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat A*) and GTR2^{KO} strains tested on tested on MA medium as well as 100 μ M choline containing MA medium.

Total <i>csp-1</i> & GTR2 ^{wild type} progeny analyzed on race tubes	25
Controls used for the race tube analysis	- SG#26 (<i>csp-1; chol-1 ras-1^{bd} mat a</i>) - SG#361 - (<i>csp-1; chol-1 ras-1^{bd}; NCU05950^{KO} mat a</i>) - SG#80 (<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i>) - SG#213 (<i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i>) - SG#300 (<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat A</i>).
<i>csp-1; chol-1 ras-1^{bd+}; frq¹⁰</i>	7 pg#5, pg#6, pg#11, pg#13, pg#25, pg#35 & pg#42
<i>csp-1; ras-1^{bd+}; frq¹⁰</i>	1 pg#19
<i>csp-1; ras-1^{bd+}</i>	6 pg#2, pg#10, pg#15, pg#17, pg#31 & pg#41
<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰</i>	2 pg#12 & pg#23
<i>csp-1; ras-1^{bd}</i>	2 pg#8 & pg#18
<i>csp-1; chol-1 ras-1^{bd}</i>	7 pg#1, pg#9, pg#16, pg#21 , pg#24 , pg#32 & pg#39

Table 9.7: Progeny from backcross between SG#180 (*ras-1^{bd} mat a*) and pg#4 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}*) strains on tested on MA medium as well as 100 μ M choline containing MA medium.

Total progeny analyzed on race tubes	77
Controls used for the race tube analysis	- SG#26 (<i>csp-1; chol-1 ras-1^{bd} mat a</i>) - SG#361 (<i>csp-1; chol-1 ras-1^{bd}; NCU05950^{KO} mat a</i>) - SG#80 (<i>csp-1; chol-1 ras-1^{bd}; frq^{l0} mat a</i>) - SG#213 (<i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i>) - SG#300 (<i>csp-1; chol-1 ras-1^{bd}; frq^{l0} mat A</i>) - SG#180 (<i>ras-1^{bd} mat a</i>) - Pg#4 (<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd} mat A</i>)
<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd}</i>	16 pg#3, pg#4, pg#6, pg#14, pg#18, pg#19, pg#28, pg#43, pg#44, pg#52, pg#61, pg#62, pg#64, pg#65, pg#68, pg#72
<i>csp-1; gtr2^{KO}; chol⁺</i>	19

<i>ras-1^{bd}</i>	pg#1, pg#8, pg#27, pg#29, pg#30, pg#37, pg#38, pg#42, pg#46, pg#53, pg#55, pg#56, pg#58, pg#60, pg#63, pg#66, pg#71, pg#75, pg#76
<i>csp-1; gtr2⁺; chol-1</i> <i>ras-1^{bd}</i>	15 pg#2, pg#12, pg#13, pg#21, pg#25, pg#26, pg#31, pg#35, pg#36, pg#41, pg#45, pg#47, pg#67, pg#73, pg#77
<i>csp-1; gtr2⁺; chol⁺ ras-1^{bd}</i>	24 pg#5, pg#7, pg#9, pg#10, pg#11, pg#20, pg#22, pg#23, pg#24, pg#32, pg#33, pg#34, pg#39, pg#40, pg#48, pg#49, pg#50, pg#51, pg#54, pg#57, pg#59, pg#69, pg#70, pg#74

Table 9.8: Progeny from backcross between SG#180 (*ras-1^{bd} mat a*) and pg#20 (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰ mat A*) strains on tested on MA medium as well as 100 μ M choline containing MA medium.

Total progeny analyzed on race tubes	83
Controls used for the race tube analysis	- SG#26 (<i>csp-1; chol-1 ras-1^{bd} mat a</i>) - SG#361 (<i>csp-1; chol-1 ras-1^{bd}; NCU05950^{KO} mat a</i>) - SG#80 (<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i>) - SG#213 (<i>csp-1; chol-1 ras-1^{bd}; uv90; frq¹⁰ mat a</i>) - SG#300 (<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat A</i>) - SG#180 (<i>ras-1^{bd} mat a</i>) - Pg#20 (<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰ mat A</i>)
<i>csp-1; gtr2^{KO}; chol⁺ ras-1^{bd} frq¹⁰</i>	11 pg#1, pg#12, pg#18, pg#29, pg#31, pg#48, pg#63, pg#64, pg#66, pg#68, pg#74

<i>csp-1; gtr2^{KO}; chol⁺ ras-1^{bd} frq⁺</i>	12 pg#5, pg#6, pg#13, pg#15, pg#25, pg#26, pg#50, pg#55, pg#61, pg#76, pg#77, pg#81
<i>csp-1; gtr2⁺; chol⁺ ras-1^{bd} frq¹⁰</i>	15 pg#17, pg#19, pg#24, pg#30, pg#34, pg#39, pg#53, pg#57, pg#58, pg#70, pg#73, pg#75, pg#78, pg#80, pg#82
<i>csp-1; gtr2⁺; chol⁺ ras-1^{bd} frq⁺</i>	8 pg#21, pg#38, pg#47, pg#51, pg#56, pg#59, pg#60, pg#62
<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd} frq¹⁰</i>	7 pg#36, pg#37, pg#44, pg#52, pg#65, pg#69, pg#71
<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd} frq⁺</i>	10 pg#2, pg#10, pg#11, pg#14, pg#28, pg#32, pg#33, pg#40, pg#46, pg#79
<i>csp-1; gtr2⁺; chol-1 ras-1^{bd} frq¹⁰</i>	10 pg#7, pg#8, pg#9, pg#22, pg#27 pg#41, pg#43, pg#49, pg#54, pg#83
<i>csp-1; gtr2⁺; chol-1 ras-1^{bd} frq⁺</i>	10 pg#3, pg#4, pg#16, pg#20, pg#23, pg#35, pg#42, pg#45, pg#67, pg#72

Table 9.9: *csp-1* & *Stk-10^{KO}* progeny from cross between SG#300 (*csp-1*: *chol-1 ras-1^{bd}*; *frq¹⁰* mat A) and *Stk-10^{KO}* strains tested on tested on MA medium as well as 100 μ M choline containing MA medium.

Total progeny analyzed on race tubes	8
Controls used for the race tube analysis	- SG#26 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i> mat a) - SG#361 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>NCU05950^{KO}</i> mat a) - SG#80 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰</i> mat a) - SG#213 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>uv90</i> mat a) - SG#300 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰</i> mat A) - SG#552 (<i>Stk-10^{KO}</i>)
<i>csp-1 stk-10^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i>	4 pg#34, pg#41, pg#46 & pg#48
<i>csp-1 stk-10^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq⁺</i>	4 pg#11, pg#15, pg#23 & pg#44

Table 9.10: *csp-1* & *stk10*^{wild type} progeny from cross between SG#300 (*csp-1*; *chol-1 ras-1^{bd}*; *frq¹⁰* mat A) and SG#571 (*stk-10^{KO}* mat a) strains tested on tested on MA medium as well as 100 μ M choline containing MA medium.

Total progeny analyzed on race tubes	26
Controls used for the race tube analysis	- SG#26 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i> mat a) - SG#361 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>NCU05950^{KO}</i> mat a) - SG#80 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰</i> mat a) - SG#213 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>uv90</i> mat a) - SG#300 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰</i> mat A) - SG#571 (<i>stk-10^{KO}</i> mat a)
<i>csp-1 stk-10^{KO}</i> ; <i>chol-1 ras-1^{bd}</i>	4 pg#11, pg#15, pg#23 & pg#44
<i>csp-1 stk-10^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i>	4 pg#34, pg#41, pg#46 & pg#48
<i>csp-1 stk-10⁺</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq⁺</i>	4 pg#16, pg#18, pg#37 & pg#42
<i>csp-1 stk-10⁺</i> ; <i>chol⁺ ras-1^{bd+}</i> ;	4

<i>frq</i> ⁺	pg#7, pg#28, pg#39, pg#45
<i>csp-1 stk-10</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1</i> ^{bd} ; <i>frq</i> ⁺	2 pg#1 & pg#20
<i>csp-1 stk-10</i> ⁺ ; <i>chol-1 ras-1</i> ^{bd+} ; <i>frq</i> ⁺	0
<i>csp-1 stk-10</i> ⁺ ; <i>chol-1 ras-1</i> ^{bd} ; <i>frq</i> ^{l0}	5 pg#10, pg#12, pg#19, pg#40 & pg#47
<i>csp-1 stk-10</i> ⁺ ; <i>chol-1 ras-1</i> ^{bd+} ; <i>frq</i> ^{l0}	2 pg#4 & pg#26
<i>csp-1 stk-10</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1</i> ^{bd+} ; <i>frq</i> ^{l0}	2 pg#29 & pg#36
<i>csp-1 stk-10</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1</i> ^{bd} ; <i>frq</i> ^{l0}	1 pg#33

Table 9.11: *csp-1* & PIK1a^{KO} progeny from cross between SG#80 (*csp-1*: *chol-1 ras-1^{bd}; frq¹⁰ mat a*) and PIK1a^{KO} strains tested on tested on MA medium as well as 100 μ M choline containing MA medium.

Total progeny analyzed on race tubes	25
Controls used for the race tube analysis	- SG#26 (<i>csp-1</i>; <i>chol-1 ras-1^{bd} mat a</i>) - SG#80 (<i>csp-1</i>; <i>chol-1 ras-1^{bd}; frq¹⁰ mat a</i>)
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol-1 ras-1^{bd}; frq</i> ⁺	2 1b & 12b
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1^{bd}; frq</i> ⁺	1 3b
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol-1 ras-1^{bd}; frq¹⁰</i>	1 1c
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1^{bd}; frq¹⁰</i>	1 20b
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1^{bd+}; frq</i> ⁺	2 2b & 13b
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol-1 ras-1^{bd+}; frq</i> ⁺	0
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol</i> ⁺ <i>ras-1^{bd+}; frq¹⁰</i>	1 4c
<i>csp-1</i> ; <i>pik1a</i> ⁺ ; <i>chol-1 ras-1^{bd+}; frq¹⁰</i>	0

<i>csp-1 ; pik1a^{KO}; chol⁺ ras-1^{bd}; frq⁺</i>	1 22b
<i>csp-1 ; pik1a^{KO}; chol-1 ras-1^{bd}; frq⁺</i>	1 11b
<i>csp-1 ; pik1a^{KO}; chol⁺ ras-1^{bd}; frq¹⁰</i>	2 10b & 18b
<i>csp-1 ; pik1a^{KO}; chol-1 ras-1^{bd}; frq¹⁰</i>	4 2c , 5b , 7b & 19b
<i>csp-1 ; pik1a^{KO}; chol⁺ ras-1^{bd+}; frq⁺</i>	3 4b, 6b & 8b
<i>csp-1 ; pik1a^{KO}; chol-1 ras-1^{bd+}; frq⁺</i>	2 9b & 17b
<i>csp-1 ; pik1a^{KO}; chol⁺ ras-1^{bd+}; frq¹⁰</i>	2 5c & 6c
<i>csp-1 ; pik1a^{KO}; chol-1 ras-1^{bd+}; frq¹⁰</i>	0

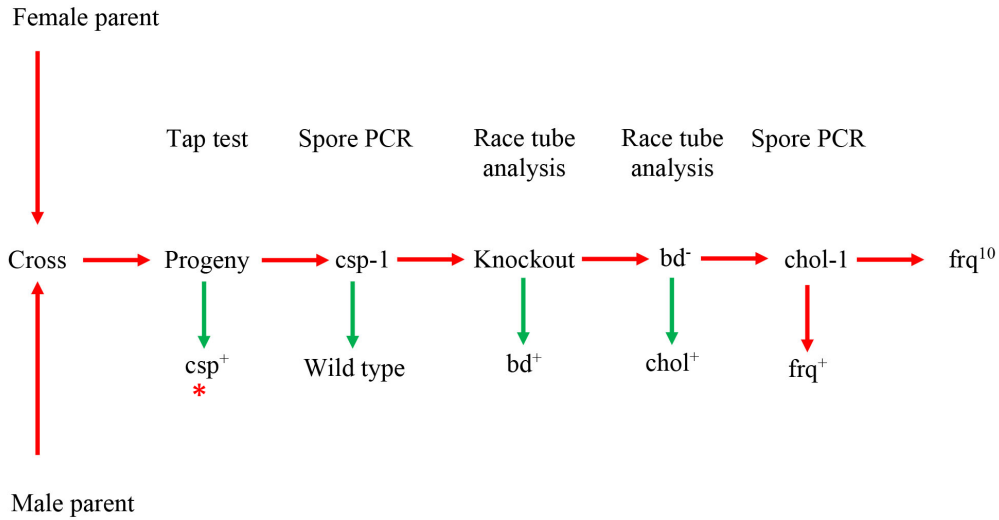


Figure 9.1: Schematic diagram showing the strain analysis process.

* Progeny class that did not assay.

KO = Knockout and W = Wild type

Expected progeny genotypes:

csp-1; chol-1 ras-1^{bd}; frq¹⁰; KO
csp-1; chol-1 ras-1^{bd}; frq⁺; KO
csp-1; chol-1 ras-1⁺; frq¹⁰; KO
csp-1; chol-1 ras-1⁺; frq⁺; KO
csp-1; chol+ ras-1^{bd}; frq¹⁰; KO
csp-1; chol+ ras-1^{bd}; frq⁺; KO
csp-1; chol+ ras-1⁺; frq¹⁰; KO
csp-1; chol+ ras-1⁺; frq⁺; KO
csp-1; chol-1 ras-1^{bd}; frq¹⁰; W
csp-1; chol-1 ras-1^{bd}; frq⁺; W
csp-1; chol-1 ras-1⁺; frq¹⁰; W
csp-1; chol-1 ras-1⁺; frq⁺; W
csp-1; chol+ ras-1^{bd}; frq¹⁰; W
csp-1; chol+ ras-1^{bd}; frq⁺; W
csp-1; chol+ ras-1⁺; frq¹⁰; W
csp-1; chol+ ras-1⁺; frq⁺; W

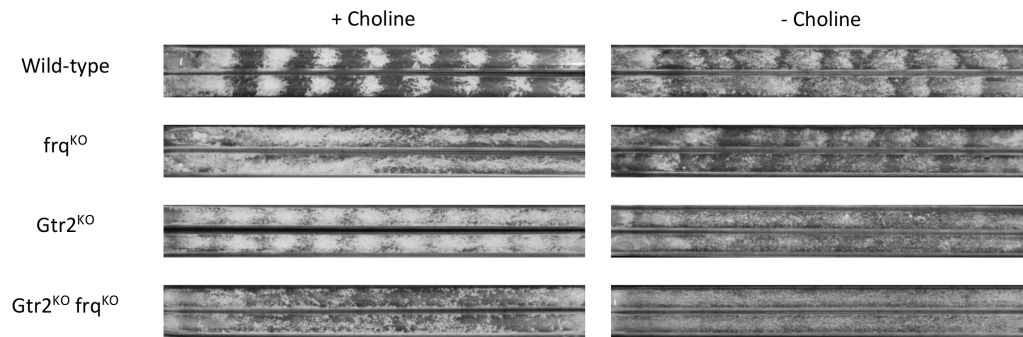
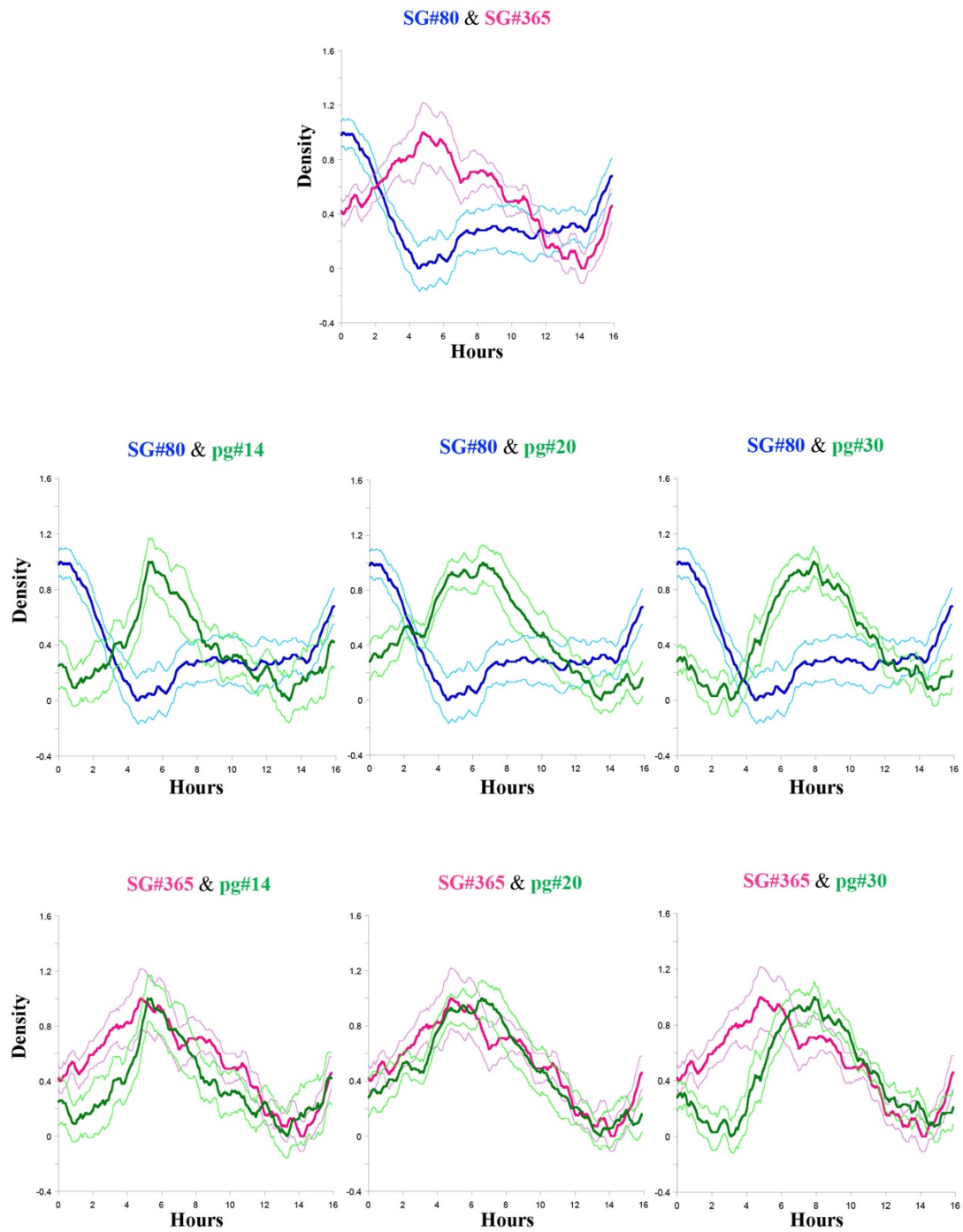


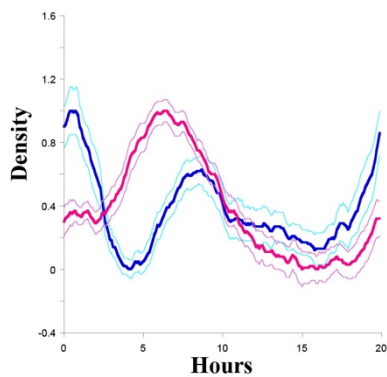
Figure 9.2: Conidiation phenotypes of selected GTR2 knockout mutant progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and FGSC#16041 (GTR2^{KO}) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Two representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Genotypes, periods and growth rates are listed in Table 9.5 & Appendix XXXV - B.

A. T=16 hrs

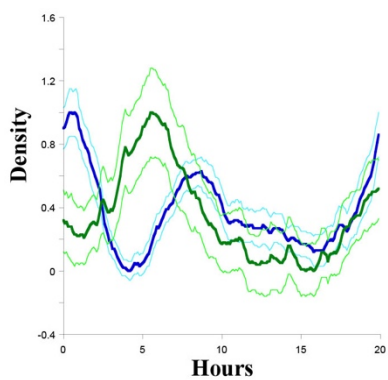


B. T=20 hrs

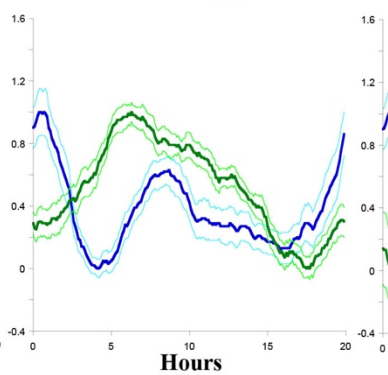
SG#80 & SG#365



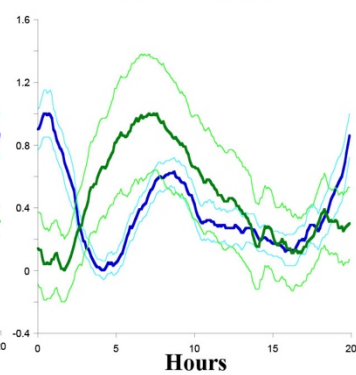
SG#80 & pg#14



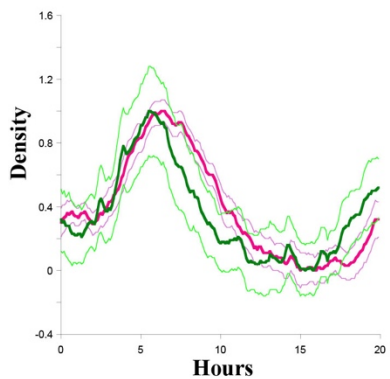
SG#80 & pg#20



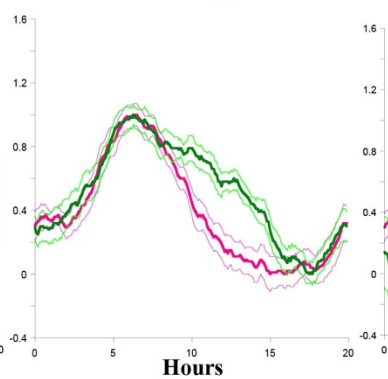
SG#80 & pg#30



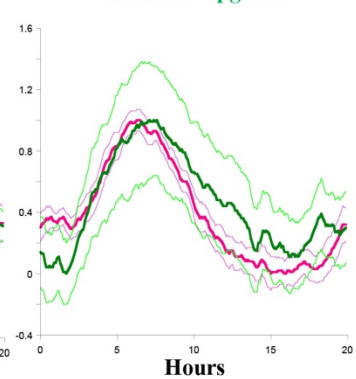
SG#365 & pg#14



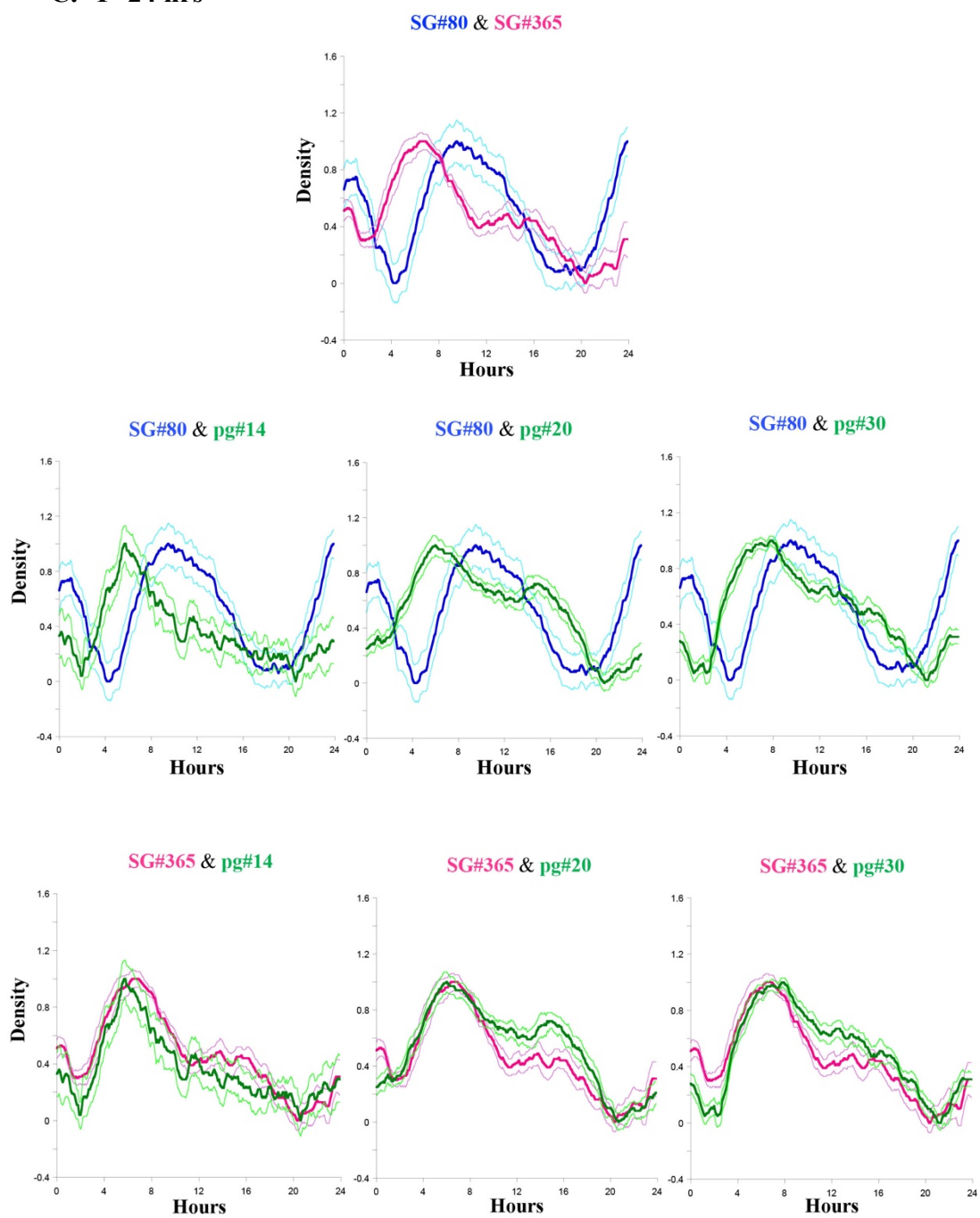
SG#365 & pg#20



SG#365 & pg#30



C. T=24 hrs



D.

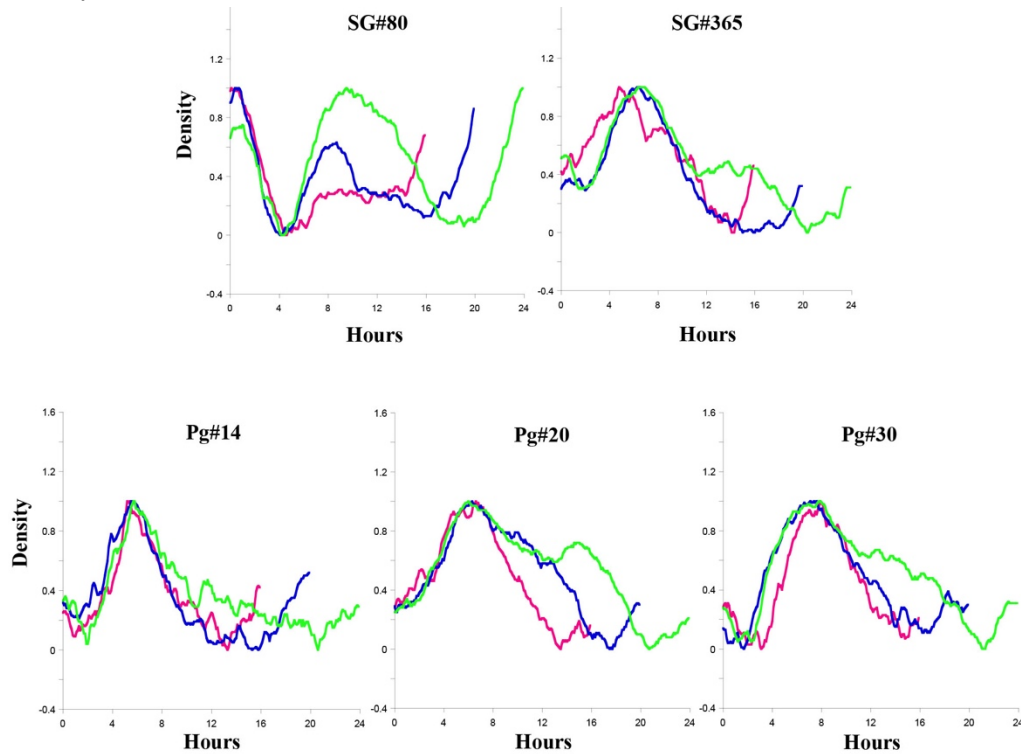


Figure 9.3: Heat pulse entrainment phenotype of GTR2 deletion mutant.

GTR2 wild type control SG#80 (blue), NCU05950^{KO} mutant SG#365 (pink) and GTR2 deletion mutant (green) were grown at 22°C and entrained to repeated 2-hour pulses of 32°C at intervals of T hours. T is the length of one cycle from one pulse start to the next pulse start. The normalized conidiation density of an average “day” (one T-cycle) is plotted against time after the beginning of the heat pulse. T-cycles were 16 hrs (A), 20 hrs (B) or 24 hrs (C). Thin lines are ± 1 S.E.M. Five replicate tubes were averaged for each trace, 2 cycles from each tube, so N = 10 cycles averaged. All strains, including control, are also *csp-1*; *ras^{bd} chol-1*; *frq^{null}*. Growth media contained 100 μ M choline to repair the *chol-1* defect, which is not relevant to this experimental design.

(D). Comparison of different T-cycles for the heat pulse entrainment of GTR2 wild type, NCU05950^{KO} mutant and GTR2 deletion mutants. Data are re-plotted from (A, B and C). T-cycles were 16 hrs (pink), 20 hrs (blue) or 24 h (green).

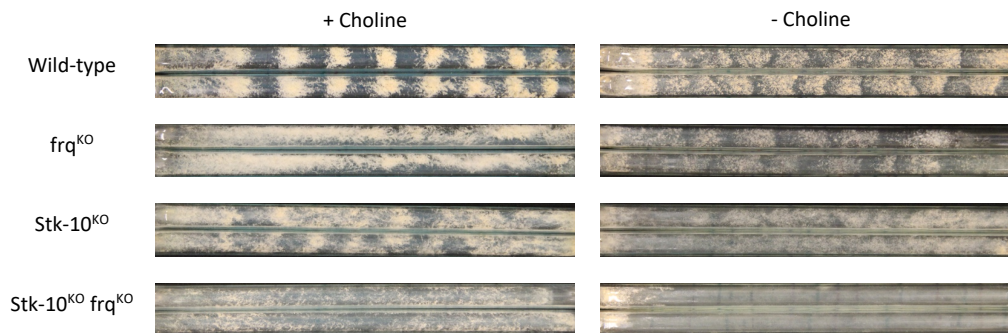
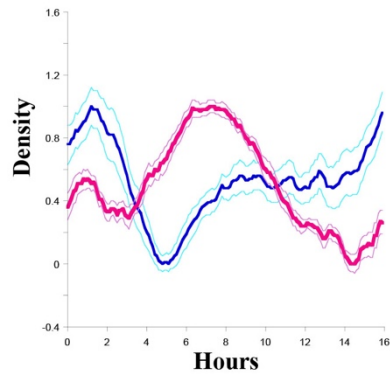


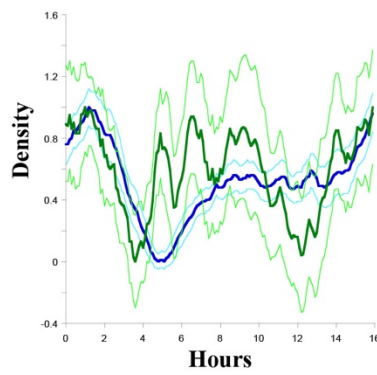
Figure 9.4: Conidiation phenotypes of selected progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and STK-10^{KO} (FGSC#17939 = SG#571) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Two representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Periods and growth rates are listed in Table 9.9 & Appendix XXXVII - B.

A. T = 16 hrs

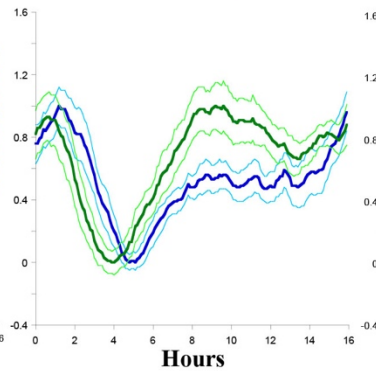
SG#80 & SG#365



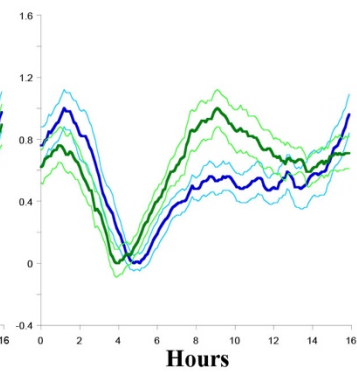
SG#80 & pg#34



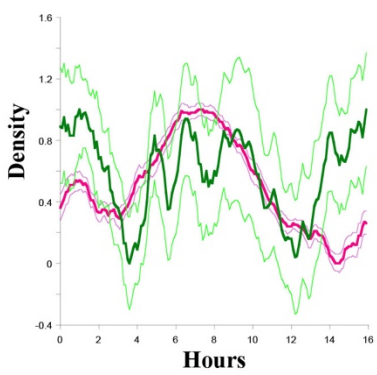
SG#80 & pg#46



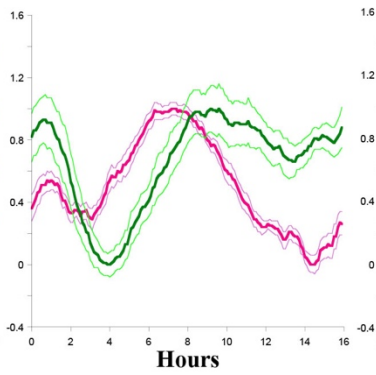
SG#80 & pg#48



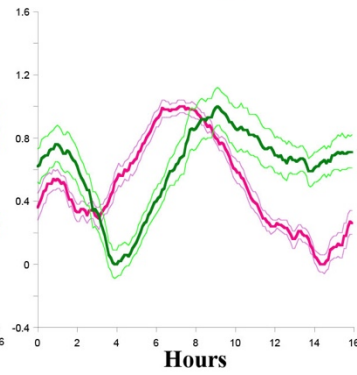
SG#365 & pg#34



SG#365 & pg#46

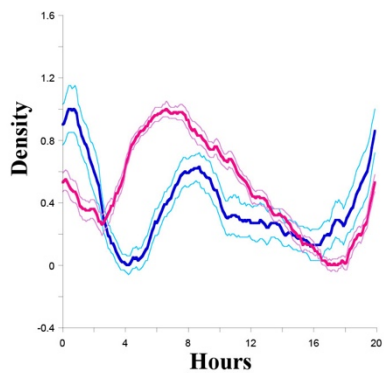


SG#365 & pg#48

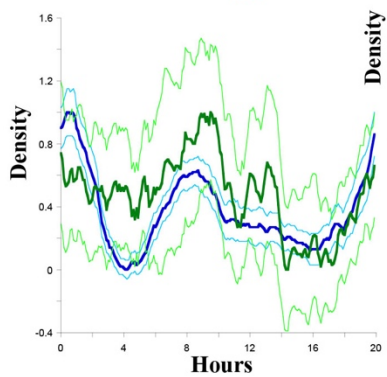


B. T=20 hrs

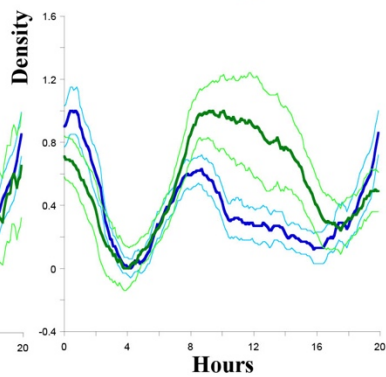
SG#80 & SG#365



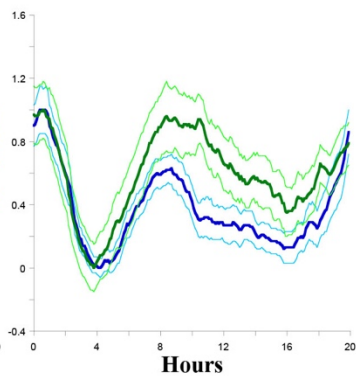
SG#80 & pg#34



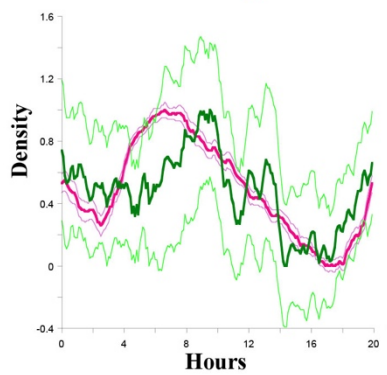
SG#80 & pg#46



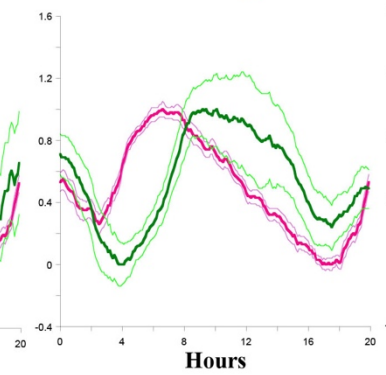
SG#80 & pg#48



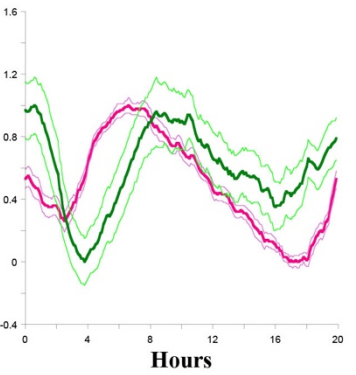
SG#365 & pg#34



SG#365 & pg#46

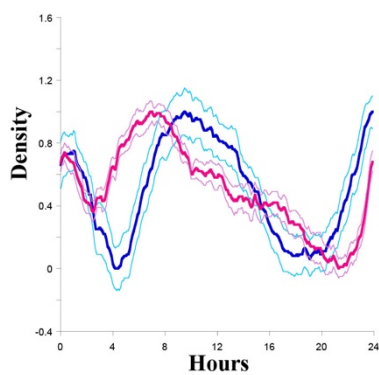


SG#365 & pg#48

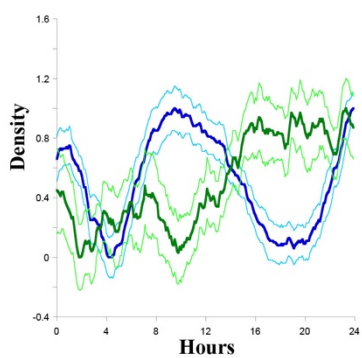


C. T=24 hrs

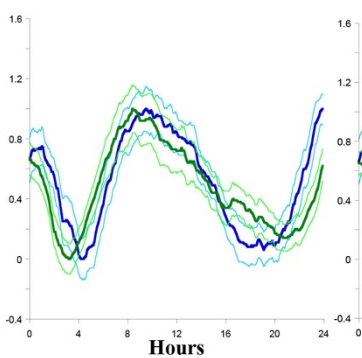
SG#80 & SG#365



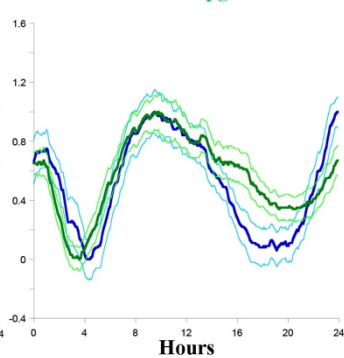
SG#80 & pg#34



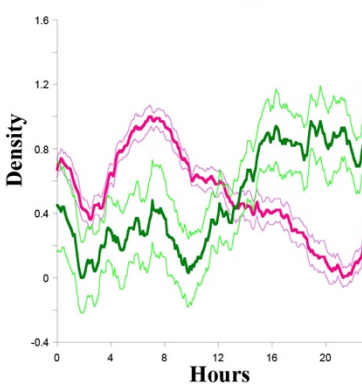
SG#80 & pg#46



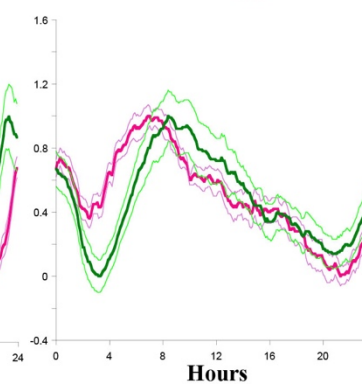
SG#80 & pg#48



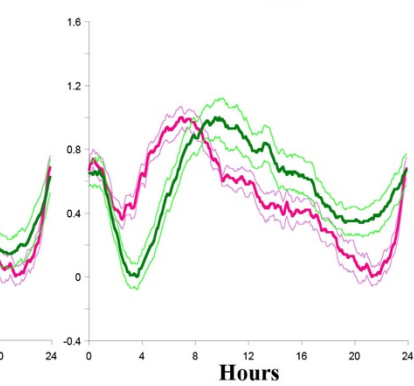
SG#365 & pg#34



SG#365 & pg#46



SG#365 & pg#48



D.

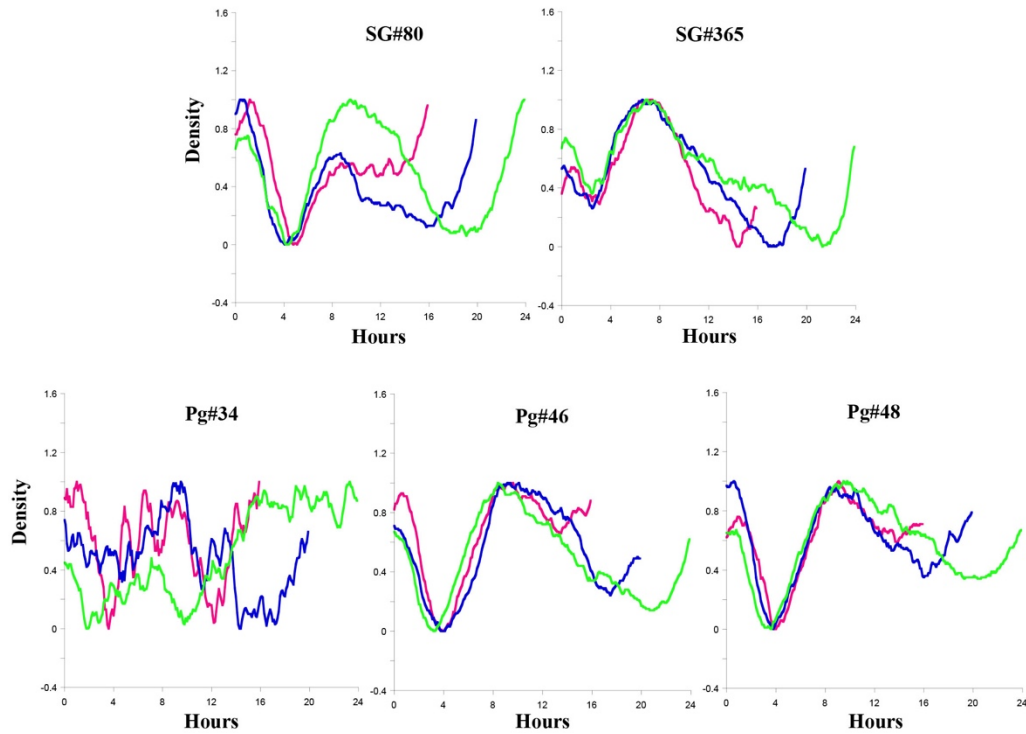


Figure 9.5: Heat pulse entrainment phenotype of Stk-10 (NCU03200) deletion mutants.

Stk-10 wild type control SG#80 (blue), NCU05950^{KO} mutant SG#365 (pink) and Stk-10 (NCU03200) deletion mutant (green) were grown at 22°C and entrained to repeated 2- hour pulses of 32°C at intervals of T hours. T is the length of one cycle from one pulse start to the next pulse start. The normalized conidiation density of an average “day” (one T-cycle) is plotted against time after the beginning of the heat pulse. T-cycles were 16 hrs (A), 20 hrs (B) or 24 hrs (C). Thin lines are ± 1 S.E.M. Five replicate tubes were averaged for each trace, 2 cycles from each tube, so N = 10 cycles averaged. All strains, including control, are also *csp-1*; *ras^{bd} chol-1*; *frq^{null}*. Growth media contained 100 μ M

choline to repair the chol-1 defect, which is not relevant to this experimental design.

(D). Comparison of different T-cycles for the heat pulse entrainment of Stk-10 wild type, NCU05950^{KO} mutant and Stk-10 (NCU03200) deletion mutants.

Data are re-plotted from (A, B and C). T-cycles were 16 hrs (pink), 20 hrs (blue) or 24 h (green).

CHAPTER 10: Effects of VTA in nutrient sensing

10.1: Introduction

Target of Rapamycin (TOR) is a highly evolutionarily conserved serine/threonine kinase, that acts as a central regulator of anabolic processes such as protein synthesis, lipid biosynthesis, nucleotide synthesis, intracellular trafficking, actin cytoskeletal organization, cell growth, proliferation, motility and metabolism (González and Hall 2017). It also represses catabolic processes such as autophagy, to ultimately promote cell growth (González and Hall 2017). The TOR signaling pathway has been well studied in yeast and mammalian cells (Loewith and Hall 2011; Shimobayashi and Hall 2014). TOR functions in response to both extracellular and intracellular signals such as energy status (oxygen and ATP levels), cellular stresses, growth factors such as insulin in mammals, neurotransmitters (glutamate and neuropeptides) and nutrient availability (glucose and amino acids). TOR kinase forms the catalytic subunit of two structurally and functionally distinct multi-protein complexes, TORC1 and TORC2 (González and Hall, 2017; Hill *et al.*, 2018). These complexes are sensitive to distinct stimuli and only TORC1 is sensitive to rapamycin (Loewith *et al.*, 2002). TORC1 regulates protein translation through its downstream effectors yeast Sch9 or mammalian p70S6 kinase (S6K) and its direct target yeast Tap42 or mammalian 4E-binding protein 1 (4E-BP1) (Bröer and Bröer 2017). Furthermore, TORC1 also interacts with the yeast Atg1-Atg-13-Atg17 or mammalian ulk1 (UNC51-like kinase 1)-atg13 (autophagy 13)-FIP200 (focal adhesion kinase-interacting protein 200 kDa) complex to regulate autophagy (Bröer and Bröer 2017; Yang and Klionsky 2009). In general, under nutrient deprived conditions (e.g. when the supply of precursor amino acids is insufficient) or in the presence of rapamycin, TORC1 becomes inactivated and

ensures that protein synthesis is repressed and autophagy is induced (Bröer and Bröer 2017).

Therefore, we have carried out experiments to discover any nutritional effects in NCU05950 (ortholog of yeast EGO1 or mammalian LAMTOR1) , NCU00376 (ortholog of yeast GTR2 or mammalian Rag GTPases - Rag A/B and C/D) and NCU03200 (Stk-10- ortholog of yeast Sch9 or mammalian S6K) related to TOR signaling in *Neurospora crassa* by supplementing wild type and NCU05950 knockout, GTR2 knockout and Stk-10 knockout strains with different amino acids as well as carbon sources to measure the linear growth rate and dry weight. Furthermore, effects of rapamycin on NCU05950 were tested using wild type and FKR-2 (NCU04140) deletion mutant as control strains.

10.2: Methods

10.2.1: Linear growth rates measurement

For assaying linear growth rates on different growth media, race tubes were used as culture vessels and cultures were incubated at 22°C in DD. Growth rates were calculated for each day of growth. Since the growth rates increased until cultures reached the maximum or steady growth rates at day 4 for agar or at day 6 for Phytigel which grew more slowly, growth rates were reported for day 4 only or day 6 for phytigel. (Most of the experiments were conducted by Chanhee Seo.)

10.2.2: Growth rate measurement by obtaining dry weights

For assaying growth rates in liquid media, dry weights were obtained from cultures grown in 1mL of appropriate media in 24 well microtiter plates.

Strains used were the OR wild type (SG#259) along with gene deletion mutants of NCU05950 (SG#357), NCU00376, NCU03200 and NCU02455 with no additional mutations. Conidiospore suspensions were used as inoculum. To obtain the spore suspensions, conidia from culture stock tubes were mixed with 2-5ml of sterile distilled water and the number of spores was counted using the haemocytometer to obtain the concentration of the spore suspension. Spore suspensions were diluted to get 500 /1000 spores in 0.1mL and were inoculated into 1 mL of media in each well. Cultures were grown at 25°C in a 12:12 LD cycle. Preliminary time-course experiments were conducted to find the best time to harvest and it showed that the cultures were growing linearly at 48 hours. Therefore, 48 hours was chosen as the time point at which the cultures were harvested. The mycelia were harvested over nylon net filters, washed with water and dried for 48 hours at 80°C in pre-dried and pre-weighed microfuge tubes or tubes made out of aluminum foil. The dried samples were weighed and the weights of the empty tubes were subtracted to obtain the net dry weights. Three replicates of each condition were averaged to give one value for each independent experiment and means were calculated from three or four independent experiments. Means were compared using the unpaired two-tailed Student's T-test. (Most of the experiments were conducted by Chanhee Seo.)

10.3: Results & Discussion

10.3.1: The NCU05950 deletion mutant is defective in nutrient sensing for growth.

If the NCU05950 gene product functions as a component of the TOR signaling pathway, a deletion mutant has the potential to affect the growth response of *Neurospora crassa* to different nutrition states. Therefore, linear

growth rate on solid agar in race tubes as well as total growth rate (by measuring dry weight gain) in liquid cultures were assayed with various media.

When assaying linear growth rate, SG#1 (*csp-1: ras-1^{bd}*) was used as the wild type control and SG#363 (*csp-1: ras-1^{bd}: NCU05950^{KO}*) was used as the test strain. SG#259 (Oak Ridge standard) strain was used as the wild type control and SG#357 (NCU85950 deletion mutant) with no additional mutations was used as the test strain for the growth experiments in liquid media. These strains were *csp-1⁺* strains, which give uniform conidial suspensions for inoculation of liquid cultures. In every experiment in which the phenotype of *uvr90* mutant or the NCU05950 knockout is assayed, the control strains carried the same genetic background. This allowed us to conclude that the effects we observed are due to the NCU05950 gene and not due to variable genetic background.

The NCU05950 gene product is a component of the TOR signaling pathway, which is the most well-known amino acid sensing pathway in mammals and yeast. Nitrogen sources (typically amino acids) stimulate TORC1 activity and active TORC1 promotes protein synthesis and inhibit autophagy to encourage growth (Jewell *et al.*, 2013). In mammals and yeast, glutamine, leucine and arginine stimulate TORC1 activity (Loewith and Hall 2011; Broer and Broer 2017). Glutamine is the preferred nitrogen source for yeast and in *Neurospora crassa*, it is also the preferred amino acid nitrogen source for growth (Stracka *et al.*, 2014; Davis 2000). Arginine is also a prominent nitrogen source for *Neurospora crassa*. It stores large amounts of arginine in vacuoles and releases it when glutamine is depleted (Legerton and Weiss 1984). Therefore, I chose to assay the effects on growth of three amino acids: 5mM glutamine (gln), 5mM leucine (leu) and 5mM arginine (arg).

The Vogel's minimum liquid medium contains ammonium nitrate (NH_4NO_3) and it is the only nitrogen supplier. Therefore, when assaying the growth on media containing amino acids (gln or leu or arg) as the only nitrogen source, the amino acids were added to Vogel's minimum liquid medium without ammonium nitrate (NH_4NO_3).

The growth rates on solid agar in race tubes with various media were assayed and differential responses of the NCU05950 wild type (SG#1: *csp-1*; *ras-1^{bd}*) and the NCU05950 deletion mutant (SG#363: *csp-1*; *ras-1^{bd}*; *NCU05950^{KO}*) strains were found. As shown in Figure 10.1, on water agar (2%), which did not have any added nutrition, NCU05950 wild type strain (SG# 1) grew faster; on phytagel (2%) with no added nutrition, the mutant SG#363 was faster. But, when yeast extract (2%) was added to agar, both the strains grew at the same rate. This indicates that the NCU05950 deletion mutant does not have a generalized growth defect that slows growth under all conditions, but rather it responds atypically to nutritional state.

When using the standard Vogel's minimal medium (VM) with 2% agar and 2 % glucose, both strains could grow at the same rate without added nitrogen (no NH_4NO_3) sources (Figure 10.1). The wild type strain SG#1 increased its growth rate with added 0.5% casein hydrolysate (a source of amino acids) or the standard ammonium nitrate source, while the mutant strain SG#363 did not respond to these nitrogen sources.

This analysis was repeated with a new set of strains that had the *chol-1* mutation in the background with a sufficient amount of choline added to repair the *chol-1* defect. The following strains were included for the assay: wild type control (SG#26 - *csp-1*; *chol-1 ras-1^{bd} mat a*), the original *uv90* mutant (SG#224 - *csp-1*; *chol-1 ras-1^{bd} ; uv90 mat a*), NCU05950 deletion mutant (SG#361- *csp-1*; *chol-1 ras-1^{bd} ; NCU05950^{KO} mat a*) and two transformants

carrying wild-type copies of NCU05950 at the *his-3* locus in either the NCU05950 deletion background (SG#430 - *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; NCU05950^{KO} mat a*) or the *uv90* mutant background (SG#468- *csp-1 his-3⁺::NCU05950; chol-1 ras^{bd}; uv90*). As shown in Figure 10.2, the results indicated that in most cases the *uv90* mutant phenotype was similar to the NCU05950 deletion and the ectopic copy of the wild-type gene could reverse the growth phenotypes of the mutants. The presence of *chol-1* mutation did not alter the phenotypes as long as there was sufficient choline supplementation for the cultures.

The results obtained from the cultures grown on solid agar medium were complicated due to the presence of unknown nutrients in agar. Therefore, to further refine this analysis, a wild-type control (SG#259) and the NCU05950 deletion mutant (SG#357) were grown in liquid VM medium (without agar to avoid the unknown impurities that allow growth on water agar alone) with or without ammonium nitrate (which is the standard nitrogen source), 2 % glucose and different amino acids.

Both extra glutamine and arginine added to VM medium increased the growth of the wild-type strain (SG#259) but had no significant effect on the growth of the NCU05950 deletion mutant strain (SG#357) (Figure 10.3). In VM medium without ammonium nitrate (VM - No NH₄NO₃), arginine was significantly better than glutamine at supporting the growth of the wild-type (SG#259), but there was no significant difference in the SG#357 which is the NCU05950 deletion mutant (Figure 10.3). Leucine inhibited the wild type (SG#259) in VM medium but had little effect on the NCU05950 deletion mutant (SG#357), and leucine was a poor nitrogen source for growth of both strains in the absence of ammonium nitrate (Figure 10.3).

Leucine and glutamine activate TORC1 through different pathways in both mammalian cells and yeast (Gonzalez and Hall 2017; Varlakhanova *et al.*, 2017; Ukai *et al.*, 2018). Activation of TORC1 by leucine depends on the EGO complex in yeast (Gtr1, Gtr2, Ego1 and Ego3) or the functionally homologous Ragulator-Rag complex in mammalian cells (RagA/ B, RagC/D and Ragulator) while glutamine activates TORC1 by another poorly-characterized pathway that involves Pib2, a FYVE-containing PI3P-binding protein (Stracka *et al.*, 2014; Jewell *et al.*, 2015; Varlakhanova *et al.*, 2017; Ukai *et al.*, 2018). Our results may also indicate separate pathways for different amino acids in *N. crassa*.

We next tested the effects on growth rate of varying glucose concentration (0.125%, 0.25%, 0.5%, 1%, 2% and 4%) by measuring the dry weights of fungal mycelia grown in liquid media as described in section 10.2.2. The NCU05950 deletion mutant grew at the same rate as the wild type at low glucose concentrations but grew more slowly than wild type at higher concentrations of glucose (Figure 10.4).

Taken together, these results indicate that the NCU05950 deletion mutant is defective in its growth response to added nutrition, both nitrogen and carbon sources, and therefore the TOR pathway is implicated in growth responses in *N. crassa*.

10.3.2: The NCU00376 (GTR2) deletion mutant is also defective in nutrient sensing for growth.

If the NCU00376 gene product functions in the same pathway as NCU05950, a deletion mutant might phenocopy the growth response of NCU05950 deletion mutant to different nutrition states. Therefore, total growth rate (by measuring dry weight gain) in liquid cultures was assayed.

A wild-type control (SG#259) and the NCU00376 (GTR2^{KO}) deletion mutant (SG#510) were grown in liquid VM medium with or without added ammonium nitrate (which is the standard nitrogen source), 2 % glucose and different amino acids.

Both extra glutamine and arginine added to VM medium increased the growth of the wild-type strain (SG#259) but had no significant effect on the growth of the NCU00376 deletion mutant strain (SG#510) (Figure 10.5). In VM medium without ammonium nitrate (VM - No NH₄NO₃), arginine was significantly better than glutamine at supporting the growth of the wild-type, but there was no significant difference in the NCU00376 deletion mutant (Figure 10.5). Leucine inhibited the wild type (SG#259) in VM medium but had little effect on the NCU00376 deletion mutant (SG#510), and leucine was a poor nitrogen source for growth of both strains in the absence of ammonium nitrate (Figure 10.5).

The effects on growth rate of varying glucose concentration (0.125%, 0.25%, 0.5%, 1%, 2% and 4%) were tested by measuring the dry weights of fungal mycelia grown in liquid media as described in section 10.2.2. The NCU00376 deletion mutant grew at the same rate as the wild type at low glucose concentrations but grew more slowly than wild type at higher concentrations of glucose (Figure 10.6).

The effects of amino acids and glucose concentrations on NCU00376 deletion mutant were comparable to that of the NCU05950 deletion mutant. Therefore, these results support our prediction, which is NCU05950 and NCU00376 are involved in the nutrient sensing TOR signaling pathway.

10.3.3: The NCU03200 (STK-10) deletion mutant displayed distinct nutrient sensing and growth patterns.

NCU03200 (STK-10) was identified as a homolog of the yeast Sch9 (homologous to the TOR kinase substrate S6K in mammals), which is a downstream effector of the TORC1 (Park *et al.*,2011). The same study found that the NCU03200 deletion strain had impaired growth and osmoregulation. The NCU03200 deletion strain is sensitive to sodium chloride and the NCU03200 gene product is involved in basal hyphae development as well as asexual development (aerial hyphae development) (Park *et al.*,2011).

We assayed the total growth rate (by measuring dry weight gain) in liquid cultures. A wild-type control (SG#259) and the NCU03200 (STK-10^{KO}) deletion mutant (SG#571) were grown in liquid VM medium with or without added ammonium nitrate (which is the standard nitrogen source), 2 % glucose and different amino acids.

Both extra glutamine and arginine added to VM medium increased the growth of the wild-type (SG#259) and also had significant effects on the growth of the NCU03200 deletion mutant (SG#571) strain (Figure 10.7). In VM medium without ammonium nitrate (VM - No NH₄NO₃), arginine was significantly better than glutamine at supporting the growth of the wild-type as well as the NCU03200 deletion mutant (Figure 10.7). Leucine inhibited the wild type (SG#259) in VM medium and also the NCU03200 deletion mutant (SG#571), and leucine was a poor nitrogen source for growth of both strains in the absence of ammonium nitrate (Figure 10.7).

The effects on growth rate of varying glucose concentration (0.125%, 0.25%, 0.5%, 1%, 2% and 4%) were tested by measuring the dry weights of fungal mycelia grown in liquid media as described in section 10.2.2. The

NCU03200 deletion mutant grew more slowly than wild type and growth rate was nearly same for all the tested glucose concentrations (Figure 10.8).

In order to assay the effect of different carbon sources, a wild-type control (SG#259) and the NCU03200 (STK-10^{KO}) deletion mutant (SG#571) were grown in liquid as well as solid (with 2% agar) VM medium that substituted 0.5% maltose for 2% glucose. Maltose-arginine (MA) growth medium (containing 1x Vogel's salts, 0.5% maltose, 0.01% L-arginine) is normally used for race tube analysis and therefore, maltose was selected. NCU03200 (STK-10^{KO}) deletion mutant (SG#571) grew more slowly than the wild-type control (SG#259) on all the tested media (Figure 10.9 & Figure 10.10). There was no significant effect on growth rates from different carbon sources on VM medium with 2 % agar for both the strains (Figure 10.9). However, on liquid VM medium glucose was significantly better than maltose at supporting the growth of the wild-type (SG#259) as well as the NCU03200 deletion mutant (Figure 10.10).

Unlike the NCU05950 deletion mutant and NCU00376 deletion mutant, NCU03200 deletion mutant was able to respond to addition of amino acids as nitrogen source, but it did not respond to changes in glucose levels. This result suggests that NCU03200 gene product functions in the glucose (Carbon) sensing pathway.

10.3.4: NCU05950 gene product does not participate in rapamycin induced growth inhibition.

The effect of rapamycin on *Neurospora crassa* wild type strain (SG#259) and NCU05950 knockout strain (SG#357) was examined by measuring the linear growth rate on solid agar medium in race tubes as well as

total growth rate (by measuring dry weight gain) in liquid cultures at different concentrations of rapamycin. There was no significant difference in linear growth rate between the NCU05950 deletion mutant strain and wild type strain (Figure 10.11). Total growth rate was obtained by measuring dry weight gain in VM liquid cultures with different concentrations of rapamycin using FKR-2 (NCU04140) deletion mutant as the control strain. FK506-binding protein 1A or Peptidyl-prolyl cis-trans isomerase fkr-2 has been identified as a rapamycin binding protein in *Neurospora crassa* (Solscheid and Tropschug 2000; Pinto *et al.*, 2008). As shown in Figure 10.12, rapamycin inhibits the growth of wild type (SG#259) and NCU05950 knockout (SG#357) strains, but there is no significant effect on the FKR-2 (NCU04140) deletion mutant strain except at the highest concentrations. The growth inhibitory effect of rapamycin became saturated at concentrations above ~1000 ng /mL and increased concentrations of rapamycin below this correlated with increased growth inhibition.

This result suggests that in *Neurospora crassa*, rapamycin induced growth inhibition occurs without participation of NCU05950 gene product, which is homologous to human p18/LAMTOR1 and yeast EGO1/Meh1p/Gse2p. In yeast, EGO1 is localized to the vacuolar membrane and serves as an anchor for the EGO complex (Gtr1, Gtr2, Ego1 and Ego3). Human p18/LAMTOR1 is localized to the surface of late endosomes/lysosomes to anchor the functionally homologous Ragulator-Rag complex (RagA/ B, RagC/D and Ragulator) in mammalian cells. These complexes function upstream of TORC1. Similar to yeast (Evans *et al.*, 2014), in *Neurospora crassa* additional EGO complex / Ragulator-Rag complex -independent mechanisms must exist for maintaining activity of TORC1 in the presence of rapamycin.

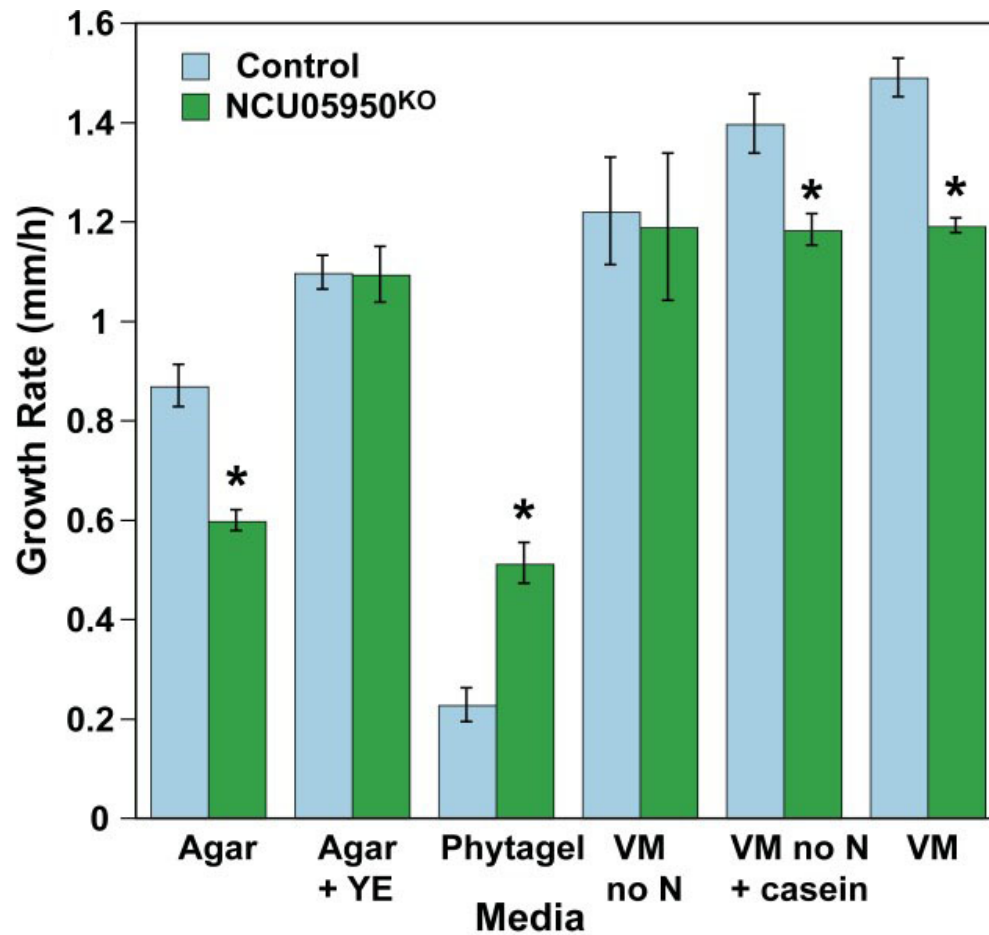


Figure 10.1: Effects of nutrients on growth rate. Cultures of control (*csp-1*; *ras^{bd}*) and NCU05950 deletion mutant (*csp-1*; *ras^{bd}*; *NCU05950^{KO}*) were grown on race tubes. Left three sets of columns: Media contained either agar or Phytigel as a gelling agent, and no additional nutrients except Yeast Extract (YE) in one set. Right three sets of columns: Media contained Vogel's minimal salts plus 2% glucose and 2% agar, and either no nitrogen source (VM no N), casein hydrolysate as nitrogen source (VM no N + casein), or the usual NH_4NO_3 nitrogen source (VM). Values are the mean of 6-9 replicate race tubes and error bars are $\pm$ S.E.M. Stars (*) indicate statistically significant difference between control and mutant, $p < 0.01$. (Ratnayake *et al.*, 2018)

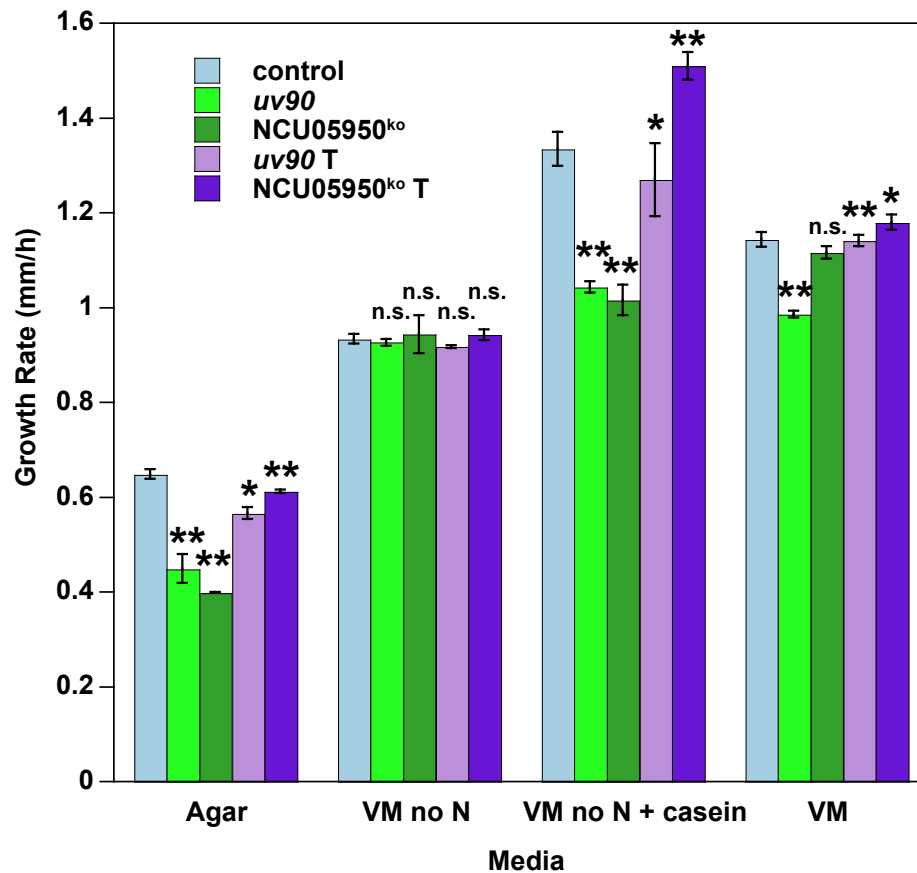


Figure 10.2: Reversal of nutrient growth effects by transformation with wild-type NCU05950 gene. Strains were grown on race tubes containing various growth media as described in Figure 10.1. All strains carried the *csp-1*; *ras^{bd}* *chol-1* background and were grown with 100 μ M choline added to the media. *uv90* strains are the original *uv90* mutant. NCU05950^{KO} strains are the deletion mutant. Strains marked T (for transformant) also carried a wild-type copy of NCU05950 inserted at the *his-3* locus. Values are the mean of three replicate race tubes and error bars are $\pm$ S.E.M. Stars (*) indicate statistically significant difference between mutant and control, or between transformant and corresponding mutant; *, $p < 0.05$; **, $p < 0.01$; n.s., not significantly different. (Ratnayake *et al.*, 2018)

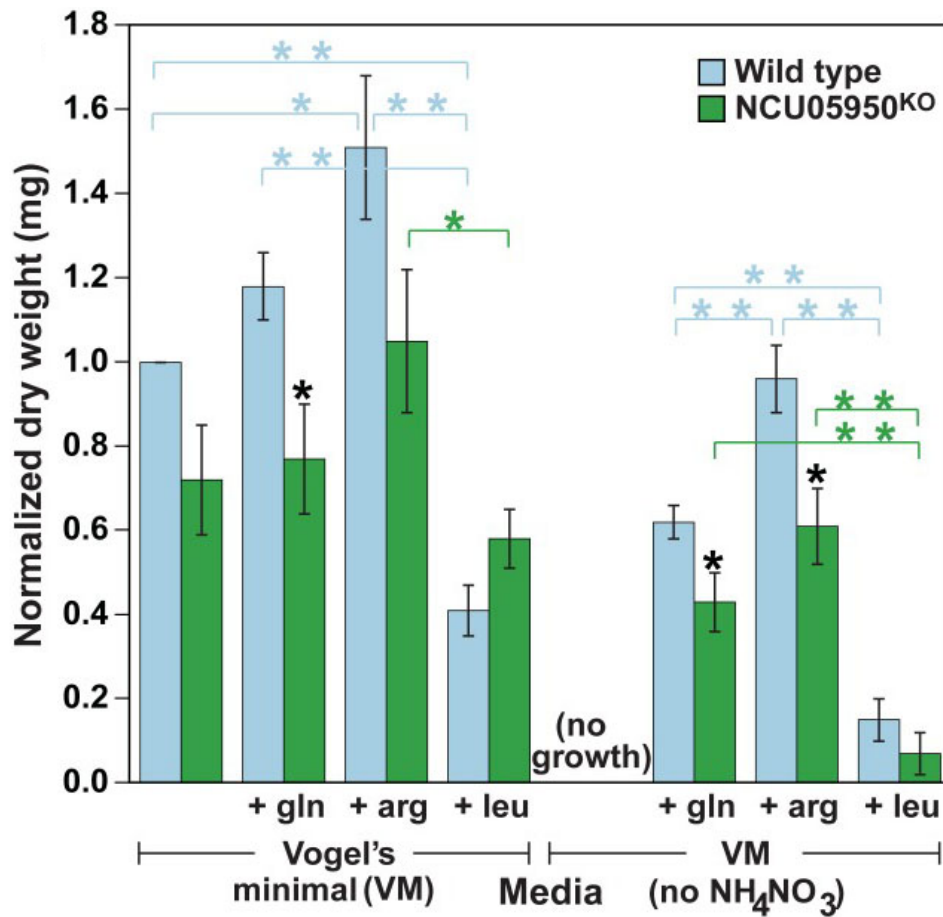


Figure 10.3: Effects of nutrients on dry weight. Cultures of wild type or NCU05950 deletion mutant (carrying no additional mutations) were grown in liquid media with Vogel's minimal medium (VM) and 2% glucose, with or without added NH_4NO_3 and added amino acids. Error bars are mean of four replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild-type weight on VM = 1.0. Black stars (*) indicate statistically significant difference between wild type and mutant for the same growth condition, $p < 0.05$. Colored stars indicate statistically significant difference between growth conditions for the same strain, $p < 0.05$ (*) or $p < 0.01$ (**). (Ratnayake *et al.*, 2018)

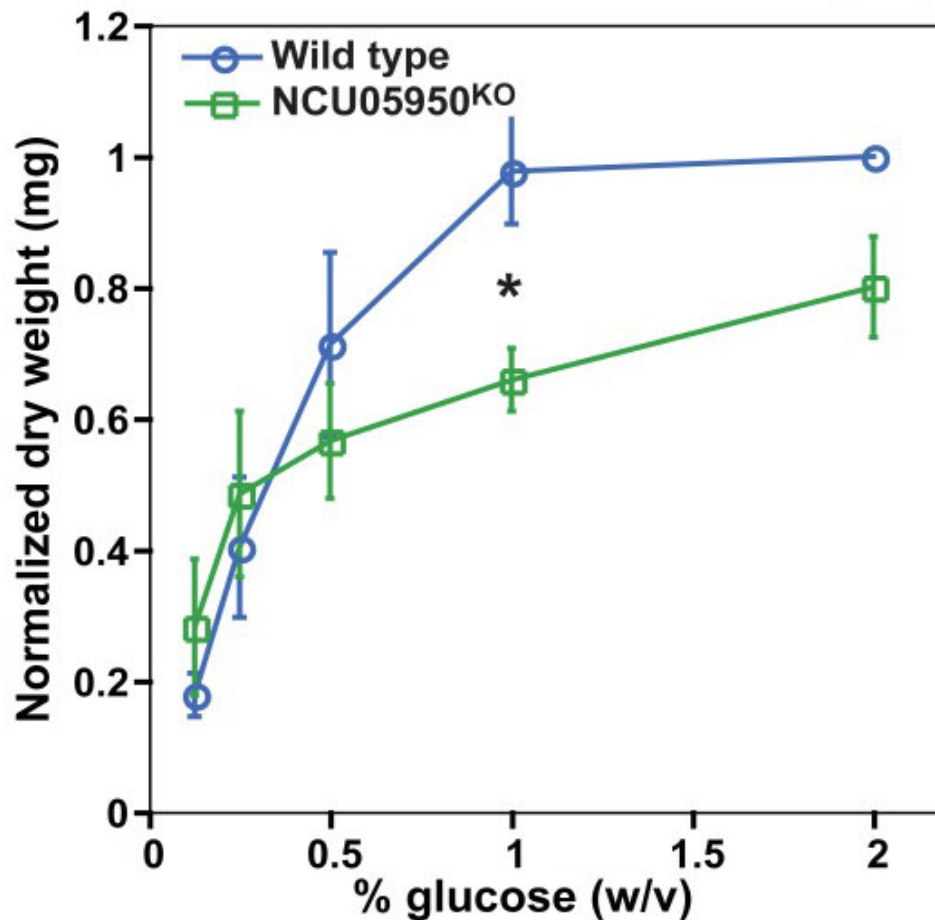


Figure 10.4: Effects of glucose concentration on growth rate. Cultures were grown in VM medium with NH_4NO_3 and various concentrations of glucose. Strains as for Figure 10.3. Error bars are mean of three replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild-type weight on 2% glucose = 1.0. Black star (*) indicates statistically significant difference between wild type and mutant, $p < 0.05$.

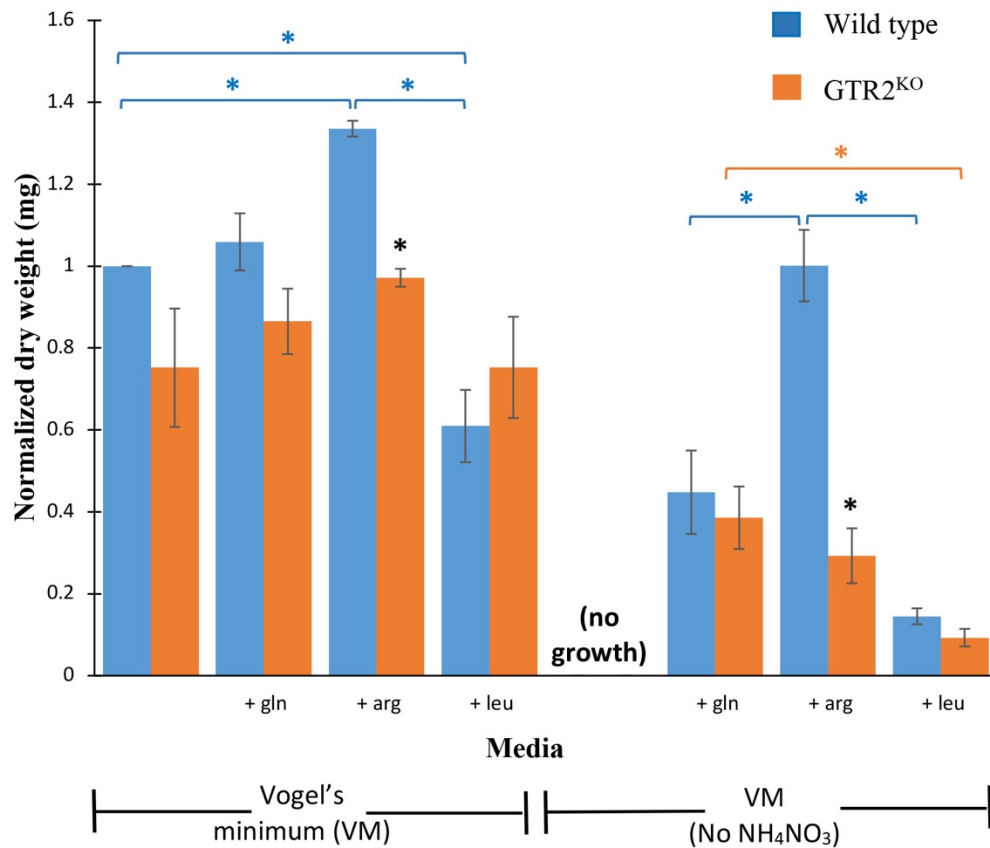


Figure 10.5: Effects of nutrients on dry weight of GTR2 deletion mutant. Cultures of wild type or GTR2 deletion mutant (carrying no additional mutations) were grown in liquid media with Vogel's minimal medium (VM) and 2% glucose, with or without added NH_4NO_3 and added amino acids. Error bars are mean of four replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild-type weight on VM = 1.0. Black stars (*) indicate statistically significant difference between wild type and mutant for the same growth condition, $p < 0.01$. Colored stars indicate statistically significant difference between growth conditions for the same strain, $p < 0.05$.

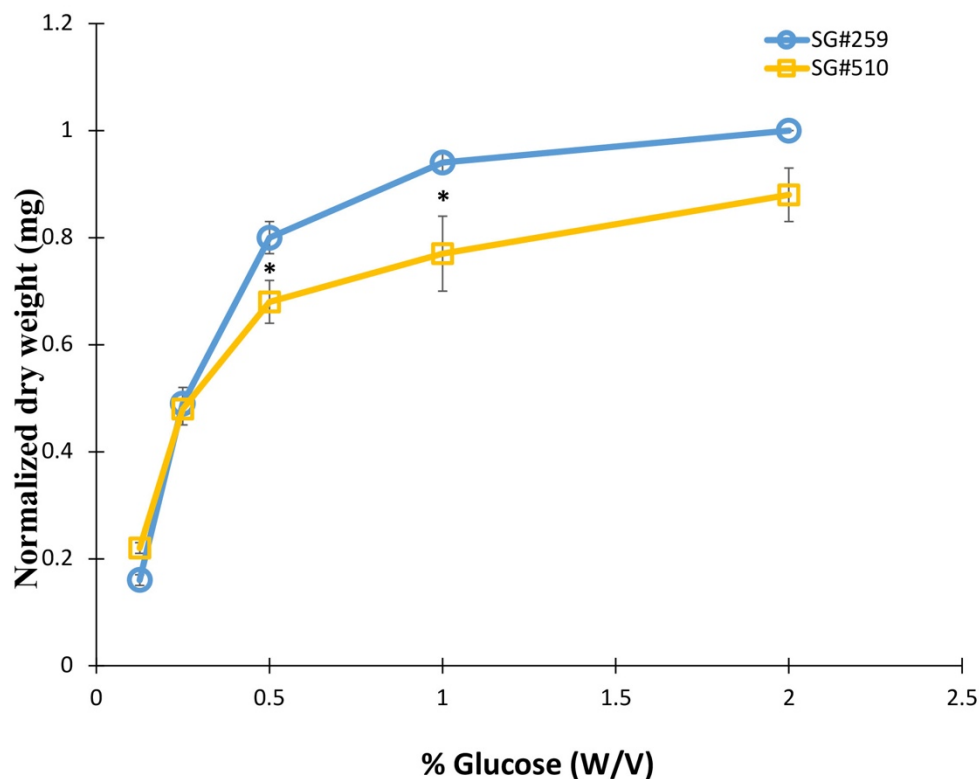


Figure 10.6: Effects of glucose concentration on growth rate of GTR2^{KO}. Cultures were grown in VM medium with NH₄NO₃ and various concentrations of glucose. Strains as for Figure 10.5. Error bars are mean of three replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild-type weight on 2% glucose = 1.0. SG#259 = Wild type strain and SG#510 = GTR2^{KO}. Black star (*) indicates statistically significant difference between wild type and mutant, $p < 0.01$.

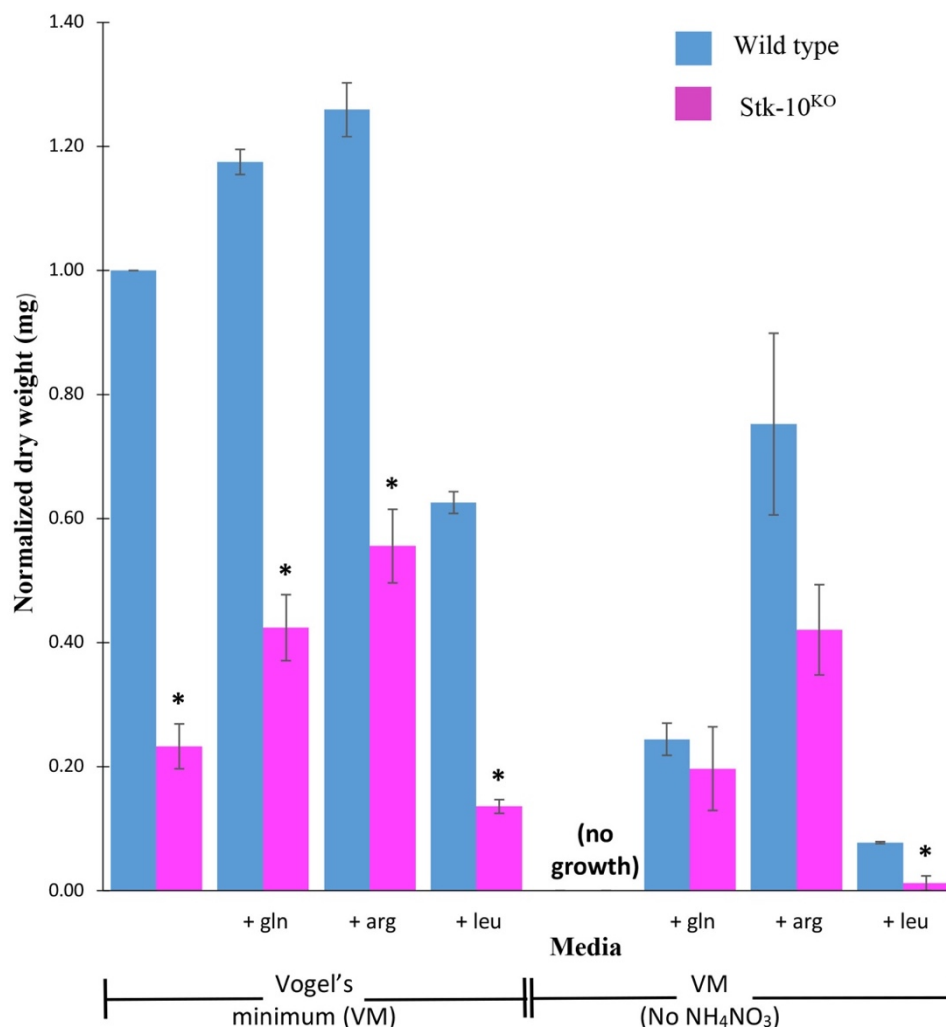


Figure 10.7: Effects of nutrients on dry weight of Stk-10 deletion mutant.

Cultures of wild type or Stk-10 deletion mutant (carrying no additional mutations) were grown in liquid media with Vogel's minimal medium (VM) and 2% glucose, with or without added NH_4NO_3 and added amino acids. Error bars are mean of four replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild-type weight on VM = 1.0. Stars (*) indicate statistically significant difference between control and mutant, $p < 0.01$.

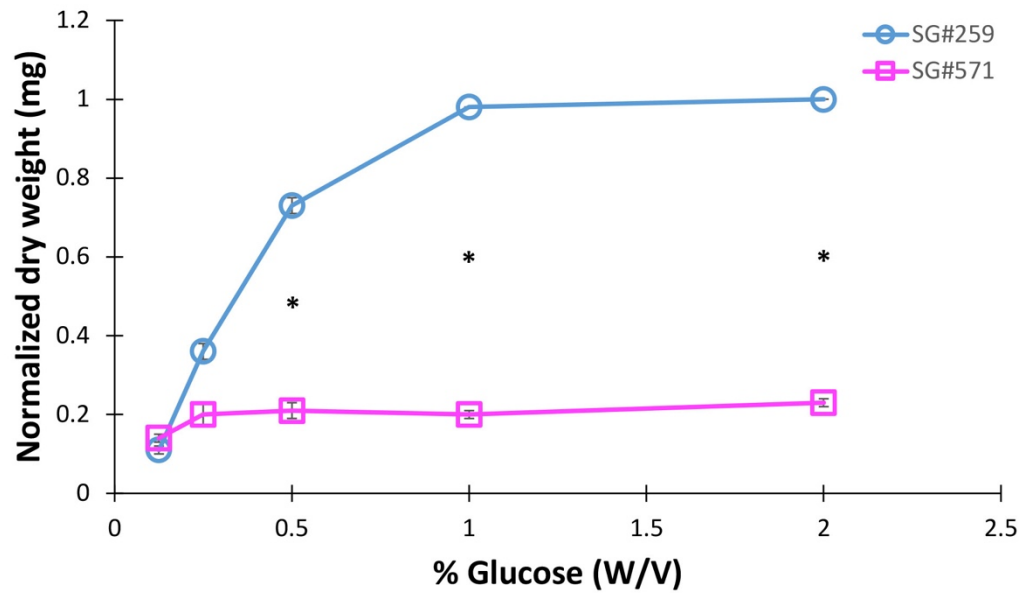
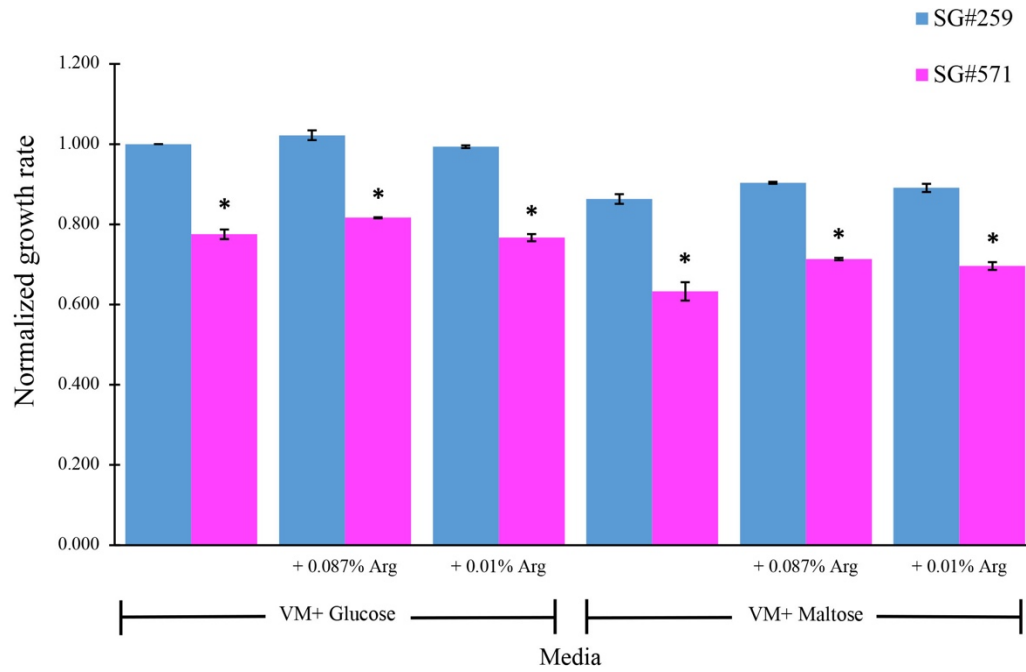


Figure 10.8: Effects of glucose concentration on growth rate of Stk-10^{KO}.

Cultures were grown in VM medium with NH_4NO_3 and various concentrations of glucose. Strains as for Figure 10.7. Error bars are mean of three replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild-type weight on 2% glucose = 1.0. SG#259 = Wild type. SG#571 = Stk-10^{KO}. Black star (*) indicates statistically significant difference between wild type and mutant, $p < 0.01$.

A.



B.

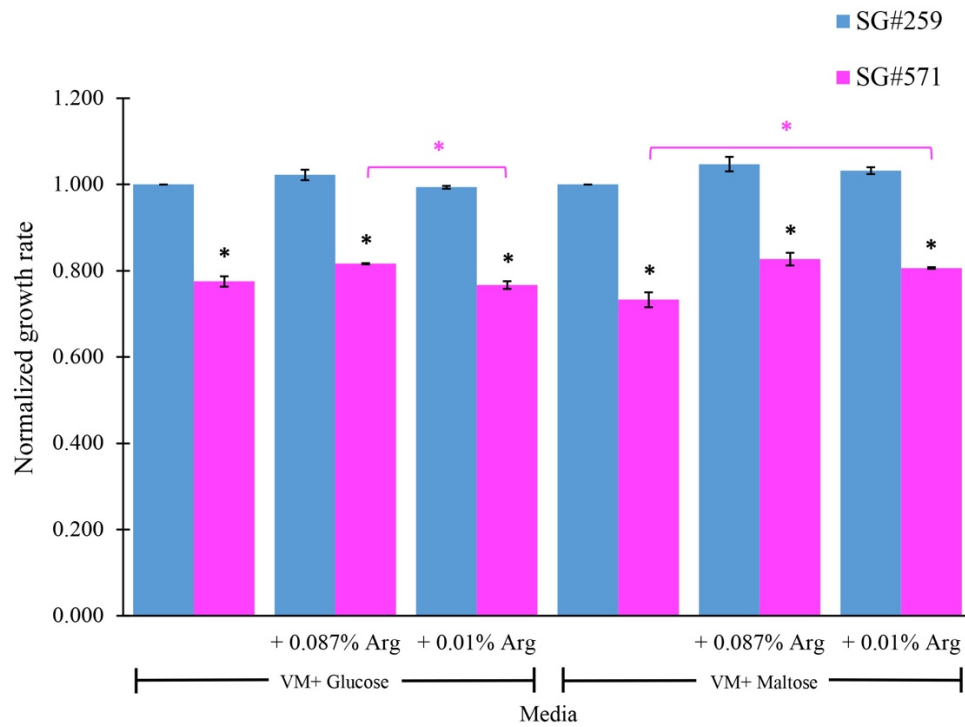


Figure 10.9: Effects of different carbon sources on growth rate of Stk-10 deletion mutant. Cultures of wild type control strain SG#259 and Stk-10 deletion mutant strain SG#571 were grown on race tubes. Left three sets of columns: Media contained Vogel's minimal salts plus 2% glucose, 2% agar and either 0.087 % (5 mM) Arginine or 0.01% Arginine. Right three sets of columns: Media contained Vogel's minimal salts plus 0.5 % maltose, 2% agar and either 0.087 % (5 mM) Arginine or 0.01% Arginine. Values are the mean of 3 replicate race tubes and error bars are $\pm$ S.E.M. Black star (*) indicates statistically significant difference between wild type and mutant, $p < 0.05$. Colored stars indicate statistically significant difference between growth conditions for the same strain, $p < 0.05$. A. growth rates were normalized to the wild-type growth rate on 2% glucose = 1.0. B. Growth rates were normalized to the wild-type growth rate on 2 % glucose (Left three sets of columns) and 0.5 % maltose (Right three sets of columns) = 1.0.

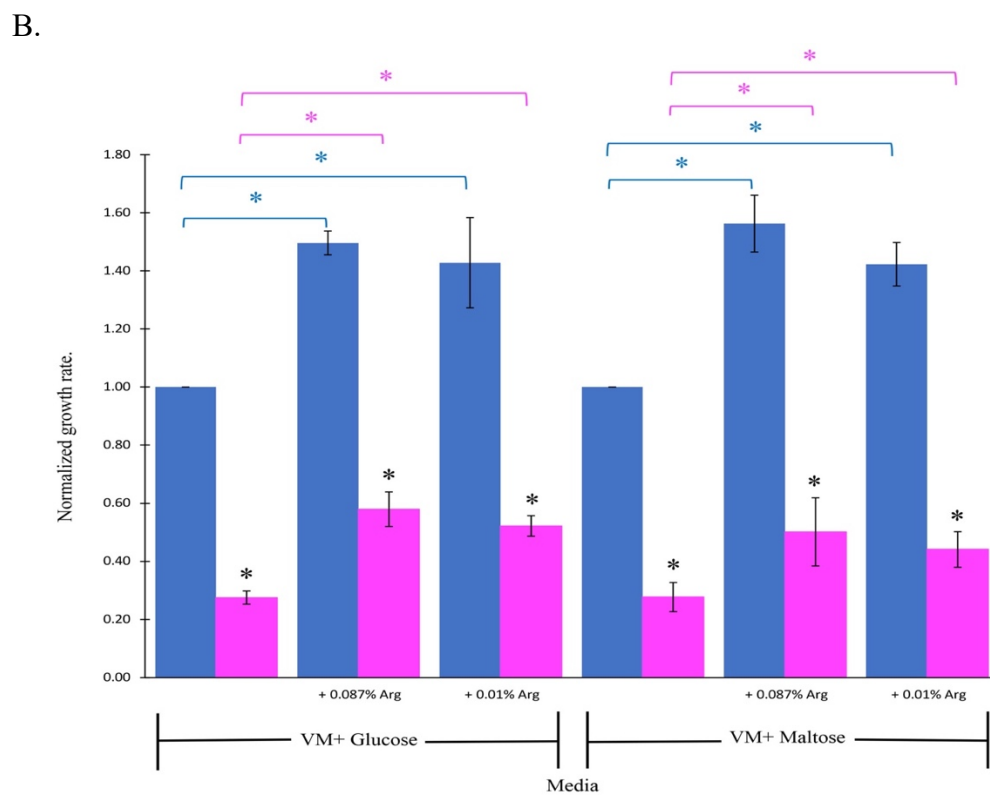
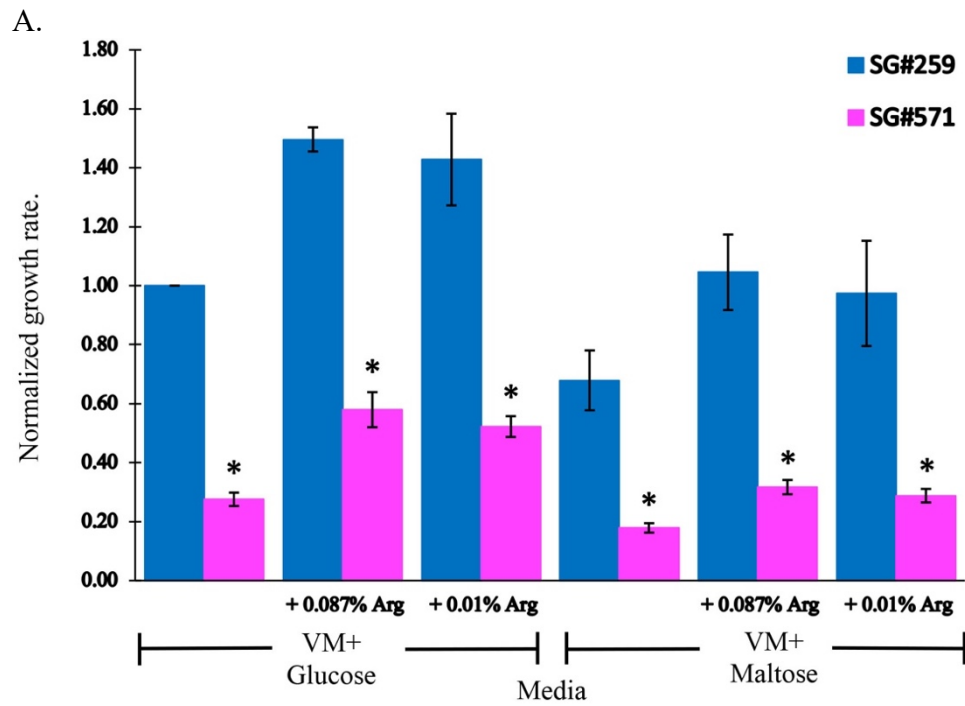


Figure 10.10: Effects of different carbon sources on dry weight of Stk-10 deletion mutant. Cultures of wild type control strain SG#259 and Stk-10 deletion mutant strain SG#571 (*NCU03200.2^{KO}*) were grown in liquid media. Left three sets of columns: Media contained Vogel's minimal salts plus 2% glucose and either 0.087 % (5 mM) Arginine or 0.01% Arginine. Right three sets of columns: Media contained Vogel's minimal salts plus 0.5 % maltose and either 0.087 % (5 mM) Arginine or 0.01% Arginine. Error bars are mean of the three replicate experiments $\pm$ S.E.M. Black star (*) indicates statistically significant difference between wild type and mutant, $p < 0.05$. Colored stars indicate statistically significant difference between growth conditions for the same strain, $p < 0.05$. A. Dry weights within each experiment were normalized to the wild-type weight on 2% glucose = 1.0. B. Dry weights within each experiment were normalized to the wild-type weight on 2% glucose (Left three sets of columns) and 0.5 % maltose (Right three sets of columns) = 1.0.

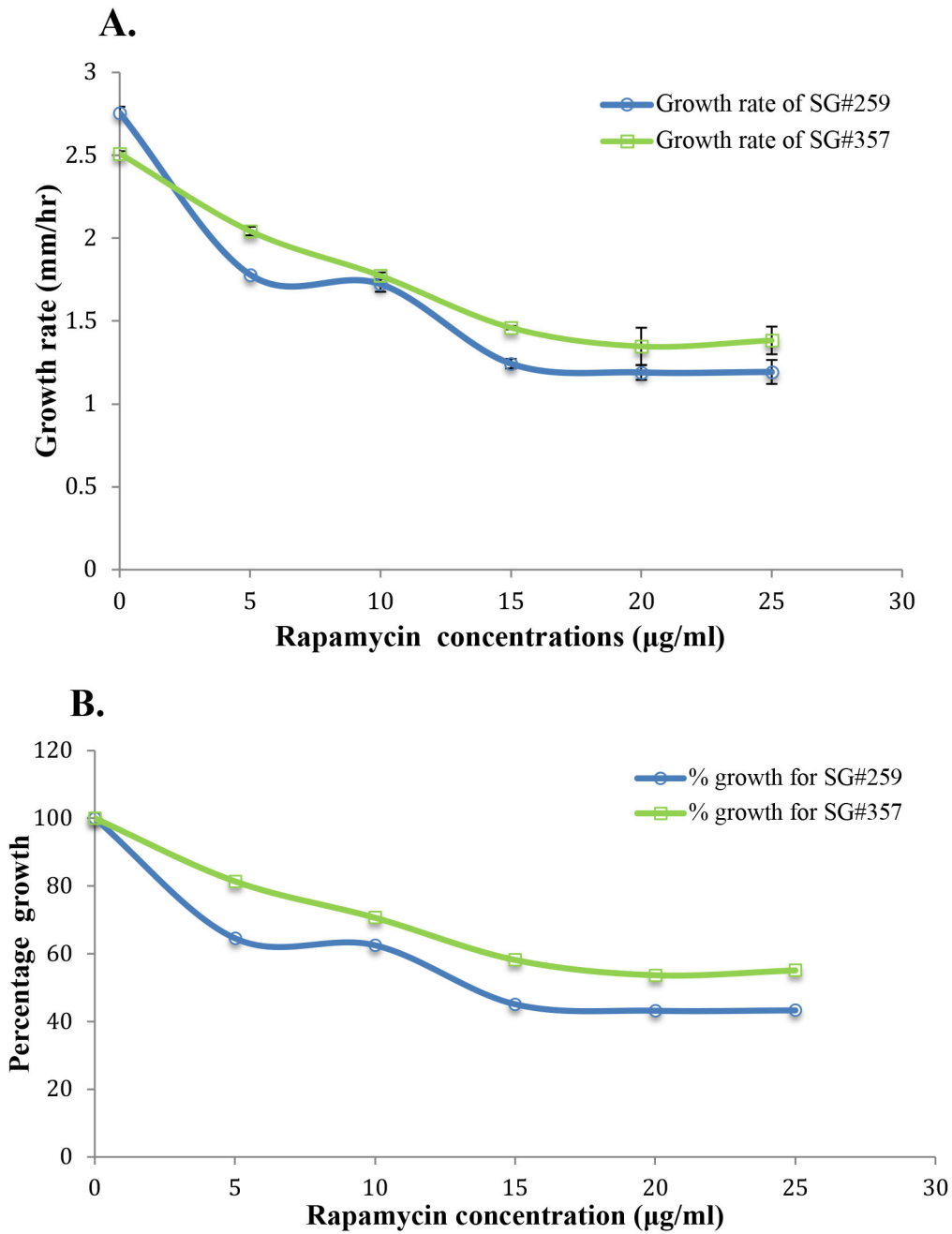


Figure 10.11: Growth rate (A) and percentage growth (B) differences between wild type (SG#259) and NCU05950^{KO} (SG#357) strains on Rapamycin containing agar medium in race tubes.

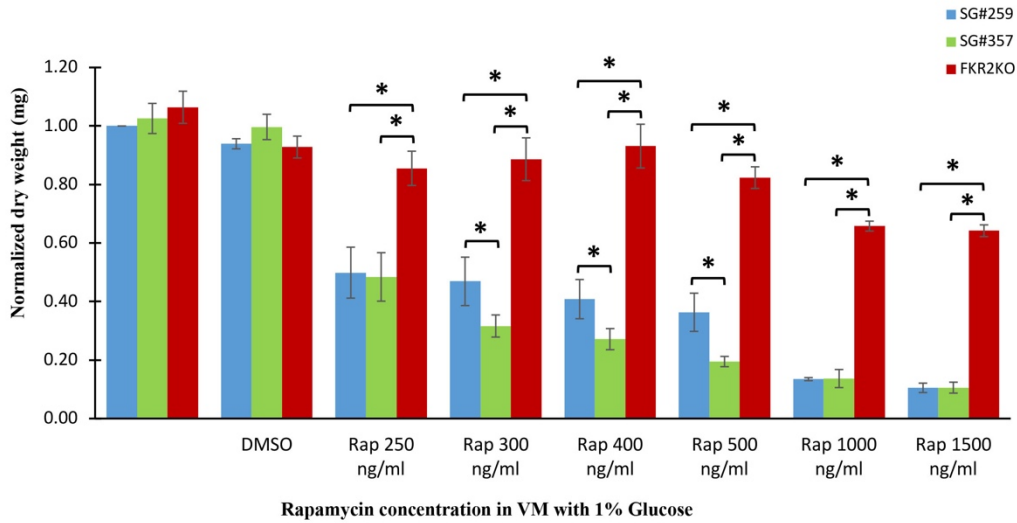


Figure 10.12: The effect of rapamycin on *Neurospora crassa* wild type strain (SG#259), NCU05950 knockout strain (SG#357) and FKR2 knockout strain. Cultures of wild type (SG#259), NCU05950 deletion mutant (SG#357) and FKR2^{KO} (carrying no additional mutations) were grown in liquid media with Vogel's minimum (VM) and 1% glucose with different rapamycin concentrations. DMSO was used as the solvent for rapamycin. Error bars are means of three replicate experiments $\pm$ S.E.M. Dry weights within each experiment were normalized to the wild type weight on VM=1.0. Stars (*) indicate statistically significant different between strains for the same growth condition, $p < 0.05$.

CHAPTER 11: Discussion

In *Neurospora crassa*, the core of a transcription/translation feedback loop (TTFL) is comprised of the *frequency* (*frq*) gene, two *white-collar* genes (*wc-1* and *wc-2*) and their protein products. *frq* transcription is positively regulated by a transcription complex (WCC or white color complex) which consists of WC-1 and WC-2. FRQ in turn negatively regulates WCC activity. Rhythms have been observed in the absence of a functional FRQ/WCC TTFL. These are called FRQ-less rhythms and are assumed to be driven by one or more FRQ-less oscillators (FLOs). Our laboratory has identified three mutations (*prd-1*, *prd-2* and *uv90*) that disrupt FLO. *prd-1* and *prd-2* mutations were previously known to affect periods in strains that contain functional FRQ and the *uv90* mutation containing strain was created by our laboratory through UV mutagenesis (Li and Lakin-Thomas, 2010; Li *et al.*, 2011).

The *uv90* mutation compromises FRQ-less rhythmicity of the conidiation or spore formation rhythms in *Neurospora crassa* under two different assay systems: free running rhythmicity in *chol-1* (choline requiring) strains growing in choline depleted cultures and rhythmic entrainment to short heat pulses (Li *et al.*, 2011). In the FRQ wild-type background, this *uv90* mutation dampens the free-running rhythm of conidiation, the amplitude of the FRQ protein rhythm and the amplitude of the entire circadian oscillator as measured by responses to phase-resetting by light or heat pulses (Li *et al.*, 2011). Therefore, the target of the *uv90* mutation was identified as a gene that is necessary for robust FRQ-less rhythmicity and also for maintaining the amplitude of the entire circadian system when FRQ is present (Li *et al.*, 2011; Ratnayake *et al.*, 2018).

Studying the function of the gene affected by the *uv90* mutation will help us to understand the mechanism of circadian rhythms and other cellular processes. The gene affected by the *uv90* mutation was initially mapped to the right arm of linkage group (LG) VI by crossing the original *uv90* mutant strain SG#213 (*csp-1: chol-1 ras-1^{bd}; uv90; frq¹⁰ mat a*) with known mutant strains (Li *et al.*, 2011). Former lab members have further mapped it as the NCU05950 gene in the *Neurospora crassa* genome by using Cleaved Amplified Polymorphic Sequence (CAPS) markers and screening the growth phenotype of gene knock out strains (Ratnayake *et al.*, 2018).

I have further mapped the *uv90* mutation and identified it as a deletion of a 35128 bp region of linkage group (LG) VI (3321068 to 3356197). Two strategies were used to confirm that the deletion of NCU05950 gene and only this gene is responsible for the *uv90* phenotype: assaying the circadian rhythm phenotype of an NCU05950 gene deletion strain and rescuing the phenotype of the *uv90* mutant strain by transformation with a wild-type copy of NCU05950 gene. An ectopic wild type copy of NCU05950 gene rescued the phenotypic defects of the *uv90* mutation and results from various experiments outlined in Chapter 3 confirmed that the phenotype of the *uv90* mutant is due to a loss-of-function (null) mutation in the NCU05950 gene and not due to the disruption of neighboring genes.

The NCU05950 gene does not have a functional annotation in the *Neurospora crassa* genome database (FungiDB, <http://fungidb.org/fungidb/>; Stajich *et al.* 2012) and it is listed as a “Hypothetical protein”. As an initial attempt to determine the function of the NCU05950 gene product, the hypothetical protein product of NCU05950 was compared against other proteins using bioinformatical methods. A number of similar proteins from other filamentous fungi were retrieved, but none of these had functional

annotations. Therefore, a search for putative protein domains was performed and identified a conserved domain in the LAMTOR family, which includes human p18/LAMTOR1 and yeast EGO1/Meh1p/Gse2p. These proteins are found on lysosomal / vacuolar membranes and anchor protein complexes that function upstream of TORC1 to activate TOR (Target of Rapamycin) in response to nutritional signals, specifically amino acids.

As predicted, a GFP fusion of NCU05950 protein localized to the vacuolar membrane, in agreement with the known localization of the orthologous human and yeast proteins. This membrane association appears to be dependent on an N-terminal amino acid modification and deletion of putative acylation sites abolished vacuolar membrane localization. This finding suggests that the NCU05950 gene product is a LAMTOR-like protein and fatty acids anchor NCU05950 protein to the vacuolar membrane as for the mammalian and yeast homologs. The ability of NCU05950 to associate with the vacuolar membrane is critical for its clock / circadian rhythm related function, because deletion mutations in these putative acylation sites fail to rescue the NCU05950 deletion mutant phenotype. In contrast, deletion of only the putative site for myristoylation at the N-terminal did not disrupt vacuolar localization of the protein and did not disrupt the rhythm-related function of the protein, as this deletion was able to rescue the phenotype of the gene deletion mutant.

A functional FLAG-tagged NCU05950 fusion protein was found at nearly constant levels across two circadian cycles and expression of NCU05950 protein from a constitutive promoter (*ccg-1*) as well as the native NCU05950 promoter rescued the rhythmic phenotype in the NCU05950 deletion mutant background. This indicates that the native promoter is not required for the clock function of NCU05950, in agreement with our finding that rhythmicity of the

protein levels is not required for clock function. NCU05950 mRNA levels are also constitutively expressed across two circadian cycles without obvious rhythmicity and it indicates a correlation between the expression levels of NCU05950 mRNA and protein. Therefore, NCU05950 is not a typical clock-controlled gene (Vitalini *et al.*, 2006).

Co-Immunoprecipitation and Mass-spectrometry analysis was used to identify the protein partners associated with NCU05950. I expected to find homologs of subunits of the vacuolar localized EGO-Rag GTPase-complex (EGO1, EGO2, EGO3, GTR1 and GTR2) in yeast and Ragulator- Rag GTPase complex (p18/LAMTOR1, p14/LAMTOR2, MP1/LAMTOR3, p10/LAMTOR4/C7orf59, HBXIP/LAMTOR5, RagA/B and RagC/D) in mammals, that function upstream of TORC1 (Yonehara *et al.*, 2017; Powis *et al.*, 2015; Loewith and Hall, 2011; Eltschinger and Loewith, 2016). As expected, GTR1 (NCU01099- GTP-binding protein GTR1) and GTR2 (NCU00376 - GTR/RAG family GTPase Gtr2) were found and those are homologous to Rag-GTPase found in yeast EGO-Rag GTPase-complex and mammalian Ragulator- Rag GTPase complex. I also found some unexpected protein interactions, which includes ribosomal proteins as well as some unnamed / hypothetical proteins. Identifying ribosomal proteins as the protein partners associated with NCU05950 suggests a role in ribosome associated biological processes for NCU05950 protein.

Highly conserved Ras-related GTPase Gtr1 and Gtr2 were identified as proteins that physically interact with NCU05950. Several lines of evidence suggest that this interaction is of physiological relevance. I found that GFP fusion GTR2 localizes to the older vacuoles and some small vesicles, that could be endosomes. The localization of GTR2 is dependent upon the presence of NCU05950, a result that I have found by microscopic analysis of GTR2::GFP

fusion protein in live cell cultures. In the absence of NCU05950, GTR2 appears to be localized to the cytosol, less on vesicles and none on the large vacuoles. Similar results have been reported for the yeast EGO1/ Meh1p/Gse2p and GTR1 by Gao *et al.*, 2005. The GTR2 deletion mutant phenocopies the conidiation rhythms of NCU05950 deletion mutant. Similar to the NCU05950 deletion mutant or *uvr90* mutant, the GTR2 deletion mutation severely compromises the function of the FRQ-less oscillator by completely disrupting conidiation rhythms in the *chol-1; frq¹⁰* background under choline deficient conditions, and GTR2 deletion mutant damps the amplitude of the conidiation rhythms in FRQ wild type strains that contain a functional FRQ-WCC TTFL. Fourth, similar to NCU05950, loss of GTR2 function disables the oscillator that is revealed by the heat pulse entrainment. GTR2 gene product has a greater effect on rhythmic behavior in the absence of functional FRQ (in the *frq¹⁰* strain). Therefore, NCU05950 and GTR2 are likely to be components of a single pathway that is responsible for circadian rhythmicity and GTR2 also affects the FLO in *Neurospora crassa*.

I have also tested other proteins in the TOR signaling pathway to determine their influence on rhythmicity and obtained deletion mutants from the *Neurospora crassa* knockout project of genes homologous to yeast FPR2, TOR1, TOR2, VAM6, and the TORC1 targets Sch9/S6K and Tap42. The deletion mutant strains were crossed to our standard laboratory strains to introduce *csp-1*, *chol-1*, *ras^{bd}* and *frq¹⁰* mutations. STK-10 is homologous to yeast Sch9 and mammalian S6K. According to preliminary results, the STK-10 deletion mutant did not precisely phenocopy NCU05950. Therefore, further investigation is required to determine the rhythm related function of STK-10 as well as for other potential components of the TOR signaling pathway.

If the NCU05950 protein functions in the TOR pathway, as predicted, a null mutation might be expected to affect the growth response of the fungus to different nutritional states. Therefore, to find the role of NCU05950 in growth control and nutrition sensing, we assayed the growth rate on solid agar in race tubes with various media and dry weight gain in liquid VM medium without agar with or without ammonium nitrate, which is the standard nitrogen source. Nitrogen sources (typically amino acids) stimulate TORC1 activity and active TORC1 promotes protein synthesis and inhibits autophagy to encourage growth (Jewell *et al.*, 2013). Therefore, growth on media containing different amino acids (glutamine, leucine and arginine) was assayed. Furthermore, we tested the effects on growth rate of varying glucose concentration. The results of our growth experiments indicated differential responses of the NCU05950 wild type and the NCU05950 deletion mutant and the NCU05950 deletion mutant is defective in its growth response to added nutrition, both nitrogen and carbon sources. The GTR2 deletion mutant also phenocopied the growth response of NCU05950 deletion mutant to different nutrition states. STK-10 deletion mutant was able to respond to addition of amino acid as nitrogen source. However, STK-10 deletion mutant did not respond to changes in glucose levels and this finding suggests that STK-10 functions in the glucose (Carbon) sensing pathway. Therefore, all these findings implicate the TOR pathway in growth responses in *Neurospora crassa*.

Our work suggests that in *N. crassa*, the TOR signaling pathway is important in nutrient sensing and growth control as in other eukaryotes and in addition plays a role in regulating circadian rhythmicity. Since the *uvr90* / NCU05950 gene product is homologous in structure and function to the yeast EGO1 and mammalian LAMTOR1, and the protein is found on the vacuolar

membrane, we proposed naming this gene as “*vta*”, vacuolar TOR-associated protein (Ratnayake *et al.*, 2018).

There are reports of connections between TOR signaling pathway and rhythmicity in other organisms. In general, if a signaling pathway is important for the activity of the circadian clock, it is usually regulated by the circadian oscillator and therefore exhibits rhythmic activities under constant conditions (Cao, 2018). Furthermore, extracellular signals regulate signaling pathways and these signaling pathways can affect expression of genes that are also under circadian control. Therefore, a feedback loop exists between the signaling pathways and circadian oscillator, that can be regulated or entrained by environmental cues (Cao, 2018). The TOR signaling pathway is a typical example that couples environmental cues to the circadian clock in cells and its network (Cao, 2018). The TOR signaling pathway acts as a one-way signaling pathway to promote increased translation in response to extracellular signals of nutritional availability. However, there are indications that the TOR pathway responds to internal nutritional status and translational rate through a negative feedback loop (Eltschinger and Loewith, 2016). This suggests the interesting possibility of the existence of a TOR signaling pathway linked feedback loop that might provide the substrate for the evolution of the oscillator which functions in the absence of the canonical TTFL.

There is evidence showing a multidimensional role of TOR signaling on the circadian clock; TOR signaling can be part of the photic or non-photic entrainment pathways that sense environmental cues and transmit to the central circadian oscillator; TOR signaling can be a part of output pathways that take rhythmic information from the canonical transcription – translation feedback loop to transmit rhythmicity to output rhythms (Ramanathan *et al.*, 2018; Cao *et al.*, 2011; Huang *et al.*, 2013; Zhang *et al.*, 2018) ; TOR signaling can regulate

network properties of coupled circadian oscillators; it may be directly involved in generating or sustaining rhythmicity and regulating autonomous clock properties such that disrupting TOR signaling affects the circadian oscillator (Zheng and Sehgal 2010; Walton *et al.*, 2018; Liu *et al.*, 2018; Cao *et al.*, 2011; Cao *et al.*, 2013; Ramanathan *et al.*, 2018; Lipton *et al.*, 2017). Furthermore, Ratnayake *et al.*, 2018 has shown a link between TOR signaling pathway and non-canonical oscillators. Cao, 2018 and Lakin-Thomas, 2018 have given detailed reviews about the connection between TOR signaling pathway and circadian rhythms.

Characterization of the *uvr90* gene has provided evidence that the TOR pathway and nutritional sensing play a role in the mechanism of rhythmicity in *N. crassa*, both in the presence and absence of a functional FRQ/WCC TTFL. Previous work in our lab has also identified another clock-affecting gene product PRD-1 and it is an RNA helicase located in the nucleus (Adhvaryu *et al.*, 2016). As reported in Emerson *et al.*, 2015 the localization of PRD-1 is responsive to glucose concentration in the growth medium and the *prd-1* mutant is defective in nutritional compensation of the circadian period. Recent co-IP results in our lab showed that PRD-1 binds ribosomal protein S6. Both *uvr90* and *prd-1* mutations compromise rhythmicity in FRQ-less conditions and therefore nutritional sensing and metabolism are associated in this system (Li *et al.*, 2011; Li and Lakin-Thomas 2010). In *Neurospora crassa*, rhythmicity can be studied in the absence of the TTFL. Therefore, it can be used as a model system for dissecting the role of the TOR pathway in rhythmicity as well as identifying the molecular mechanisms of the circadian metabolic network.

CHAPTER 12: Future directions

Our hypotheses are that the *Neurospora* circadian system is comprised of mutually interacting multiple components such as the FRQ-less oscillator (FLO) and the FRQ/WCC oscillator; and VTA is a component of the FLO. Therefore, the main long-term objectives are to define the architecture of the *Neurospora* circadian system at the biochemical/molecular level by describing the components of the FLO (s), the interactions between FRQ/WCC oscillator and FLO at the molecular as well as phenotypic level and to investigate the contribution of the TOR (Target of Rapamycin) signaling pathway to clock function.

During this study a connection between circadian rhythms and TOR signaling pathway was identified. The targets and role of the TOR signaling pathway in *Neurospora crassa* are not known and there are few reports of connections between the TOR pathway and rhythms in other organisms. Therefore, it is important to investigate the role of TOR signaling pathway as well as VTA in the circadian system of *Neurospora crassa* and thereby to investigate how the TOR pathway might be involved in the FLO.

A. What are the TOR signaling pathway components in *Neurospora crassa* and what is the role of VTA in TOR signaling pathway?

A1. What are VTA's binding partners?

To define the TOR signaling pathway in *Neurospora crassa* and the role of VTA in that pathway, we can focus on exploring proteins in the TOR pathway associated with VTA. This can be achieved by continuing co-immunoprecipitation (co-IP) and mass spectrometry (MS) analysis using the

strain expressing NCU05950::3xFLAG fusion protein. As expected I found some homologs of subunits of the yeast vacuolar localized EGO complex and mammalian Ragulator complex that function upstream of TORC1 along with some unexpected proteins. Therefore, to confirm the associations reciprocal co-immunoprecipitation (using FLAG tagged GTR2 & GTR1) can be performed with suspected binding partners. Alternatively, a different epitope tag can be used for the identified binding partner and it can be put into the strain expressing NCU05950::3xFLAG and Western blotting followed by immunodetection can be used to analyze the interactions instead of MS.

Furthermore, in order to identify the transient or weak protein-protein interactions, crosslinking the proteins with formaldehyde can be performed before immunoprecipitation to capture weakly interacting proteins in a complex by facilitating covalently binding them together as they interact. Identification and analysis of unexpected interactors could give clues to clock related functions of VTA.

During this study, NCU04811 in the *Neurospora crassa* genome was identified as a VTA interacting protein and it was an unexpected binding partner of VTA, which was not previously predicted based on the knowledge of known complexes in yeast as well as in mammals. It is listed as a hypothetical protein and its function is currently unknown, but the gene ontology (GO) term suggests oxidative stress response. Based on the amino acid sequence analysis, I would like to propose it as a homolog of mammalian c17orf59, which interacts with Ragulator, but not with Rag-GTPases, disrupts the Rag-Ragulator interactions and when over-expressed, it inhibits mTORC1 and prevents its lysosomal recruitment. Further sequence analysis and IP-MS analysis could be performed with strains overexpressing NCU04811 protein to see whether there is a change in binding partners.

In yeast, C-terminal deletion in Ego1 abolishes the interaction with both Ego2 and Ego3. Variants of C-terminal deletion mutants of VTA tagged with GFP/FLAG could be constructed to check whether the C-terminal of VTA is important for its function, localization and binding with other proteins. These strains could be initially used to assay the rhythmic phenotype on race tubes as well as vacuolar localization. Then at a later stage these strains could be used as tools to determine any changes to VTA interacting partners and also localization changes of binding partners.

A2. What are the epistatic relationships between proposed TOR pathway components?

In order to investigate whether VTA and STK-10 are in the same pathway, a VTA^{KO} and STK-10^{KO} double mutant strain could be created to analyze the rhythmic phenotype on race tubes to look for epistasis. Furthermore, comparing the effect of different media on STK-10^{KO} and VTA^{KO} by measuring the dry weight can be performed.

Prd-1 is another clock gene which affects Frq-less rhythms in *Neurospora crassa* and our lab is working to characterize the function of PRD-1. VTA affects nutritional sensing for growth, PRD-1 grows slowly and its localization responds to glucose levels and both proteins affect rhythmicity in *Neurospora crassa*. To investigate whether VTA is required for effects of glucose on PRD-1 localization and expression, GFP tagged PRD-1 could be introduced to VTA^{KO} strain to see if deleting VTA blocks the effects of glucose on PRD-1. To investigate whether VTA and PRD-1 interact in the same pathway and to check whether VTA and PRD-1 interact genetically, double mutant strains carrying both *prd-1* mutant and VTA^{KO} could be created to assay

rhythmic as well as growth phenotypes. This analysis may show whether PRD-1 acts upstream or downstream of VTA or whether they are in independent pathways.

A3. What is the role of VTA in growth control and nutrient sensing?

What other nutrients link with VTA?

The growth assays could be continued using various carbon and nitrogen sources, comparing wild type and VTA knock out mutant, to find the role of VTA in growth control and nutrient sensing.

Since VTA plays a role in growth control and nutritional sensing, co-IP-MS analysis could be performed on samples collected from strains grown in different media, different growth environments as well as under stress conditions to detect any changes in binding partners.

A4. Is the vacuolar localization of VTA required for it to associate with other proteins in a complex?

In order to check whether the vacuolar localization of VTA is required for it to associate with other proteins in a complex, the deletion mutant strains of VTA that do not localize to vacuoles could be used for co-IP and MS analysis to see if other proteins still associate with VTA or if the mutants fail to pull down the same proteins as the wild type FLAG tagged protein. For this task, the 3xFLAG tagged deletion mutant strains that I constructed could be used. The GFP fusions of these deletions do not localize to the vacuole. As an alternative method, localization of GFP or RFP tagged TOR pathway proteins in VTA^{KO} and VTA wild type background could be analyzed to see whether VTA is required for localization of other proteins in the TOR pathway. Furthermore,

GFP tagging of the VTA interactors could be performed to see if VTA is required for their localization.

B. What is the role of TOR in rhythmicity?

B1. What other components of the TOR pathway contribute to clock function?

In order to check the contribution of other proteins in the TOR pathway to clock function, race tube experiments could be performed with gene deletion strains. We have obtained gene deletion strains from the *Neurospora* Knockout project, which are available at the Fungal Genetic Stock Center. During my study period, I started analyzing deletion mutants of genes homologous to yeast GTR2, PIK1a and FKR5, which are upstream regulators of TORC1, and Sch9/S6K (Stk-10) and Tap42, which are downstream TORC1 targets. Genetic crosses have been made to put the deletion mutations into the same genetic background as our standard laboratory strains. I completed the rhythmic phenotype assays with GTR2 knockouts and further assays need to be performed with Stk-10 and Tap42 knockout strains to check whether these knock out strains give the same phenotype as the VTA mutant strain. With the information from race tube experiments, we will be able to confirm the interaction partners of the *vta* gene product and also can predict the point at which the *vta* gene product is involved in the TOR signaling network in *N. crassa*. In addition to the knockout strains I analysed, other TOR pathway related genes in *Neurospora crassa* could be identified and their knockout phenotypes could be assayed (eg: yeast LST8, GTR1, AVO1, Pib2). For those genes that are essential for which deletions are not available, such as the putative TOR1 homolog, attempts could be made to knock down the activity

with RNAi (RNA interference) or functional domains of those proteins could be identified and mutation could be introduced to alter activity of the protein. The rhythmic phenotypes could be assayed by performing race tube experiments.

Once VTA interacting partners are identified, knockout or knockdown strains could be used to look for effects on rhythmicity.

If the knockout of NCU04811 gene is available, its clock phenotype could be assayed to see if it is involved in the clock.

B2. What is the effect of TOR activity on circadian rhythms?

I have tested the growth inhibitory effects of TORC1 inhibitor rapamycin which inhibits TORC1 activity indirectly via rapamycin binding proteins and VTA does not participate in rapamycin induced growth inhibition as there was no significant difference in dry weight and linear growth rate between the VTA deletion mutant strain and wild type strain. To determine the effect of rapamycin on circadian rhythms, wild type strains with and without functional FRQ-WCC TTFL could be assayed on rapamycin containing media on race tubes to measure the period and amplitude of rhythms. These experiments should be conducted under conditions where both the FRQ/WCC and FLO rhythms can be assayed. Further experiments could be performed with the newer inhibitor Torin that inhibit TORC1 directly to look for effects on rhythmicity in the wild type and any differences in growth inhibitory effects between wild type and VTA knockout mutants. Results of these experiments could indicate whether the activity of TOR complex affects the circadian system of *Neurospora crassa*.

Experiments could be performed to look for effects on rhythmicity (conidiation phenotype) by different carbon and nitrogen sources (media) using wild type and VTA knockout mutant strains. The VTA deletion mutant may

have lost metabolic compensation or the ability to maintain the rhythmicity under different nutritional conditions. Therefore, the period and amplitude of the rhythms may be affected by metabolic state in VTA deletion mutant, unlike the wild type. Wild type strains with and without functional FRQ-WCC TTFL could be grown on different media with various carbon and nitrogen sources, on race tubes to measure the period as well as amplitude of rhythms and thereby to determine the effect of nutrients/metabolic state on circadian rhythms.

B3. Is TOR complex activity rhythmic?

To investigate the rhythmic activity of TOR signaling pathway, rhythmic TORC1 activity in both Frq^+ and Frq^{KO} strains could be studied. The most widely used assay for TORC1 activity is measuring the phosphorylation of one of its targets, S6K (mammals) or Sch9 (yeast) or Stk-10 (*Neurospora crassa*), which phosphorylate ribosomal protein S6. If the sequence of the peptides that were used to make commercially available Anti-phospho-S6K (TOR substrate) antibodies matches the *Neurospora crassa* sequence, commercially available Anti-phospho-S6K could be used on Western blots to look for rhythmic Stk-10 phosphorylation. Alternatively, STK-10::3xFLAG expressing strains could be constructed to investigate the different phosphorylated forms on a gel.

Co-IP and MS analysis could be performed with samples collected at different times in the circadian cycle to identify the proteins interacting with VTA at different times of the day.

To check whether the components in the TOR signaling pathway are rhythmic in abundance and /or activity, Western blotting of FLAG epitope tagged proteins (GTR2::3xFLAG, STK-10::3xFLAG and TAP42::3xFLAG, etc.) in this pathway could be used. Furthermore, phosphorylation rhythms

could be investigated by analysing the differential migration pattern on gels by phosphorylated and un-phosphorylated forms of protein by adding phosphatase to see if there is a change in migration.

B4. How does TOR interact with FRQ?

To see if TOR might participate in the mechanism of the FLO, the heat pulse entrainment method could be used with both FRQ wild type and FRQ-less (*frq* null mutant) strains. Furthermore, to look for interaction between the TOR signaling pathway and FRQ-WCC TTFL, Western blotting of FRQ protein could be carried out with gene knockout strains of TOR signaling pathway components (such as *GTR2*^{KO}, *STK-10*^{KO}, etc) to see if the FRQ protein rhythm is affected as it is by the *uvr90* mutant, which damps the rhythm of FRQ protein.

C. What is the role of VTA in FLO and what are the other components of the FLO?

During this study the majority of co-IP and MS analysis was conducted with a strain expressing NCU05950::3xFLAG fusion protein that has a functional FRQ / WCC oscillator. In order to investigate the role of VTA in FLO as well as to identify other components of the FLO, VTA interacting proteins can be identified using the strains I constructed which express NCU05950::3xFLAG fusion protein in the *frq*¹⁰ background, so the FRQ/WCC TTFL is non-functional. If there is a difference in proteins that associate with VTA, it will demonstrate that FRQ/WCC TTFL affects the TOR pathway.

D. What is the role of VTA in autophagy?

To check whether VTA has a role in autophagy, electron microscopy could be used to compare any difference in vacuolar structures and to identify autophagosomes in the *uv90* original mutant, VTA deletion and wild type strains under minimal media, nutrient rich media as well as rapamycin/torin containing media. Furthermore, GFP or RFP tagged autophagosomes marker protein VAM3 could be used to localize the autophagosome in confocal microscopy. Apart from that, GFP tagged VTA expressing and RFP tagged VAM3 expressing strains could be used to check whether VTA co-localizes with VAM3 on autophagosomes during autophagy.

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Appendix I

The *Neurospora crassa* strains used in this study and plasmids constructed during this study.

A.

The *Neurospora crassa* strains used in this study.

Strain identification number in the lab	Strain genotype	Comments
SG#1	<i>ras-1^{bd} mat a</i>	
SG#4	<i>ras-1^{bd} mat A</i>	
SG#26	<i>csp-1; chol-1 ras-1^{bd} mat a</i>	Constructed in the lab.
SG#59	<i>ras-1^{bd}; frq¹⁰ mat A</i>	
SG#80	<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰ mat a</i>	Constructed in the lab.
SG#180	<i>ras-1^{bd} mat a</i>	
SG#198	<i>csp-1 his-3::ccg1-Bml-sgfp; chol-1 ras-1^{bd}; int⁻</i>	GFP-tubulin strain.
SG#213	<i>csp-1; chol-1 ras-1^{bd}; uv90; frq¹⁰ mat a</i>	Constructed in the lab.
SG#224	<i>csp-1; chol-1 ras-1^{bd}; uv90 mat a</i>	Constructed in the lab.
SG#259	Oak Ridge standard	From FGSC.

SG#274	<i>csp-1 his-3; chol-1 ras-1^{bd}</i> <i>mat a</i>	Constructed in the lab.
SG#300	<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰</i> <i>mat A</i>	Constructed in the lab.
SG#301	<i>csp-1; chol-1 ras-1^{bd}; frq¹⁰</i> <i>mat A</i>	Constructed in the lab. Spore PCR with Frq specific primers confirmed that this strain is not a <i>frq¹⁰</i> and contains the <i>frq</i> gene.
SG#328	<i>csp-1 his-3⁺::p ccg-1::hH1⁺::tdimerRedN; chol-1 ras^{bd}; inl</i>	Constructed in the lab. Adhvaryu <i>et al.</i> , 2016
SG#357	NCU05950 ^{KO} <i>mat A</i>	From FGSC.
SG#359	<i>csp-1 his-3; chol-1 ras^{bd};</i> NCU05950 ^{KO} <i>mat a</i>	Constructed in the lab.
SG#361	<i>csp-1; chol-1 ras^{bd};</i> NCU05950 ^{KO}	Constructed in the lab.
SG#365	<i>csp-1; chol-1 ras^{bd};</i> NCU05950 ^{KO}	Constructed in the lab.
SG#370	<i>csp-1; chol-1 ras^{bd}; uv90;</i> <i>wc-1^{KO}</i>	Constructed in the lab. Isolate #146-2
SG#420 – SG#425	Heterokaryon <i>csp-1 his-3⁺:: NCU05950;</i> <i>chol-1 ras^{bd}; NCU05950^{KO}</i> <i>mat a</i>	Constructed during this study. Strain IDs: SG#420 - L1a SG#421 - L1c SG#422 - L1

		SG#423 - L2a SG#424 - L3b SG#425 - L1e
SG#427	<i>csp-1; chol-1 ras^{bd}; uv90; wc-1^{KO}</i>	Constructed in the lab. Isolate #160 - 20
SG#428 – SG#432	Homokaryon <i>csp-1 his-3⁺:: NCU05950 ; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#428 - L1a-micro SG#229 - L1e-micro SG#430 - L3b-micro SG#431 - L1c-micro SG#432 - L2b-micro
SG#433	FGSC#10158 <i>his-3⁺::pccg-1::nca-1⁺::sgfp⁺ mat A</i>	From FGSC. Gene: <i>nca-1</i> Locus: NCU03305 Protein: Ca ²⁺ -ATPase (SERCA-type ATPase present in endoplasmic reticulum, nuclear envelope and in other membranes) Bowman <i>et al.</i> , 2009
SG#434	FGSC#10167 <i>his-3⁺pccg-1::vps-52⁺::sgfp⁺ mat A</i>	From FGSC. Gene: <i>vps-52</i> Locus: NCU05273

		Protein: Golgi body-associated retrograde protein. Bowman <i>et al.</i> , 2009
SG#435	FGSC#10168 <i>his-3⁺::pccg-1::tdimer2(12)::vam-3⁺ mat A</i>	From FGSC. Gene: <i>vam-3</i> Locus: NCU06777 Protein: Vacuole-associated SNARE protein. Bowman <i>et al.</i> , 2009
SG#436	FGSC#10170 <i>his-3⁺pccg-1::vma-5⁺::sgfp⁺ mat A</i>	From FGSC. Gene: <i>vma-5</i> Locus: NCU09897 Protein: Subunit C of vacuolar ATPase Bowman <i>et al.</i> , 2009
SG#437	FGSC#10171 <i>his-3⁺::pccg-1::arg-4⁺ ::sgfp⁺ mat A</i>	From FGSC. Gene: <i>arg-4</i> Locus: NCU10468 Protein: Mitochondrial acetylornithine-glutamate transacetylase. Bowman <i>et al.</i> , 2009
SG#438	FGSC10377 <i>rid^Δ::hph⁺ his-3::lpl^Δ (5192-</i>	From FGSC.

	<i>6040)::hph⁺::tk⁺::flag-pcna ; inl</i>	
SG#439 – SG#443	<i>csp-1 his-3⁺:: pccg- I::NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#439 - FX-5-b SG#440 - FX-5-d SG#441 - FX-22-a SG#442 - FX-22-c SG#443 - FX-22-d
SG#444 – SG#447	<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly:: 3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#444 - FN-16-a SG#445 - FN-16-c SG#446 - FN-16-d SG#447 - FN-26-b
SG#448 – SG#453	<i>csp-1 his-3⁺:: Native promoter -NCU05950:: Gly:: GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#448 - GN-1-1-1 SG#449 - GN-1-1-2 SG#450 - GN-1-1-4 SG#451 - GN-10-2 SG#452 - GN-10-3 SG#453 - GN-10-4

SG#454 – SG#464	<i>csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP;</i> <i>chol-1 ras^{bd}; NCU05950^{KO}</i> <i>mat a</i>	Constructed during this study. Strain IDs: SG#454 - GX-1-3-1 SG#455 - GX-1-3-2 SG#456 - GX-1-3-3 SG#457 - GX-1-3-4 SG#458 - GX-1-8-1 SG#459 - GX-1-8-2 SG#460 - GX-1-8-3 SG#461 - GX-1-8-4 SG#462 - GX-3-2 SG#463 - GX-3-3 SG#464 - GX-3-4
SG#465 – SG#467	<i>csp-1 his-3⁺ :: NCU05950;</i> <i>chol-1 ras^{bd}; uv90 ; frq⁹</i>	Constructed during this study. Strain IDs: SG#465 - 188-3 SG#466 - 188-6 SG#467 - 188-8
SG#468 – SG#470	<i>csp-1 his-3⁺ :: NCU05950;</i> <i>chol-1 ras^{bd}; uv90</i>	Constructed during this study. Strain IDs: SG#468 - 188-21 SG#469 - 188-22 SG#470 - 188-25
SG#508	<i>NCU02455.2^{KO}</i>	From FGSC.

		FGSC#13000 (FKR5 ^{KO})
SG#509	<i>NCU05608.2^{KO}</i> (Heterokaryon)	From FGSC. FGSC#13432 (TOR2 ^{KO})
SG#510	<i>NCU00376.2^{KO}</i>	From FGSC. FGSC#16041 (GTR2 ^{KO})
SG#511	<i>NCU01539.2^{KO}</i> (Heterokaryon)	From FGSC. FGSC#18870 (VAM6 ^{KO})
SG#512	<i>NCU10397.2^{KO}</i>	From FGSC. FGSC#19786 (PIK1a ^{KO})
SG#513	<i>NCU08268.2^{KO}</i>	From FGSC. FGSC#21741 (TAP42 ^{KO})
SG#514	<i>csp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺: chol-1 ras-1^{bd} mat a</i> (Heterokaryon)	Constructed during this study. Strain ID: RFP-Vam-3-a-12
SG#515	Heterokaryon with nuclei of <i>csp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺: chol-1 ras-1^{bd} mat a</i> AND <i>csp-1 his-3⁺::</i> Native promoter -	Constructed during this study. Constructed by co-inoculating spores of a heterokaryon expressing RFP::Vam-3 protein

	<i>NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	(SG#514: RFP-Vam-3-a-12) and a homokaryon expressing NCU05950::GFP protein (SG#449 : GN-1-1-2).
SG#516	Heterokaryon with nuclei of <i>csp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺; chol-1 ras-1^{bd} mat a</i> AND <i>csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Constructed by co-inoculating spores of a heterokaryon expressing RFP::Vam-3 protein (SG#514: RFP-Vam-3-a-12) and a homokaryon expressing NCU05950::GFP protein (SG#462 : GX-3-2).
SG#517	Heterokaryon with nuclei of <i>csp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺; chol-1 ras-1^{bd} mat a</i> AND <i>csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Constructed by co-inoculating spores of a heterokaryon expressing RFP::Vam-3 protein (SG#514: RFP-Vam-3-a-12) and a homokaryon expressing NCU05950::GFP protein (SG#463 : GX-3-3).
SG#518	Heterokaryon with nuclei of	Constructed during this study.

	<i>csp-1 his-3⁺::pccg-1::tdimer2(12)::vam-3⁺: chol-1 ras-1^{bd} mat a</i> AND <i>csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed by co-inoculating spores of a heterokaryon expressing RFP::Vam-3 protein (SG#514: RFP-Vam-3-a-12) and a homokaryon expressing NCU05950::GFP protein (SG#454 : GX-1-3-1).
SG#521- SG#527	Heterokaryon <i>csp-1 his-3⁺:: pccg-1:: Δ 7-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#521 - Δ 7GX - 2 SG#522 - Δ 7GX - 4 SG#523 - Δ 7GX - 6 SG#524 - Δ 7GX - 7 SG#525 - Δ 7GX - 9 SG#526 - Δ 7GX - 12 SG#527 - Δ 7GX - 19
SG#528 – SG#530	Heterokaryon <i>csp-1 his-3⁺:: pccg-1:: Δ 10-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#528 - Δ 10GX - 1 SG#529 - Δ 10GX - 2 SG#530 - Δ 10GX - 6
SG#531 – SG#535	Heterokaryon	Constructed during this study.

	<i>csp-1 his-3⁺:: pccg-1:: Δ 1-NCU05950::Gly::3XFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Strain IDs: SG#531 - Δ 1FX - 1 SG#532 - Δ 1FX - 3 SG#533 - Δ 1FX - 5 SG#534 - Δ 1FX - 7 SG#535 - Δ 1FX - 9
SG#536 – SG#539	Heterokaryon <i>csp-1 his-3⁺:: pccg-1:: Δ 7-NCU05950::Gly::3XFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#536 - Δ 7FX - 2 SG#537 - Δ 7FX - 3 SG#538 - Δ 7FX - 4 SG#539 - Δ 7FX - 7
SG#540 – SG#543	Heterokaryon <i>csp-1 his-3⁺:: pccg-1:: Δ 10-NCU05950::Gly::3XFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#540 - Δ 10FX - 3 SG#541 - Δ 10FX - 6 SG#542 - Δ 10FX - 10 SG#543 - Δ 10FX - 11
SG#544 – SG#546	<i>csp-1 his-3⁺:: pccg-1:: Δ 10-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#544 - Δ 10GX - 1-x SG#545 - Δ 10GX - 1-y SG#546 - Δ 10GX - 1-z

SG#547	<i>csp-1 his-3⁺:: pccg-1:: Δ 7-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain ID: SG#547 - Δ 7GX – 12 - zN
SG#548 – SG#550	<i>csp-1 his-3⁺:: pccg-1:: Δ 10-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#548 - Δ 10GX – 1 - xN SG#549 - Δ 10GX – 1 - yN SG#550 - Δ 10GX – 1 - zN
SG#551 – SG#553	<i>csp-1 his-3⁺:: pccg-1:: Δ 7-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#551 - Δ 7GX – 12 - xO SG#552 - Δ 7GX – 12 - yO SG#553 - Δ 7GX – 12 - zO
SG#554 – SG#555	<i>csp-1 his-3⁺:: pccg-1:: Δ 10-NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#554 - Δ10GX – 1 – xO SG#555 - Δ10GX – 1 – yO
SG#556 – SG#558	<i>csp-1 his-3⁺:: Native promoter - Δ1-NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#556 - Δ1FN – 9 – c SG#557 - Δ1FN – 15 – e SG#558 - Δ1FN – 22 – d

SG#559 – SG#565	<i>csp-1 his-3⁺</i> :: Native promoter – $\Delta 7$ - <i>NCU05950::Gly::3xFLAG</i> ; <i>chol-1 ras^{bd}</i> ; <i>NCU05950^{KO}</i> <i>mat a</i>	Constructed during this study. Strain IDs: SG#559 – $\Delta 7$ FN – 3 – a SG#560 – $\Delta 7$ FN – 3 – b SG#561 – $\Delta 7$ FN – 3 – c SG#562 – $\Delta 7$ FN – 3 – d SG#563 – $\Delta 7$ FN – 3 – e SG#564 – $\Delta 7$ FN – 6 – d SG#565 – $\Delta 7$ FN – 6 – e
SG#571	<i>NCU03200.2^{KO}</i>	From FGSC. FGSC#17939 (STK10 ^{KO})
SG#572 – SG#577	<i>csp-1 his-3⁺</i> :: Native promoter - <i>NCU05950::Gly::3xFLAG</i> ; <i>chol-1 ras^{bd}</i> ; <i>frq¹⁰</i> ; <i>NCU05950^{KO}</i>	Constructed during this study. Strain IDs: SG#572 - 59x444-10 (KOFF-10) SG#573 - 59x444-17 (KOFF-17) SG#574 - 59x444-28 (KOFF-28) SG#575 - 59x444-40 (KOFF-40) SG#576 - 59x444-46 (KOFF-46)

		SG#577 - 59x444-51 (KOFF-51)
SG#578 – SG#581	<i>csp-1 his-3⁺::</i> Native promoter - <i>NCU05950::Gly::3xFLAG</i> ; <i>chol-1 ras^{bd}; frq¹⁰</i>	Constructed during this study. Strain IDs: SG#578 - 59x444-27 (WFF-27) SG#579 - 59x444-29 (WFF-29) SG#580 - 59x444-50 (WFF-50) SG#581 - 59x444-76 (WFF-76)
SG#582 – SG#585	<i>csp-1 his-3⁺::</i> Native promoter – $\Delta 10$ - <i>NCU05950::C-</i> <i>Gly::3xFLAG</i> ; <i>chol-1 ras^{bd}</i> ; <i>NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#582 – $\Delta 10$ FN – 13 – y SG#583 – $\Delta 10$ FN – 2 – a SG#584 – $\Delta 10$ FN – 2 – b SG#585 – $\Delta 10$ FN – 2 – c
SG#590 – SG#592	<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i>	Constructed during this study. Progeny of the cross between SG#300 (<i>csp-1</i> : <i>chol-1 ras-1^{bd}; frq¹⁰ mat A</i>) and GTR2 ^{KO} strain (FGSC#16041 = SG#510)

		Strain IDs: SG#590 - Pg#3 SG#591 - Pg#4 SG#592 - Pg#26
SG#593 – SG#597	<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i>	Constructed during this study. Progeny of the cross between SG#300 (<i>csp-1</i> : <i>chol-1 ras-1^{bd}</i> ; <i>frq¹⁰</i> mat A) and GTR2 ^{KO} strain (FGSC#16041 = SG#510) Strain IDs: SG#593 - Pg#14 SG#594 - Pg#20 SG#595 - Pg#30 SG#596 - Pg#34 SG#597 - Pg#37
SG#598 – SG#600	Heterokaryon <i>csp-1 his-3⁺:: pccg-1:: Δ 1-NCU05950::Gly::GFP</i> ; <i>chol-1 ras^{bd}</i> ; <i>NCU05950^{KO}</i> mat a	Constructed during this study. Strain IDs: SG#598 - Δ1GX - 3 SG#599 - Δ1GX - 8 SG#600 - Δ1GX - 12
SG#601 – SG#603	Heterokaryon <i>csp-1 his-3⁺:: Native promoter -Δ1-</i>	Constructed during this study. Strain IDs: SG#601 - Δ1GN - 16

	<i>NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	SG#602 - Δ1GN - 17 SG#603 - Δ1GN - 27
SG#604 – SG#606	Heterokaryon <i>csp-1 his-3⁺::</i> Native promoter –Δ7- <i>NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#604 – Δ7GN - 7 SG#605 – Δ7GN - 8 SG#606 – Δ7GN - 12
SG#607 – SG#609	Heterokaryon <i>csp-1 his-3⁺::</i> Native promoter –Δ10- <i>NCU05950::Gly::GFP; chol-1 ras^{bd}; NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#607 – Δ10GN - 1 SG#608 – Δ10GN - 4 SG#609 – Δ10GN - 8
SG#610 – SG#612	Heterokaryon <i>csp-1 his-3⁺:: pccg-1:: NCU00376::Gly::GFP; chol-1 ras^{bd} :: NCU05950^{KO} mat a</i>	Constructed during this study. Strain IDs: SG#610 – NCU05950 ^{KO} GTR2-GFP-2 = KOGG2 SG#611 – NCU05950 ^{KO} GTR2-GFP-6 = KOGG6 SG#612 – NCU05950 ^{KO} GTR2-GFP-7 = KOGG7

SG#613 – SG#615	Heterokaryon <i>csp-1 his-3⁺:: pccg-1::NCU00376::Gly::GFP;</i> <i>chol-1 ras^{bd} mat a</i>	Constructed during this study. Strain IDs: SG#613 – NCU05950 ^W GTR2-GFP-10 = WGG10 SG#614 – NCU05950 ^W GTR2-GFP-15 = WGG15 SG#615 – NCU05950 ^W GTR2-GFP-18 = WGG18
SG#616 – SG#618	<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd};</i> <i>frq¹⁰</i>	Constructed during this study. Progeny of the cross between SG#180 (<i>ras-1^{bd}; mat a</i>) and SG#594 (pg#20 - <i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰ mat A</i>) Strain IDs: SG#616 - Pg#36 SG#617 - Pg#44 SG#618 - Pg#65
SG#619 – SG#621	<i>csp-1; gtr2^{KO}; chol-1 ras-1^{bd}</i>	Constructed during this study. Progeny of the cross between SG#180 (<i>ras-1^{bd};</i>

		<i>mat a</i>) and SG#594 (pg#20 - <i>csp-1</i>; <i>gtr2^{KO}</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰ mat A</i>) Strain IDs: SG#619 - Pg#2 SG#620 - Pg#28 SG#621 - Pg#40
SG#622 – SG#624	<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol⁺ ras-1^{bd}</i> ; <i>frq¹⁰</i>	Constructed during this study. Progeny of the cross between SG#180 (<i>ras-1^{bd}</i>; <i>mat a</i>) and SG#594 (pg#20 - <i>csp-1</i>; <i>gtr2^{KO}</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰ mat A</i>) Strain IDs: SG#622 - Pg#18 SG#623 - Pg#48 SG#624 - Pg#68
SG#625 – SG#627	<i>csp-1</i> ; <i>gtr2^{KO}</i> ; <i>chol⁺ ras-1^{bd}</i>	Constructed during this study. Progeny of the cross between SG#180 (<i>ras-1^{bd}</i>; <i>mat a</i>) and SG#594 (pg#20 - <i>csp-1</i>; <i>gtr2^{KO}</i>; <i>chol-1 ras-1^{bd}</i>; <i>frq¹⁰ mat A</i>) Strain IDs: SG#625 - Pg#6

		SG#626 - Pg#25 SG#627 - Pg#55
SG#628 – SG#630	<i>csp-1 stk-10^{KO}; chol⁺ ras-1^{bd}; frq¹⁰</i>	Constructed during this study. Progeny of the cross between SG#300 (<i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A</i>) and SG#552 (FGSC#17939 = <i>stk-10^{KO} mat a</i>) Strain IDs: SG#628 - Pg#34 SG#629 - Pg#46 SG#630 - Pg#48
SG#631 - SG#633	<i>csp-1 stk-10^{KO}; chol⁺ ras-1^{bd}</i>	Constructed during this study. Progeny of the cross between SG#300 (<i>csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A</i>) and SG#552 (FGSC#17939 = <i>stk-10^{KO} mat a</i>) Strain IDs: SG#631 - Pg#11 SG#632 - Pg#23 SG#633 - Pg#44

B.

Plasmids constructed during this study.

All plasmids are stored as glycerol stocks in -80°C.

1. pBM61-1kb up stream-NCU05950-1kb down stream
2. pUC19-1kb up stream-NCU05950-1kb down stream
3. pNative promoter-NCU05950::C-Gly::GFP
4. *pccg-I*-NCU05950::C-Gly::GFP
5. pNative promoter-NCU05950::C-Gly::3xFLAG
6. *pccg-I*-NCU05950::C-Gly::3xFLAG
7. pNative promoter- Δ 1-NCU05950::C-Gly::GFP
8. pNative promoter- Δ 7-NCU05950::C-Gly::GFP
9. pNative promoter- Δ 10-NCU05950::C-Gly::GFP
10. *pccg-I*- Δ 1-NCU05950::C-Gly::GFP
11. *pccg-I*- Δ 7-NCU05950::C-Gly::GFP
12. *pccg-I*- Δ 10-NCU05950::C-Gly::GFP
13. pNative promoter- Δ 1-NCU05950::C-Gly::3xFLAG
14. pNative promoter- Δ 7-NCU05950::C-Gly::3xFLAG
15. pNative promoter- Δ 10-NCU05950::C-Gly::3xFLAG
16. *pccg-I*- Δ 1-NCU05950::C-Gly:: 3xFLAG
17. *pccg-I*- Δ 7-NCU05950::C-Gly:: 3xFLAG
18. *pccg-I*- Δ 10-NCU05950::C-Gly:: 3xFLAG
19. *pccg-I*-NCU00376::C-Gly::GFP

Appendix II

Growth media, buffers and solutions

Vogel's 50x stock

Add successively to 250 mL flask, stirring continuously:

188 mL DI water stir and heat

32.8 g $\text{Na}_3\text{-citrate}\cdot 2\text{H}_2\text{O}$, let dissolve

62.5 g KH_2PO_4 anhydrous, let dissolve

25.0 g NH_4NO_3 anhydrous, let dissolve

Turn off heat and add water to 250 mL.

Add the following while stirring:

2.5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, let dissolve

1.25 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ dissolved in 5 mL water *

1.25 mL trace elements solution

0.625 mL biotin solution (Make fresh)

Allow to cool, add about 2 mL chloroform, cap tightly.

*Add calcium very slowly and carefully to avoid precipitation, using pasture pipette submerged below the surface of stirring solution to avoid precipitation.

Allow solution to cool completely, add 2 mL chloroform and cap tightly. Store at room temperature.

Fresh biotin solution

1 mg per 10 mL of 50/50 ethanol / water

(Don't try to weigh out 1 mg; weigh a small amount (close to 10mg) of biotin and add enough 50/50 ethanol/water to bring the final concentration to 0.1 mg/mL.)

Trace Elements

Add successively to 9.5 mL water (ddH₂O), while stirring:

0.5 g citric acid. H₂O

0.5 g ZnSO₄·7H₂O

0.1 g Fe (NH₄)₂(SO₄)₂·6H₂O

0.025 g CuSO₄·5H₂O

0.066 g MnSO₄·4H₂O (Or: 0.050 g MnSO₄·H₂O)

0.005 g H₃BO₃ anhydrous

0.0037 g (NH₄)₆Mo₇O₂₄·4H₂O

Store in the refrigerator and wrapped in foil to keep dark.

2x Synthetic crossing medium (SCM) stock

Add successively to 250 mL water, with stirring:

0.46 g K₂HPO₄·3H₂O (Or: 0.35 g K₂HPO₄ anhydrous)

0.25 g KH₂PO₄

0.5 g KNO₃

0.05 g NaCl

0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ dissolved in 1 mL water

1.25 mL fresh biotin

0.05 mL trace elements

Store in refrigerator with chloroform.

Liquid minimum medium

(For micro-titer wells or shaker flasks)

2 mL - Vogel's 50x stock

2 g - D-Glucose

100 mL - Water

Minimal agar medium

(For stock tubes and baby tubes; allow 3 mL per stock tube and 1 mL per baby tube)

2 mL - Vogel's 50x stock

2 g - D-Glucose

2 g - Agar

100 mL - Water

Maltose-arginine (MA) medium

(MA medium for race tubes; allow 6 mL per short race tube, 8.5 mL per long race tube and 17 mL per square tube)

2 mL	-	Vogel's 50x stock
0.5 mL	-	L-arginine (20 mg/mL)
0.5 g	-	Maltose
2 g	-	Agar
100 mL	-	Water

Synthetic crossing medium (SCM)

(Allow 3 mL per tube)

50 mL	-	2x Synthetic crossing medium (SCM) stock
2 g	-	Sucrose
2 g	-	Agar
50 mL	-	Water

Growth supplements:

Storage: In fridge at 4°C with chloroform.

L-Arginine: 2%= 20 mg/mL in water. Add 0.5 mL per 100 mL = 0.01%.

Inositol (myo-, meso- or i-inositol): 5 mg/mL in water. Add 0.9 mL per 100 mL = 250 µM.

Choline for stock tubes: approximately 2 mg/mL, weighed out from wet choline. Add 1.5 mL per 100 mL = 215 μ M.

Choline for exact concentrations: stocks must be weight out of dried choline.

1.0 M = 139.6 mg/mL. Make sterile stock and dilute in sterile Eppendorf tubes for working stocks.

Add 1 mL of 10 mM stock per 100 mL = 100 μ M.

Choline 10 mM stock – 139.6 mg in 100 mL water.

Histidine: 25 mg/mL L-His HCl. Use 2mL per 100 mL = 500 μ g/mL final concentration.

Leucine: 4 mg / mL stock. Use 4 mL per 100 mL medium.

Hygromycin: Final concentration 200 μ g/mL. Use 0.4 mL per 100 mL media from 50 mg/mL stock.

Testing for histidine requirement

Media:

Make 1 mL per strain of liquid minimal + glucose

- a) plus choline (+ inositol or whatever is needed for growth)
- b) plus choline (+ inositol etc.) + histidine

Choline = 100 μ M final

Histidine = 0.2 mL/10 mL of 25 mg/mL

Aliquot 1 mL per stock tube with plastic cap.

Autoclave and inoculate with spores, one of each kind of media per strain.

Incubate at 22°C on shaker in incubator or at room temperature for 2-3 days.

Look for growth or no growth.

Testing for hygromycin resistance

Media:

Make 2 sets of 1 mL per strain of liquid minimal medium + glucose

Set a) 1 mL plus choline (or whatever is needed for growth)

Set b) 1 mL plus choline (or whatever) + hygromycin

To make media:

Make 2 flasks, each with volume needed for one set, add choline if needed.

Prepare two stock tubes per strain to test, with caps.

Autoclave media flasks and capped tubes.

After autoclaving, add sterile hygromycin to one flask. (Do not autoclave hygromycin).

Dispense 1 mL sterile media into sterile tubes.

Choline = 100 μ M final = 100 μ L per 10 mL of 10mM stock.

Hygromycin = final 200 μ g/mL = 40 μ L per 10 mL from 50 mg/mL stock.

Stock is already filter-sterilized; do not autoclave.

Inoculation and growth:

Inoculate with few spores from baby tubes, one tube of each set per strain.

Include controls with a hygromycin – sensitive (normal) strain, and a hygromycin – resistant strain (any knockout will do).

Incubate at 22°C on shaker in incubator at a fast shaking speed for 2-3 days.

Look for growth or no growth.

Sorbose Plating Media

(To restrict growth to form colonies)

A:

0.1 g - Fructose (0.1%)

0.1 g - Glucose (0.1%)

2 g - Sorbose (2%)

50 mL - Water

B:

2 mL - Vogel's 50x stock

2 g - Agar (2%)

1.5 mL - Choline (if required) from 2 mg/mL stock (215 µM)

50 mL - Water

Autoclave two solutions separately and mix after autoclaving for 100 mL total.

(NH₄ causes carmelization of sorbose)

After mixing A & B, keep at 55°C in oven or water bath until needed.

Measure out 20 mL into sterile 50 mL conical tube, add spores, mix thoroughly and pour into 10 cm petri plates. Allow solidifying the media.

Slant tubes and babies

Volume of media:

Slant tubes (Stock tubes): 3 mL minimum medium

Babies: 1 mL minimum medium

Crossing tubes: 3 mL SC medium

(Add appropriate growth supplements to all of the above)

Make medium in a flask and melt agar in microwave. (Caution: do not put cotton plugs into microwave). Aliquot medium into tubes with non-sterile pipette, cap slants and cross tubes with plastic caps and babies with cotton plugs and autoclave. When tubes are still hot, lay tube rack on its side to produce slants while agar cools.

Petri plates

20 mL per small petri plate (10 cm) and 50 mL per large petri plate (14 cm). At sterile bench, pour approximate amount of the media into sterile plastic dishes, avoiding air bubbles. When cool, shake off condensation from lid.

Race tubes

Maltose-arginine for race tubes: Mix all ingredients together. Sterilize media separately from race tubes sealed with cotton plugs at both ends. Allow 6 mL

per short race tube (30 cm), 8.5 mL per long race tube (40 cm) and 17 mL per square tube.

Cellophane

Cut into circles the same size as petri dish, using non-sterile dish as template. (For cellophane, cut it larger than plate by using lid as template. Use scalpel and trace around cellophane on top of cardboard.) Soak circles in large volume of RO water and cover with distilled water. Layer circles in glass petri dish full of distilled water, alternating circles with strips of aluminum foil. Be sure circles are covered with water. Wrap dish in foil and autoclave, keeping dish flat, 30 minutes slow exhaust.

When tubing and media are cool, at sterile bench use sterile flat forceps to transfer tubing to medium, smoothing out air bubbles. Tilt plates up to drain, shake off water and dry flat overnight. Do not leave fan on or plates will dry out.

4% agar

2 g - Difco Bacto-Agar

50 mL - dd H₂O

Mix together agar and water, sterilize and pour contents into two sterile small (10 cm) Petri plates.

1 M sorbitol

9.1 g - Sorbitol

Dissolved in about 30 mL water, then top up volume to 50 mL.

Recovery medium

0.2 mL - Vogel's 50x stock

1.82 g - Sorbitol

9 mL - ddH₂O

Choline (150 µL of 2 mg/ mL) or any other supplement as needed (No histidine)

Autoclave, allow to cool, add 1 mL FIGS.

Top Agar (Plating media)

91 g - Sorbitol (Dissolved in about 350 mL water, then add water to 450 mL total)

10 mL - Vogel's 50x stock

14 g - Agar

Choline (7.5 mL of 2 mg/ mL) or other supplement as needed (No histidine)

Autoclave, cool in oven to 50-60°C, add 50 mL FIGS.

After mixing top agar and FIGS, keep at 55°C in oven or water bath until needed. Measure out 20 mL into sterile 50 mL conical tube, add spores, mix thoroughly and pour into 10 cm petri plates. Allow solidifying the media.

FIGS

10 g	-	Sorbose
0.25 g	-	Fructose
0.25 g	-	Glucose
50 mL	-	ddH ₂ O

Autoclave, cool to 50-60°C and mix with top agar plating medium.

100 ml medium on 1L flask for macro-conidia induction

2 g	-	Glucose
2g	-	Agar
2 mL	-	Vogel's 50x stock
2 mL	-	Histidine (25 mg/ml)
100 mL	-	ddH ₂ O

Plus, any other supplement needed, such as choline (1.5 mL of 2 mg/ mL)

Swirl the flasks after autoclaving to coat the sides of flask to increase the surface area for conidial growth.

Microconida induction media

For 100 mL (5 plates):

5 mL	-	2x Synthetic crossing medium (SCM) stock (0.1x SCM)
0.5 g	-	Sucrose (0.5%)
2 g	-	Agar (2%)

1.5 mL - Choline (if required) from 2 mg/mL stock (215 μ M)

100 mL - ddH₂O

After autoclaving add;

1 mL - Na-iodoacetate (20.8 mg/mL) from filter-sterilized 0.1

M stock (1.0 mM)

400 μ L - Hygromycin B (if required) from 50 mg/mL stock (200 μ M)

DNA Extraction Buffer

1.21 g - Tris (final concentration 100 mM)

1.86 g - EDTA (final concentration 50 mM)

1 mL - 10% SDS

Dissolve Tris and EDTA with stirring in 80 mL dd H₂O. Adjust pH to 8.0. Add water (ddH₂O) for a total volume of 100 mL. Store at room temperature.

To make DNA extraction buffer with 1% SDS, mix 1 mL 10% SDS with 9 mL DNA extraction buffer.

Tris-EDTA (TE) Buffer

0.121 g - Tris (final concentration 10 mM)

0.0372 g - EDTA (final concentration 1 mM)

Dissolve Tris and EDTA with stirring in 80 mL dd H₂O. Adjust pH to 8.0. Add water (ddH₂O) for a final volume of 100 mL.

10X TAE Buffer

48.4 g	-	Tris (40 mM)
3.72 g	-	EDTA disodium salt, dehydrate (1mM)
11.4 mL	-	Acetic Acid (20mM)
~ 600 mL	-	ddH ₂ O

Measure out approximately 600 ml water in a beaker. Add one at a time of Tris, EDTA and Acetic Acid, stirring to dissolve completely after each. Adjust pH to 8.0. Pour into a graduated cylinder and add water to up to 1 L total.

Luria-Bertani (LB) Broth

Make 1 L, autoclave and use as required

10 g	-	NaCl (1%)
5 g	-	Yeast Extract (0.5%)
10 g	-	Tryptone/peptone (1%)
1 L	-	ddH ₂ O

Luria-Bertani (LB) agar plates

Make 1 L:

10 g	-	NaCl (1%)
5 g	-	Yeast Extract (0.5%)
10 g	-	Tryptone/peptone (1%)
15 g	-	Agar (1.5%)

1 L - ddH₂O

Dissolve first three ingredients in ddH₂O and add agar. Autoclave and cool it to 60°C in oven then add any antibiotics (if require). Add 1 mL ampicillin (100 mg/mL) for 1000 ml LB agar.

60% Glycerol

60 mL - Glycerol

40 mL - ddH₂O

Make sure mixture is very homogeneous by mixing by pipetting, shaking, vortexing.

Autoclave on liquid cycle and store in the refrigerator.

20x SSC

Dissolve the following in 800 mL of ddH₂O.

175.3 g - NaCl (3 M)

88.2 g - Sodium citrate (0.3 M)

Adjust the pH to 7.0 with a few drops of 1M HCl.

Adjust the volume to 1L with ddH₂O.

Sterilize by autoclaving.

DIG-Easy-Hyb buffer

64 mL of ddH₂O was added to a dry bottle of DIG-Easy-Hyb granules received with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche).

Maleic Acid Buffer

0.1 M Maleic Acid (pH 7.5)

0.15 M NaCl

pH adjusted with NaOH.

Stored at room temperature.

Stringency Wash buffer 1 (SW#1)

2X SSC

0.1% SDS

Stored at room temperature.

Stringency Wash buffer 2 (SW#2)

0.5X SSC

0.1% SDS

Stored at room temperature.

Washing buffer

0.1 M Maleic Acid (pH 7.5)

0.15 M NaCl

0.3% Tween 20 (v/v)

Stored at room temperature.

Blocking solution

10x Blocking solution received with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was diluted to get 1x working solution in Maleic Acid Buffer.

5 mL	-	10x Blocking solution
45 mL	-	Maleic Acid Buffer

Antibody solution

Anti-Digoxigenin-AP conjugate (from sheep, Fab-fragment, conjugate to alkaline phosphatase) received with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was diluted 1:10000 in 1x Blocking solution.

2.5 µL	-	Anti-Digoxigenin-AP conjugate
25 mL	-	1x Blocking solution

Freshly prepared.

Detection buffer

0.1 M	-	Tris (pH 9.5)
0.1 M	-	NaCl

Stored at room temperature.

10% APS

10mg	-	Ammonium persulfate
100µl	-	Water

10% SDS

10 g - SDS
100 mL - Water

0.1% SDS

100 µl - 10% SDS
9.9 mL - Water

0.5 M Tris - HCl

6.06 g - Tris-HCl
Up to 100 ml - Water
pH to 6.8

0.4% Bromphenol Blue stock

4 mg - Bromphenol Blue
1 mL - Water

Store at 4°C.

0.1 M PMSF

17 mg/ml dissolved in 100% ethanol.

Store few months at 4°C.

SDS-Protein Extraction Buffer

To make 10 ml of extraction buffer:

50 mM tris pH 6.8 - 1 ml of 0.5 M tris pH 6.8 (stored at 4°C)

2% SDS	-	2 ml of 10% SDS (stored at RT)
10% Glycerol	-	1 ml glycerol (add by weight = 1.25 g)
5 mM EDTA- Na_2	-	100 μl of 0.5 M EDTA
Water	-	5.9 ml

Divide into 10 x 1 ml before use. Freeze extra at -20°C .

Add to 1 ml extraction buffer just before use:

1 mM PMSF	-	10 μl of 0.1 M PMSF (or 30 μl of 5 mg/ml stock)
-----------	---	---

15 % acrylamide separating gel

Final acrylamide %:	-	15% acrylamide	for 2 * 15% gels
Total volume	-	5.0 ml	10 ml
Water	-	1.79 ml	3.58 ml
1.5M Tris pH 8.8	-	1.25 ml	2.50 ml
10% SDS	-	0.05 ml	0.10 ml
40% acrylamide	-	1.88 ml	3.76 ml

Add just before pouring:

10% APS	-	25 μl	50 μl
TEMED	-	2.5 μl	5 μl

7.5% acrylamide separating gel

Final acrylamide %:	-	7.5% acrylamide	for 2 * 7.5% gels
Total	-	5 ml	10 ml

1.5M Tris pH 8.8	-	1.25 ml	2.5 ml
Water	-	2.958 ml	5.917 ml
10% SDS	-	0.05 ml	0.10 ml
40% acrylamide	-	0.9375 ml	1.875 ml

De-gas under vacuum to get rid of oxygen. Add:

10% APS	-	25 μ l	50 μ l
TEMED	-	2.5 μ l	5 μ l

Pour the gel into the apparatus, add 0.1% SDS on the top of the gel (0.1 ml 10% SDS +10 ml water) to make a level top.

After 30 minutes, pour off the 0.1% SDS, rinse with water.

Stacking gel

Before preparing the stacking gel, immerse the comb into 0.1%SDS to avoid bubbles.

		One gel	Two gels
Total	-	2.5 ml	5.0 ml
Water	-	1.55 ml	3.11 ml
0.5 M Tris pH 6.8	-	0.647 ml	1.29 ml
10% SDS	-	25.9 μ l	51.8 μ l
40% acrylamide	-	259 μ l	518 μ l

De-gas under vacuum. Add:

10% APS	-	12.9 µl	25.9 µl
TEMED	-	2.59 µl	5.18 µl

Wet comb in 0.1%SDS. Pour some of the stacking gel on top of the separating gel, put the comb in. Fill up space with stacking gel and insert comb to full depth. Allow at least 30 min to polymerize.

Running buffer (Tris-glycine-SDS buffer)

Total volume	-	2 L
Glycine	-	28.8 g
Tris base	-	6.0 g
SDS	-	2.0 g
Water	-	up to 2 L

Anode buffer I

34.3 g	-	Tris base (0.3 M)
100 ml	-	Methanol (10%)
Up to 1000 ml	-	Water

pH 10.4

(check pH on meter, adjust if necessary)

Anode buffer II

3.03 g	-	Tris base (25 mM)
100 ml	-	Methanol (10%)

Up to 1000 ml - Water

pH 10.4

(check pH on meter, adjust if necessary)

Cathode buffer

3.03 g - Tris base (25mM)

3.00 g - Glycine (40mM)

100 ml - Methanol (10%)

Up to 1000 ml - Water

pH 9.4

To prepare: measure Tris base and glycine into a 1 l beaker, add water to 800 ml, adjust pH using pH meter, then add 100 ml methanol, and finally transfer to 1 l graduated cylinder and add water to 1000 ml.

1X TBS

1.21g - Tris

8.5g - NaCl

Up to 1000 ml - Water

pH 7.5 with HCl.

1X TBST

To 1L of 1X TBS add 500 µl Tween-20.

Store in fridge.

10X Blocking buffer (5% BSA+5% skimmed milk powder)

Into a 50mL falcon tube weigh out 2.5g BSA+2.5g skimmed milk powder.

Add 1xTBST up to 50mL.

Dissolve on rotating wheel at RT for 1hr.

Centrifuge for 5 min at 4000rpm to remove un-dissolved particles.

Filter the supernatant through filter paper in funnel.

Store in fridge for up to one month.

Dilute this 1 in 10 with TBST to make 1xBlocking buffer.

0.1% DEPC-treated water

1ml of DEPC was added into 1000 ml double distilled water and kept it at room temperature for at least 1 hour before autoclaving.

Allowed to cool down before use.

10X MOPS

41.86 g - MOPS (0.2M)

4.10 g - NaOAc (50 mM)

20 mL - EDTA 0.5 M stock (10mM)

Dissolved in about 800 mL DEPC treated water and pH was adjusted to 7.0 with NaOH. Water was added up to 1000 mL and sterilized by autoclaving (buffer turned to yellow color). Stored in dark at room temperature.

1X MOPS (Running buffer for RNA gel)

120 mL	-	10X MOPS
1080 mL	-	DEPC treated water

Formaldehyde-agarose gel (1.2%)

1.8 g	-	Agarose
15 mL	-	10X MOPS
24 mL	-	37% formaldehyde
125 mL	-	DEPC treated Water

Denaturation Cocktail

100 µL	-	10X MOPS (1X)
500 µL	-	Formamide (50%)
200 µL	-	Formaldehyde (2.2 M)
1 µL	-	0.5M EDTA (0.5 mM)
160 µL	-	Dye solution
39 µL	-	DEPC treated Water

Make fresh, just before using.

Dye Solution

4%	-	Glycerol
0.04%	-	Bromophenol blue

Made in DEPC treated Water and stored at 4 °C.

PBS

10 mM - phosphate

137 mM - NaCl

2.7 mM - KCl

pH adjusted to 7.4.

Vacuolar preparation medium (VPM)

2.3831g - HEPES (10mM)

0.37224 g - Na₂EDTA.2H₂O (1mM)

182.2 g - Sorbitol (1M)

1 L - ddH₂O

pH adjusted to 7.5 with 10N NaOH.

Stored in the cold room or at 4 °C.

100 mM Na₂ATP

0.60524 g - Na₂ATP

10 mL - ddH₂O

pH adjusted to 7.5 with Tris base (Solid first, then 0.5M)

100 mM Na₂EDTA

3.7224 g - Na₂EDTA

100 mL - ddH₂O

pH adjusted to 7.5 with Tris base

ECA (EDTA- Chymostatin-ATP)

1 mL	-	Na ₂ EDTA from 100 mM stock (1 mM)
2 mL	-	Na ₂ ATP from 100 mM stock (2 mM)
10 µL	-	Chymostatin from 20 mg/mL stock (2 µg/mL)
50 mL	-	ddH ₂ O

pH adjusted to 7.5

100 mM PMSF (phenylmethanesulfonylfluoride)

174 mg of PMSF dissolve in 10mL of 100% ethanol)

Chymostatin

20 mg/mL stock in DMSO

100 mM HEPES

2.383 g	-	HEPES
100 mL	-	ddH ₂ O

30% sucrose mix

15 g	-	Sucrose (30%)
500 µL	-	Na ₂ EDTA from 100 mM stock (1 mM)
5 mL	-	HEPES from 100 mM stock (10 mM)
1 mL	-	Na ₂ ATP from 100 mM stock (2 mM)
5 µL	-	Chymostatin (2 µg/mL)
50 mL	-	ddH ₂ O

Stored at 4 °C.

40% sucrose mix

20 g	-	Sucrose (40%)
500 µL	-	Na ₂ EDTA from 100 mM stock (1 mM)
5 mL	-	HEPES from 100 mM stock (10 mM)
1 mL	-	Na ₂ ATP from 100 mM stock (2 mM)
5 µL	-	Chymostatin (2 µg/mL)
50 mL	-	ddH ₂ O

Stored at 4 °C.

50% sucrose mix

25 g	-	Sucrose (50%)
500 µL	-	Na ₂ EDTA from 100 mM stock (1 mM)
5 mL	-	HEPES from 100 mM stock (10 mM)
1 mL	-	Na ₂ ATP from 100 mM stock (2 mM)
5 µL	-	Chymostatin (2 µg/mL)
50 mL	-	ddH ₂ O

Stored at 4 °C.

Sucrose step gradients

Prepared on the day before the experiment in a 15 mL polycarbonate Sorvall tubes with jacket by adding 7 mL 50% sucrose mix, 2 mL 40% sucrose mix and 2 mL 30% sucrose mix. Two tubes were made and stored at 4°C.

IP lysis buffer

50 mL	-	HEPES from 100 mM stock at pH 7.5 (50 mM)
15 mL	-	NaCl from 1 M stock (150 mM)
10 mL	-	Glycerol (10% v/v)
20 µL	-	NP40 (0.02% v/v)
1 mL	-	Na ₂ EDTA 100 mM stock (1 mM)
100 µL	-	Leupeptin from 1 mg/mL stock (1 µg /mL)
100 µL	-	Pepstatin A from 1 mg/mL stock (1 µg /mL)
1000 µL	-	PMSF from 100 mM stock (1 mM)
Up to 100 mL	-	ddH ₂ O

Stored at 4°C.

1 mg/mL Leupeptin

1 mg of Leupeptin in 1 mL of ddH₂O.

1 mg/mL Pepstatin A

1 mg of Pepstatin A in 1 mL of methanol.

FLAG-rinsing buffer

5 mL	-	NH ₄ HCO ₃ from 0.5 M stock at pH 8 (50 mM)
3.75 mL	-	KCl from 1 M stock (75 mM)
500 µL	-	Na ₂ EDTA from 200 mM stock (2 mM)
Up to 50 mL	-	ddH ₂ O

Stored at 4°C.

Colloidal coomassie stain

10 g	-	(NH ₄) ₂ SO ₄ (10%)
10 mL	-	H ₃ PO ₄ (10%)
20 mL	-	Methanol (20%)
0.12 g	-	Coomassie Brilliant Blue G-250 (0.12%)
Up to 100 mL	-	ddH ₂ O

Gel drying solution

15 mL	-	Methanol
3 mL	-	Glacial acetic acid
1.5 mL	-	Glycerol
30.5 mL	-	ddH ₂ O

Appendix III

Primers used to analyze the *uvr90* mutant.

Primer Name	Sequence 5'→3'	Location on Linkage group VI and strand (+/-)
F6*	GACATTGGCCGCAGATGGAT	3,345,920- 939 (+)
F5*	GGAACCTCCGAAGAAAGGAAGG	3,346,404- 425 (+)
F4*	CCGGATGACTTCATCTTCCCTG	3,346,862- 883 (+)
F3*	CTTGGAACCGCGCAGTAACAT	3,348,428- 448 (+)
F2*	TACTTCGACGACGTCTGGCTTC	3,347,890- 911 (+)
F1*	CCGTGACACATTTGTTGCTGC	3,347,371- 391 (+)
R1*	TCCGGAACAGCATCAGGAAAG	3,348,966- 986 (-)
R2*	CATGGGTTTATCATGCGGGAC	3,348,468- 488 (-)
R3*	CCGTCGCGAAGAGATGAAACTC	3,347,852- 873 (-)

R4*	AAATGGCTGTGGTCGCATCTC	3,349,471- 491 (-)
R5*	ATGAGGAAGCCGAGAGCATCTC	3,349,938- 959 (-)
R6*	CCGGTATGAGCGTCGGAATTT	3,350,451- 471 (-)
R7*	GCCCGGATGTTTGTTCGTTC	3,350,986- 3,351,006 (-)
R8*	TCAGTCTTTTTGGTCACGGACG	3,351,543- 564 (-)
R9*	ACTCGCACATGCATACTCGCA	3,352,122- 142 (-)
UV90AFor	ACGTGGACTGTTGCGTGACAC	3,348,179- 199 (+)
UV90ARev	GTGTCACGCAACAGTCCACGT	3,348,179- 199 (-)
UV90BFor	AACTTTACCGATGCCGCTGC	3,348,687- 706 (+)
UV90BRev	GCAGCGGCATCGGTAAAGTT	3,348,687- 706 (-)
FlipR1For	CTTTCCTGATGCTGTTCCGGA	3,348,966- 986 (+)
FlipR4For	GAGATGCGACCACAGCCATTT	3,349,471- 491 (+)

FlipF5Rev	CCTTCCTTTCTTCGGAGGTTC C	3,346,425- 404 (-)
FlipF4Rev	CAGGGAAGATGAAGTCATCCG G	3,346,883- 862 (-)
FlipF1Rev	GCAGCAACAAATGTGTCACGG	3,347,391- 371 (-)
FlipR6For	AAATTCCGACGCTCATACCGG	3,350,451- 471 (+)
FlipR7For	GAACGAAACAAACATCCGGGC	3,350,986- 3,351,006 (+)
FlipR8For	CGTCCGTGACC AAAAAGACTGA	3,351,543- 564 (+)
17F1	ACGCTTCTTTCCTTATCCCACCCAC	3,344,879- 903 (+)
17F2	AGGTAGAAGTTGGAAAGGTGCGCTG	3,343,693- 717 (+)
17F3	TCGGTCTTGATCGCTGGTTCTTACG	3,342,614- 638 (+)
17F4	GTACGCCGTGTATTTGTGTGTCTGG	3,341,477- 501 (+)
17F5	TCCTCCATCACCAACGCACAATGAC	3,339,947- 971 (+)
17F6	AGCCCGCTAACGCCAATCTTATAC	3,338,357- 380 (+)

17F7	AATCTAAAGGAGGAGGACCCGGAAG	3,337,024-048 (+)
17F8	CCGGCGCAAACCATCGAAATTAAG	3,335,528-551 (+)
17F9	CTTGTGAATGGTTTGTGGCGGTTCC	3,334,358-382 (+)
17F10	TCTCCGTCTCCGTATTGGGTACATC	3,333,239-263 (+)
17F11	CATTGCCATTCCCAACAACAGGTG	3,332,263-286 (+)
17F12	GCCCAACAAACAACGAACAACGATC	3,331,005-029 (+)
17F13	CAACGGCAACAAGCTGTACGCTAG	3,329,680-703 (+)
17F14	AGTTCCGACAGAGGTGCTACATGG	3,327,929-952 (+)
17F15	AACAGCACTGACATCAGCAAGCAAG	3,326,728-752 (+)
17F16	GCTTGGCATCCTTGTACTCGTTGT C	3,325,372-396 (+)
17F17	TCTCAATCCATTCTGGGCTCATCGTC	3,324,110-134 (+)
17F18	ACTCGTATTCTCTCCATTGGGCTCG	3,323,122-146 (+)
17F19	TGGTGTGATGGAAATGTACGCGCTG	3,321,779-803 (+)

17F20	CTGGAACCCATTTCTCGCACACATC	3,320,481- 505 (+)
17F21	TTTGGTTGTCTGGTTGGCGTTATCC	3,319,264- 288 (+)
17F22	GTGAGGCCAACGACTGAGAAAGTAG	3,317,916- 940 (+)
17F23	ACAGTGACGAGACCTTTGAGAGGAG	3,316,708- 732 (+)
17F24	CCTTGCTTCACCACGTTCTTAGCTC	3,315,419- 443 (+)
17F25	TACACTCTCTCTGTTGCTCGTCGTC	3,314,341- 365 (+)
17F26	TGTTGCTATCGGATGTGGAGGTTGG	3,313,207- 231 (+)
17F27	CTCCATCGGTACAGTCCAAACCATC	3,312,220- 244 (+)
17F28	TGAAGACTGCCACCACGTTGATAC	3,310,921- 944 (+)
17F29	ATACAAGACACGACCACACGCCAAG	3,309,813- 837 (+)
17F30	GGCAAGTGTGGATTGGTGATGTGAG	3,308,466- 490 (+)
17R1	CAACGGAGAGAGAGGGTGAATGGAA	3,351,689- 665 (-)
17R2	CGACAGGCAAAGGTAAGAAAGGGAG	3,352,758- 734 (-)

17R3	GACAATTTCCACCGTGCTACCACCA	3,353,926-902 (-)
17R4	GAGGGCCGGAAAGAAGTCAAACCAA	3,355,140-116 (-)
17R5	CCACCCTCACCGTTCAGATACTTCT	3,356,189-165 (-)
17R6	CCTTCCACTCAACAGTTCGTCCGTT	3357,753-729 (-)
17R7	CATATTCCTACTTGACGCCGCTCAG	3,358,854-830 (-)
17R8	CAAGTACACCACTCCCACCACACT	3,360,081-058 (-)
17R9	CTCTGTCCTTCTCCAGCCTTCTTGA	3,361,047-023 (-)
17R10	CAAGACGGGAGTGGAAGAGTCAAAG	3,362,202-178 (-)
17R11	CATGCCATCCCACTCCCTTGAAGAT	3,363,468-444 (-)
17R12	CTCGGTCAAATGGTGGATGTGTTGC	3,364,946-922 (-)
17R13	CCAAGTCCCATGACGAAGAGCAATC	3,366,785-761 (-)
17R14	CGTCTTGCCTATTCCCACTCCTTTC	3,368,511-535 (-)
17R6FlipFor	AACGGACGAACTGTTGAGTGGAAGG	3357,729-753 (+)

17R7FlipFor	CTGAGCGGCGTCAAGTAGGAATATG	3,358,830-854 (+)
17R8FlipFor	AGTGTGGTGGGAGTGGTGTACTTG	3,360,058-081 (+)
17R9FlipFor	TCAAGAAGGCTGGAGAAGGACAGAG	3,361,023-047 (+)
17R10FlipFor	CTTTGACTCTTCCACTCCCGTCTTG	3,362,178-202 (+)
17R11FlipFor	ATCTTCAAGGGAGTGGGATGGCATG	3,363,444-468 (+)
17R12FlipFor	GCAACACATCCACCATTGACCGAG	3,364,922-946 (+)
17R13FlipFor	GATTGCTCTTCGTCATGGGACTTGG	3,366,761-785 (+)
17F6FlipRev	GTATAAGATTGGCGTTAGCGGGCT	3,338,380-357 (-)
17F7FlipRev	CTTCCGGGTCCTCCTCCTTTAGATT	3,337,048-024 (-)
17F8FlipRev	CTTAATTTGATGGTTTGCGCCGG	3,335,551-528 (-)
17F9FlipRev	GGAACCGCCACAAACCATTCACAAG	3,334,382-358 (-)
17F10FlipRev	TCTCCGTCTCCGTATTGGGTACATC	3,333,263-239 (-)
17F11FlipRev	CACCTGTTGTTGGGAATGGCAATG	3,332,286-263 (-)

17F12FlipRev	GATCGTTGTTTCGTTGTTTGTGGGC	3,331,029-005 (-)
17F13FlipRev	CTAGCGTACAGCTTGTTGCCGTTG	3,329,703-680 (-)
17F14FlipRev	CCATGTAGCACCTCTGTCGGAAC	3,327,952-929 (-)
17F15FlipRev	CTTGCTTGCTGATGTCAGTGCTGTT	3,326,752-728 (-)
17F16FlipRev	GACAACGAGTACAAGGATGCCAAGC	3,325,396-372 (-)
17F17FlipRev	GACGATGAGCCCGAATGGATTGAGA	3,324,134-110 (-)
17F18FlipRev	CGAGCCCAATGGAGAGAATACGAGT	3,323,146-122 (-)
17F19FlipRev	CAGCGCGTACATTTCCATCACACCA	3,321,803-779 (-)
17F20FlipRev	GATGTGTGCGAGAAATGGGTTCCAG	3,320,505-481 (-)
17F21FlipRev	GGATAACGCCAACCAGACAACCAAA	3,319,288-264 (-)
17F22FlipRev	CTACTTTCTCAGTCGTTGGCCTCAC	3,317,940-916 (-)
17F23FlipRev	CTCCTCTCAAAGGTCTCGTCACTGT	3,316,732-708 (-)
17F24FlipRev	GAGCTAAGAACGTGGTGAAGCAAGG	3,315,443-419 (-)

17F25FlipRev	GACGACGAGCAACAGAGAGAGTGTA	3,314,365- 341 (-)
17F26FlipRev	CCAACCTCCACATCCGATAGCAACA	3,313,231- 207 (-)
17F27FlipRev	GATGGTTTGGACTGTACCGATGGAG	3,312,244- 220 (-)
17F28FlipRev	GTATCAACGTGGTGGCAGTCTTCA	3,310,944- 921 (-)
17F29FlipRev	CTTGGCGTGTGGTCGTGTCTTGTAT	3,309,837- 813 (-)
18SeqF1	GACCCTGACACGAAATAAACGTGC	3,320,837- 860 (+)
18SeqR1	CTCTAATGCCGCTAATCCACGGAT	3,357,216- 193 (-)
18SeqF2	TCCCTATCCCACTCGAACATAATGC	3,321,025- 049 (+)
18SeqR2	GACCGGCTTCTACTCTCGTTCAA	3,356,726- 704 (-)
18SeqR3	CACACGCAAAGAGAGGAAGGAGACA	3,356,267- 243 (-)

* Primers ordered from Invitrogen and all the other primers were ordered from Integrated DNA Technologies (IDT).

Appendix IV

Primers used to clone NCU05950 gene and NCU00376 gene.

Primer Name	Sequence 5'→3'
UV90ApaIF	TCC GGGCCC TCATCTTCCCTGTTGAACTCGTG
UV90NotIR	ATAAGAAT GCGGCCGC CGAATGTTGCATGTCCTTCCA
UV90tagNotIF	ATAAGAAT GCGGCCGC TCGTGGTCCGTTCTGATG
UV90tagXbaIF	GC TCTAGA ATGGGCAACTTTTGCTCAACC
UV90tagPacIR	CC TTAATTAA ACAGCGCATGGCGGCAGCG
UV90 Δ 1XbaIF	GC TCTAGA ATGAACTTTTGCTCAACCTGCTTCGGC
UV90 Δ 7XbaIF	GC TCTAGA ATGTTTCGGCGGTAGGAGGAGCGATGAC
UV90 Δ 10XbaIF	GC TCTAGA ATGAGGAGGAGCGATGACTACGATGAG
UV90PacIR	CC TTAATTAA ACAGCGCATGGCGGCAGCGGCATCGG
GTR2XbaIF	GC TCTAGA ATGGAGGTAAGCAAAACAAGATACA
GTR2PacIR	CC TTAATTAA ACAACGACCATCACCCACCACCC

Restriction enzyme identification sites and overhang sequences on primers are in bold text.

Appendix V

Primers used to identify gene knockout strains.

Gene & expected product size from wild type strain	Primer Name	Sequence 5'→3'
NCU05950 1096 bp	UV90KO-F	CGAAGCAACTCGACTAAACC
	UV90KO-R	GGTGTGGTATATTCTGGGTC
PIK1a 3562 bp	PIK1aF	CCAAGTTAGCTTCCGTCTAG
	PIK1aR	CGTCTCCTGTTTCAGGTGAT
TOR2 8402bp	TOR2F	GGTCATTACGACTCACGAGC
	TOR2R	CCAGTTCACCTATAGCACCA
KOG1 4779 bp	KOG1F	CCTGATATAGCGCATCTACC
	KOG1R	CCTCTCGTAACTTGGAGGAT
Vam6 4582bp	Vam6F	CGAAAGATAGTCCCGCTATC
	Vam6R	GGAGGGAGGGAATAGTAATG
Tap42 1479bp	Tap42F	CCAACATCGACTCAAGACAC
	Tap42R	GGTTAATACGTAGCGAGTGG
FKR5 1161bp	FKR5F	CGAGATCACACAGGCTATA
	FKR5R	CCACTAGACATAGACCTCCA
GTR2 1935bp	GTR2F	GGACATACAAGCTAGTGCCT
	GTR2R	GCAAGTAGCCACAGAAAGTC
Stk-10 3268 bp	STK10F	CCTCGGATAGTGCTTTGACT
	STK10R	GGAGAGCTACATCAAAGCCA

Stk-10	STK10F2	CAGGTGTTATTCCAAGGACC
5601 bp	STK10R2	GACACATTACAGCACCTACC
Stk-10	STK10F3	ACCCGCTGTGTTACGTTTCCTG
4172 bp	STK10R3	GCAACACCAAGCAAGCGAGAAC

Appendix VI

Primers used to distinguish different genotypes.

Primer Name	Testing Genotype	Sequence 5'→3'
HIS-3F1	Transformants (Homokaryon or heterokaryon)	CTCATGTTCAACCCTTTGGATGG
HIS-3R		GTCATGCAGTTGGCTAAGGTTGAG
pBM61F	Transformants (Homokaryon or heterokaryon)	ATTCAGACCCCATAGCCGTGCACGCC
pBM61R		GACGGGGTAGCTTGGCCCGGGGCTACA
pBM61F2	Transformants (Homokaryon or heterokaryon)	TGTAGCCCCGGGCCAAGCTACCCCGTC
pBM61R2		GGCGTGCACGGCTATGGGGTCTGAAT
FrqF	Presence or absence of <i>Frq</i> gene	GGCCTGCTTCCTCACCATATTG
FrqR		GAGATACAAGGCCCAGATGACG
hphF	Presence or absence of <i>hph</i> cassette	GACAGAAGATGATATTGAAGGAGC
hphR		GATTTCAGTAACGTTAAGTGGAT
hphRev		GTATTGACCGATTCTTGCGGT

Appendix VII

Commercially synthesized gene fragments.

ATG – Start codon

GGGACCC – SanDI site

AGATCT – BglII

Upper case letters – Exons

Lower case letters - Introns

Δ1-NCU05950 = 611bp

GGGACCCAAC TTTTTTTTTTTTTTTTTTTTTTGG TTTTTTTTTTTGTCGGTCTGAT
CTCATTTTCGTCATCGATCGAGTTTCATCTCTTCGCGACGGTCTTTTCTCCCAA
CTTTACTTCGACGACGTCTGGCTTCAAGACGAGACTTTTTCTTGTCGCTTCGC
GCGAATTCCAATTGACAAACGTCATCCGACGACTTGATACCGACCGACCGACC
GACCACCATCCGACTGGCGACACGGCACATAAAGAAGGGAGGACAAGATAACA
AG**ATG**AAC TTTTGCTCAACCTGCTTCGGCGGTAGGAGGAGCGATGACTACGAT
GAGgtaggttttcggcatagcggccacagtccgatgacatggccatcaaaagag
gcattcgacggaacaagagaactgacgtggactggtgcgtgacacag**GAGGAC**
GAGGCCCAGCATCTCTTCGACGAGAACAACATGCACTACGGGAGCTTTGATCA
GCAACCATGATGAACCAGGAAGATCCCCAAGAGACAGAAAGGGAAATTGCGG
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Δ7-NCU05950 = 593bp

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CTCATTTTCGTCATCGATCGAGTTTCATCTCTTCGCGACGGTCTTTTCTCCCAA
CTTTACTTCGACGACGTCTGGCTTCAAGACGAGACTTTTTCTTGTCGCTTCGC
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gaactgacgtggactggtgcgtgacacag**GAGGAC**GAGGCCCAGCATCTCTTC
GACGAGAACAACATGCACTACGGGAGCTTTGATCAGCAACACATGATGAACCA
GGAAGATCCCCAAGAGACAGAAAGGGAAATTGCGGCTCTGCAGGGCGTG GTG
AACGCCACATCAAAgtatgccctcccacctgaaatgtctcccatcca**agatct**

Δ10-NCU05950 = 584bp

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CAAAgtatgccctcccacctgaaatgtctcccatccaagatct

Appendix VIII

Ligations

Plasmid	Vector size	Insert size	Molar ration of insert: vector
pBM61-1kb up stream- NCU05950-1kb down stream	6.836 kb	2.883 kb	3:1
pUC19-1kb up stream- NCU05950-1kb down stream	2.686 kb	2.87 kb	1:1
pNative promoter- NCU05950::Gly::GFP (GN)	7.556 kb	1.838 kb	3:1
pccg-1- NCU05950::Gly::GFP (GX)	8.486 kb	0.678 kb	5:1
pNative promoter- NCU05950::Gly::3xFLAG (FN)	7.096 kb	1.838 kb	3:1
pccg-1- NCU05950::Gly::3xFLAG (FX)	8.026 kb	0.678 kb	5:1
pNative promoter- Δ 1- NCU05950::Gly::GFP (Δ 1GN)	8.797 kb	0.594 kb	5:1
pNative promoter- Δ 7- NCU05950::Gly::GFP (Δ 7GN)	8.797 kb	0.576 kb	5:1
pNative promoter- Δ 10- NCU05950::Gly::GFP (Δ 10GN)	8.797 kb	0.567 kb	5:1
pccg-1- Δ 1- NCU05950::Gly::GFP (Δ 1GX)	8.486 kb	0.675 kb	5:1

pccg-1- Δ 7- NCU05950::Gly::GFP (Δ 7GX)	8.486 kb	0.657 kb	5:1
pccg-1- Δ 10- NCU05950::C-Gly::GFP (Δ 10GX)	8.486 kb	0.648 kb	5:1
pNative promoter- Δ 1- NCU05950::Gly::3xFLAG (Δ 1FN)	8.337 kb	0.594 kb	5:1
pNative promoter- Δ 7- NCU05950::Gly::3xFLAG (Δ 7FN)	8.337 kb	0.576 kb	5:1
pNative promoter- Δ 10- NCU05950::Gly::3xFLAG (Δ 10FN)	8.337 kb	0.567 kb	5:1
pccg-1- Δ 1- NCU05950::Gly::3xFLAG (Δ 1FX)	8.026 kb	0.675 kb	5:1
pccg-1- Δ 7- NCU05950::Gly::3xFLAG (Δ 7FX)	8.026 kb	0.657 kb	5:1
pccg-1- Δ 10- NCU05950::Gly::3xFLAG (Δ 10FX)	8.026 kb	0.648 kb	5:1
pccg-1- NCU00376::Gly::GFP (GTR2::GFP)	8.486 kb	2.643 kb	3:1

Appendix IX

NCU05950

Predicted Protein Sequence - 158 aa

MGNFCSTCFGGRRSDDYDEEDEAQHLFDENNMHYGSFDQQHMMNQEDPQETEREIAALQGVVER
TSNNMVDIYDMVPHDKPMQDAPAPYGFANQRYNALLSKLSTHDDMAAVARVDWGTPEDDSMEML
RKASLPTIPIKAEGGEALVGNFTDAAAAMR

Predicted RNA/mRNA Sequence (Introns spliced out; UTRs highlighted [utr]) - 695 bp

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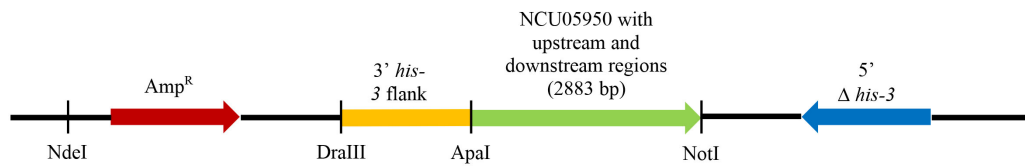
Genomic DNA sequence (Introns [intron] and UTRs [utr] highlighted)- 890 bp

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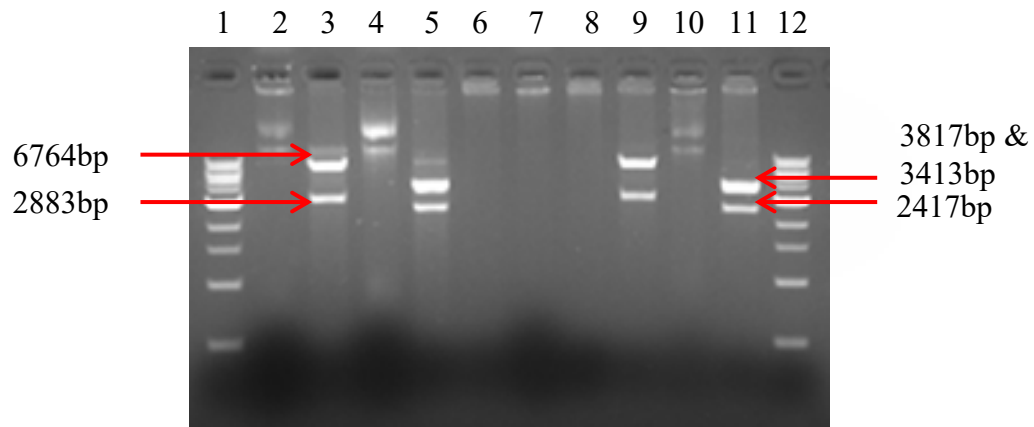
Appendix X

Cloning of NCU05950 gene to pBM61 plasmid

A. Schematic diagram of partial map of pBM61-NCU05950 plasmid (9644 bp).



B. Restriction enzyme digestion of plasmids isolated from colony PCR positive colonies (colony number 2, 9 & 28) to show the presence of insert (2883 bp) in the plasmid (6764 bp) and direction of inserts is correct.



Lane No.	Plasmid	Enzyme
2	pBM61 plasmid	Undigested
3	Plasmid DNA from colony #2	ApaI - NotI
4	Plasmid DNA from colony #2	Undigested
5	Plasmid DNA from colony #2	DraIII - HindIII
6	Plasmid DNA from colony #9	ApaI - NotI
7	Plasmid DNA from colony #9	Undigested
8	Plasmid DNA from colony #9	DraIII - HindIII
9	Plasmid DNA from colony #28	ApaI - NotI
10	Plasmid DNA from colony #28	Undigested
11	Plasmid DNA from colony #28	DraIII - HindIII

Lane No.1 & Lane No.12: 1kb ladder.

Note: Even though colony number 9 identified as a positive colony by colony PCR, no plasmid DNA was extracted from it.

Appendix XI

pBM61-NCU05950 plasmid sequence

```
1      tatagtgtcacctaaatcgatatgtgtatgatacataaggttatgtattaa
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201    caagctgtgaccgtctccgggagctgcatgtgtcagagggttttcaccgtc
      atcaccgaaacgcgcgagacgaaagggcctcgtgatacgcctatTTTTTat 300
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      cggggaaatgtgcgcggaaccctatTTTgTTTTTTTTttaaatacatTC 400
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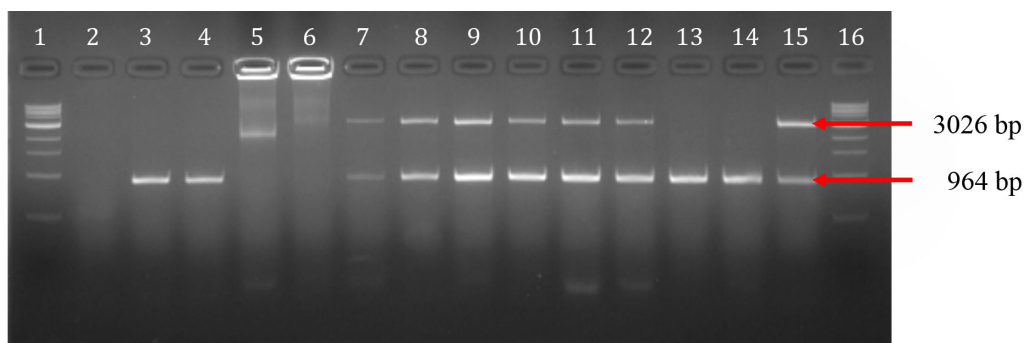
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ttgagagcagcatcgagctcctcggttagagaccttggaggcgtcgaaacg 8900
8901 tctcatggtgatcttctcaggggtggtctcctgggcggcctccttgggtga
caggggcccgaagtggcagcgagagcggacgccgcaggcttgatgccctcc 9000
9001 ttctcagcccacttacccttagcatctccagtcctgcgcttgacctcca
gctcttggcgtcaaggctcctttcgatatcggcaagagtaacgccggcag 9100
9101 caacggccctggtaagagcaaagtagaagagatcggcagcctcaaaggcg
atcttcttggggggtctgagcgggtgcagagctcctcagcctcctccatgat 9200
9201 cttggcccggactagcttctcatcggagaagagacgggcagtgtaggagc
cctcgggggagactgttctcctcgaaatcaaagtctgctcgagcttggga 9300
9301 aggcctttgagctgaccaaagcagccgactgatcgaggtggcagaaacg
acccttctgcttcacgacaaacttgagagcatcgttatcgcagtcagcg 9400
9401 agatgcggacgagctcctgagtgctcgcggaagtagcacccttgtaggag
agaccgcgcttccggctctgatagacaccagtctgtgtcctgagggcctc 9500
9501 gttcacactctcggcactgctgtagaccagaccagagcagtgctcgtgct
catcaacgacaacggtggggagaagaccatcgggacgggtcagacttccag 9600
9601 acgctcgagaggatggtggagatggcaagcttcagctgctcgagttc 9647

-  Amp^R gene
-  3' *his-3* flank region
-  pBM61 – primer F1 & pBM61 – primer R1
-  ApaI site
-  NCU05950 with upstream and downstream regions
-  NCU05950 start codon
-  NotI site
-  5' truncated *his-3* gene
-  HIS-3R primer

Appendix XII

PCR Analysis of transformed fungal strains with pBM61 vector specific primers (pBM61F2 -pBM61R2 → 3026bp).



Lane No.1 & Lane No.16: 1kb ladder

Lane No.2: Negative Control-Water PCR

Lane No.3: SG#26

Lane No.4: SG#361

Lane No.5: Negative control - PCR with pBM61 plasmid DNA

Lane No.6: Positive control - PCR with pBM61-NCU05950 plasmid DNA

Lane No.7: Transformant- L1a (SG#420)

Lane No.8: Transformant- L1c (SG#421)

Lane No.9: Transformant- L1 (SG#422)

Lane No.10: Transformant- L2a (SG#423)

Lane No.11: Transformant- L2b

Lane No.13: Transformant- L3b (SG#424)

Lane No.13: Transformant- L1b

Lane No.14: Transformant- L1d

Lane No.15: Transformant- L1e (SG#425)

Appendix XIII

Rhythmic phenotype of purified transformants

Negative control:
SG#361

* Positive control:
SG#26

* Transformant: L1a:
SG#428

* Transformant: L1c:
SG#431

* Transformant: L1

* Transformant: L2a

* Transformant: L2b:
SG#432

* Transformant: L3b:
SG#430

Transformant: L1b

Transformant: L1d

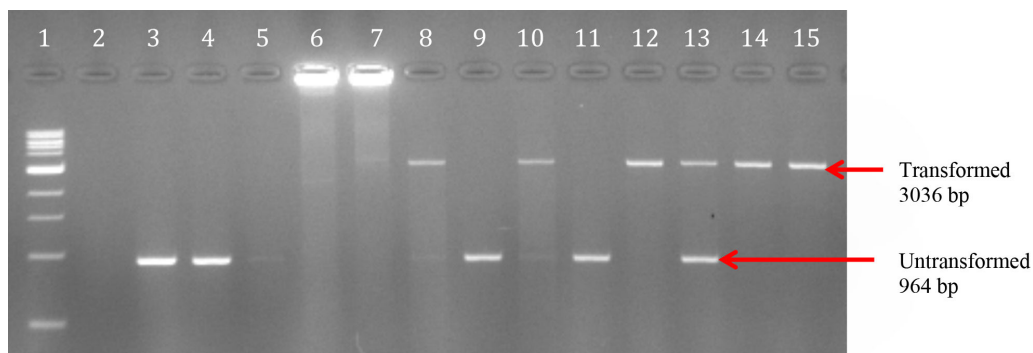
* Transformant: L1e:
SG#429



Free-running rhythmic phenotype of purified transformants carrying a wild type copy of the NCU05950 gene at an ectopic location in the *his-3* locus. Three replicate tubes grown in the presence of 100 μ M Choline on MA medium are shown. Growth is from right to left. Wild type phenotype is indicated by *.

Appendix XIV

PCR Analysis of purified transformed fungal strains.



PCR Analysis of purified transformed fungal strains with pBM61 vector specific forward primer and reverse primer (pBM61F2-pBM61R2→3026bp). Purified transformants are indicated by *.

Lane No.1: 1kb ladder

Lane No.2: Negative control - Water PCR

Lane No.3: SG#361

Lane No.4: SG#26

Lane No.5: SG#80

Lane No.6: Negative control - pBM61 plasmid DNA

Lane No.7: Positive control - pBM61-NCU05950 (BM28) plasmid DNA

Lane No.8: Transformant- L1a (SG#428) *

Lane No.9: Transformant- L1b

Lane No.10: Transformant- L1c (SG#431) *

Lane No.11: Transformant- L1d

Lane No.12: Transformant- L1e (SG#429) *

Lane No.13: Transformant- L2a

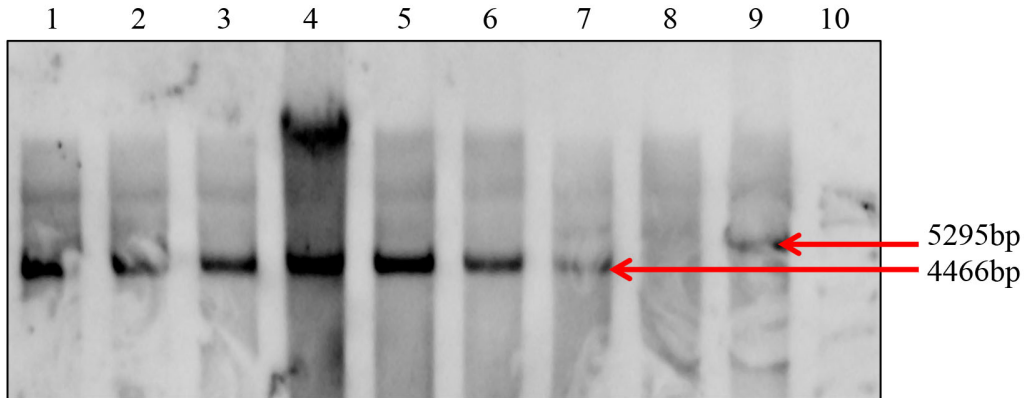
Lane No.14: Transformant- L2b (SG#432) *

Lane No.15: Transformant- L3b (SG#430) *

Note that L1b and L1d are non-transformants and L2a is a heterokaryon.

Appendix XV

Southern blot analysis of rescue progeny



Southern blotting of KpnI digested genomic DNA from NCU05950 wild type strains (SG#80), mutant type strains (SG#213) and transformants/rescue progeny (SG#430, 465,466,467,468,469 & 470).

Lane No.1: SG#470 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; uv90*)

Lane No.2: SG#469 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; uv90*)

Lane No.3: SG#468 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; uv90*)

Lane No.4: SG#467 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; uv90 ; frq⁹*)

Lane No.5: SG#466 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; uv90 ; frq⁹*)

Lane No.6: SG#465 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; uv90 ; frq⁹*)

Lane No.7: SG#430 (*csp-1 his-3⁺::NCU05950⁺ ; ras^{bd} chol-1 ; NCU05950^{KO}*)

Lane No.8: SG#213 (*csp-1 ; ras^{bd} chol-1 ; uv90 ; frq¹⁰*)

Lane No.9: SG#80 (*csp-1 ; ras^{bd} chol-1 ; frq¹⁰*)

Lane No.10: DNA Molecular Weight Marker VII, DIG-labeled (**Size**

range: 0.081 – 8.57 kbp → **17 fragments:** 81; 359; 492; 710; 718*; 992;

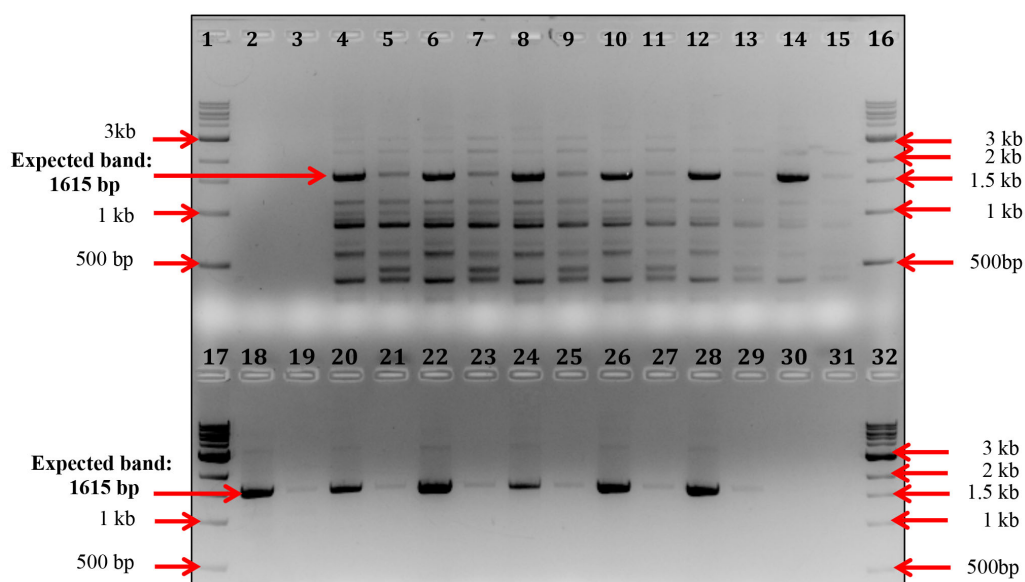
1,164; 1,482; 1,515; 1,882; 1,953; 2,799; 3,639; 4,899; 6,106; 7,427; and 8,576 bp)

Appendix XVI

Optimizing PCR conditions to obtain a product from *uv90* mutant strains

A.

PCR results showing attempts taken to find the optimum annealing temperature for F1 – R1 primers to obtain a product from *uv90* mutant strain SG#224 (*csp-1*; *ras^{bd} chol-1*; *uv90*) keeping the wild type strain SG#26 (*csp-1*; *ras^{bd} chol-1*) as the positive control. (Expected product size= 1615bp)



Lane No.1: 100bp Ladder

Lane No.2: Negative control - Water PCR with master mixture of SG#26 at 59°C

Lane No.3: Negative control - Water PCR with master mixture of SG#224 at 59°C

Even numbered lanes 4 – 28: SG#26

Odd numbered lanes 5 – 29: SG#224

Annealing temperatures of Lane No. 4 - 29: 59°C, 59.2°C, 59.7°C, 60.3°C, 61.3°C, 62.5°C, 63.8°C, 65°C, 65.8°C, 66.4°C, 66.9°C, 67°C

Lane No.16: 1kb Ladder

Lane No.17: 100bp Ladder

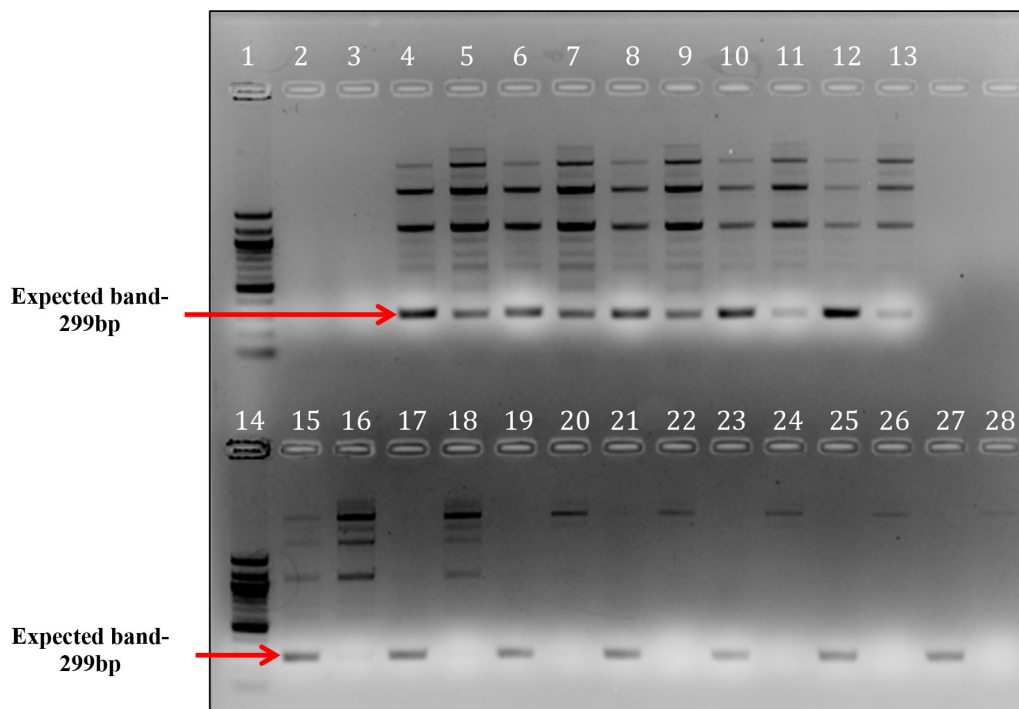
Lane No.30: Negative control - Water PCR with master mixture of SG#26 at 67°C

Lane N0.31: Negative control - Water PCR with master mixture of SG#224 at 67°C

Lane No.32: 1kb Ladder.

B.

Example of PCR results showing attempts to find the optimum annealing temperature for UV90BFor – R1 primers to obtain a product from *uv90* mutant strain SG#224 (*csp-1*; *ras^{bd} chol-1*; *uv90*) using the wild type strain SG#26 (*csp-1*; *ras^{bd} chol-1*) as the positive control. (Expected product size= 299bp)



Lane No.1 & Lane No.14: 100bp Ladder

Lane No.2: Negative control – Water PCR at 59°C

Lane No.3: Negative control - Water PCR at 67°C

Lane No.4: SG#26 at 59°C

Lane No.5: SG#224 at 59°C

Lane No.6: SG#26 at 59.2°C

Lane No.7: SG#224 at 59.2°C

Lane No.8: SG#26 at 59.7°C

Lane No.9: SG#224 at 59.7°C
Lane No.10: SG#26 at 60.3°C
Lane No.11: SG#224 at 60.3°C
Lane No.12: SG#26 at 61.3°C
Lane No.13: SG#224 at 61.3°C
Lane No.15: SG#26 at 62.5°C
Lane No.16: SG#224 at 62.5°C
Lane No.17: SG#26 at 63.8°C
Lane No.18: SG#224 at 63.8°C
Lane No.19: SG#26 at 65°C
Lane No.20: SG#224 at 65°C
Lane No.21: SG#26 at 65.8°C
Lane No.22: SG#224 at 65.8°C
Lane No.23: SG#26 at 66.4°C
Lane No.24: SG#224 at 66.4°C
Lane No.25: SG#26 at 66.9°C
Lane No.26: SG#224 at 66.9°C
Lane No.27: SG#26 at 67°C
Lane No.28: SG#224 at 67°C

Appendix XVII

Southern blot analysis results of NCU05950 wild type (SG#26& SG#80) and *uv90* mutant type (SG#224 & SG#213) strains

f- Faint bands

Restriction Enzyme	Expected band/s	Observed Band/s	Presence (✓) or absence (✗) of bands			
			SG#26	SG#22 4	SG#21 3	SG#80
EcoRI	2344 bp 6545 bp	~2.3 kb	✓	✗	✗	✓
		~6.5 kb	✓	✗	✗	✓
		~9.5 kb	✓	✓	✓	✓
EagI	2435 bp	~1.75 kb	✓	✓	✓	✓
		~2.5 kb	✓	✗	✗	✓
		~5.5 kb	✓	✓	✓	✓
		~9 kb	✓	✓	✓	✓
		~11 kb	✓	✓	✓	✓
		~13 kb	✗	✗	✓	✓
HindIII	2454 bp 9831 bp	~1 kb	✓	✓	✓	✓
		~2.5 kb	✓	✗	✗	✓
		~5 kb	✓	✓	✓	✓
		~5.5 kb	✓	✓	✓	✓
		~10 kb	✓	✗	✗	✓

		>10 kb	✓	✓	✓	✓
BglII	3186 bp 9297 bp	~3 kb	✓	✗	✗	✓
		~4 kb	✓	✓	✓	✓
		~7 kb	✓	✓	✓	✓
		~9 kb	✓	✗	✗	✓
		~10 kb	✓	✗	✗	✓
HpaII	406 bp 1140 bp 466 bp	~ 1100 bp	✓	✓f	✓f	✓
	52 bp 56 bp 293 bp	~ 900 bp	✗	✓	✓	✗
	115 bp 1078 bp	~ 40 bp	✓	✗	✗	✓
AluI	1472 bp 297 bp	~1500 bp	✓	✗	✗	✓
	952 bp 85 bp	~1100 bp	✓f	✓f	✓f	✓f
		~900 bp	✓	✗	✗	✓
XhoII	2319 bp 2237 bp 96 bp	~3000 bp	✗	✓	✓	✗
		~2300 bp	✓	✗	✗	✓
		~1600 bp	✓	✓	✓	✓
		~1400 bp	✗	✓	✓	✗
		~1100 bp	✓	✗	✗	✓
		~900 bp	✓	✓	✓	✓

		~750 bp	✖	✖	✓	✖
		~700 bp	✓	✖	✖	✓
		~500 bp	✓	✓	✓	✓
TaqI	876 bp	~1800 bp	✓f	✓f	✓f	✓f
	229 bp	~1600 bp	✓f	✓f	✓f	✓f
	148 bp	~1000 bp	✓f	✓f	✓f	✓f
	265 bp	~600 bp	✖	✓	✓	✖
	65 bp	~500 bp	✓	✖	✖	✓
	227 bp	~400 bp	✖	✓	✓	✖
	556 bp	Less than	✓	✖	✖	✓
	58 bp	400 bp	✖	✓	✓	✖
	565 bp					
	28 bp					
	319 bp					

Appendix XVIII

List of genes affected by *uv90* mutation on Linkage Group (Chromosome) VI.

Gene Number	Gene Name	Location on Oak Ridge strain, (Supercontig 6)	Genomic Sequence Length (bp)
NCU05957	Hypothetical protein	3,319,864 - 3,322,467 (+)	2604 bp
NCU05956	Beta-galactosidase (gh2-2)	3,322,517 - 3,326,426 (-)	3910 bp
NCU05955	Cel74a(gh74-1)	3,326,807 - 3,330,420 (+)	3614 bp
NCU15399	Unspecified product (tRNA(glu)-5)	3,330,833 - 3,330,904 (-)	72 bp
NCU15400	Unspecified product (tRNA(met)-4)	3,331,450 - 3,331,521 (-)	72 bp
NCU05954	Hypothetical protein	3,332,004 - 3,334,639 (-)	2636 bp
NCU05953	Hypothetical protein	3,339,700 - 3,340,998 (-)	1299 bp
NCU05952	Hypothetical protein	3,341,267 - 3,343,308 (-)	2042 bp
NCU05951	Hypothetical protein	3,344,579 - 3,346,273 (+)	1695 bp
NCU05950	Hypothetical protein	3,347,867 - 3,348,756 (+)	890 bp
NCU05949	Hypothetical protein	3,349,464 - 3,350,054 (+)	591 bp
NCU05948	Hypothetical protein, hypothetical protein, variant 1	3,353,034 - 3,355,088 (-)	2055 bp
NCU05947	Hypothetical protein	3,355,960 - 3,356,454 (-)	495 bp

Appendix XIX

Restriction enzyme digestion of recombinant plasmids

pGN and pGX

A.

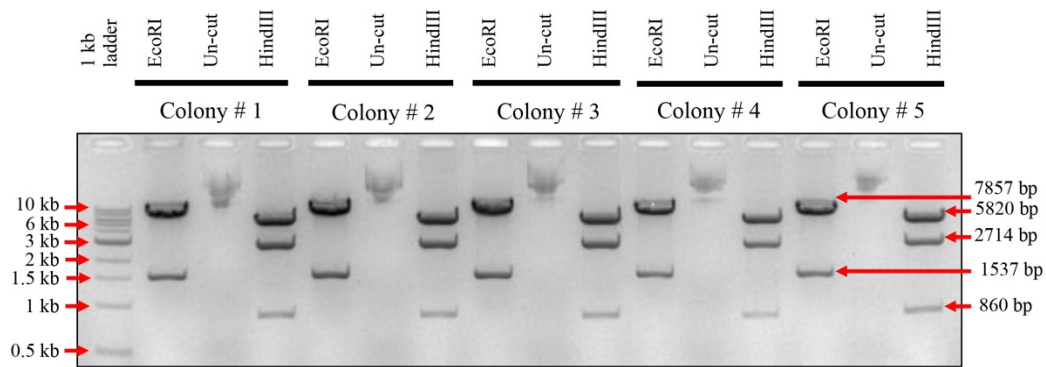
Expected fragment sizes after restriction enzyme digestion of pGN and pGX plasmids to confirm cloning.

Plasmid	Restriction enzyme	Expected fragment sizes
pGN	EcoRI	1537 bp 7857 bp
pGN	HindIII	860 bp 2714 bp 5820 bp
pGX	HindIII	775 bp 860 bp 2714 bp 4815 bp
pGX	EcoRI-XbaI	1437 bp 7727 bp

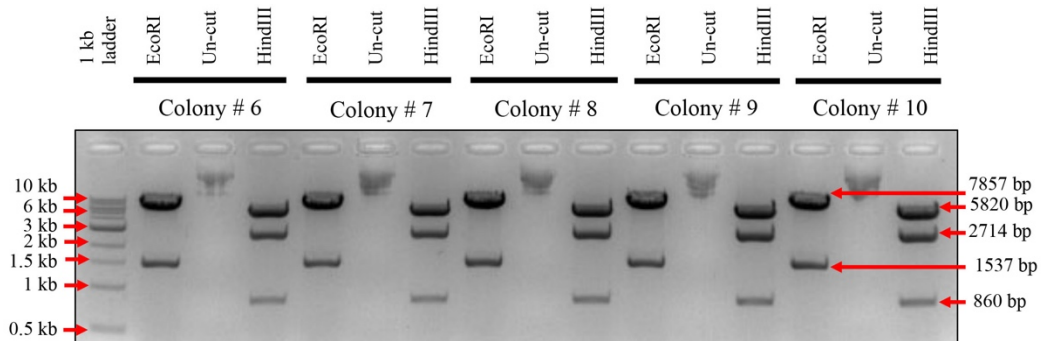
B.

Construction of recombinant plasmids with NCU05950 promoter along with NCU05950 gene coding sequence (pGN) and recombinant plasmids with *ccg-1* promoter followed by NCU05950 gene coding sequence (pGX) to introduce GFP tag. A. Restriction enzyme digestion of pGN (1-5) plasmids with EcoRI and HindIII. B. Restriction enzyme digestion of pGN (6-10) plasmids with EcoRI and HindIII. C. Restriction enzyme digestion of pGX (1-5) plasmids with EcoRI - XbaI and HindIII. D. Restriction enzyme digestion of pGX (6-10) plasmids with EcoRI - XbaI and HindIII.

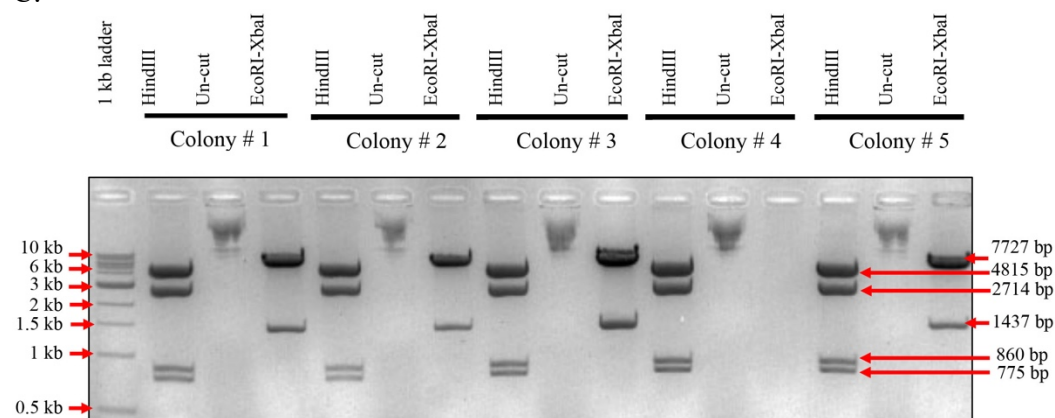
A.



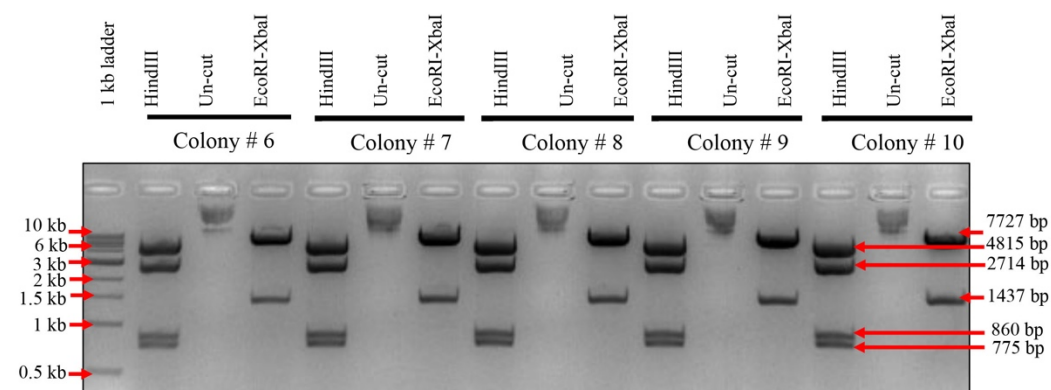
B.



C.



D.



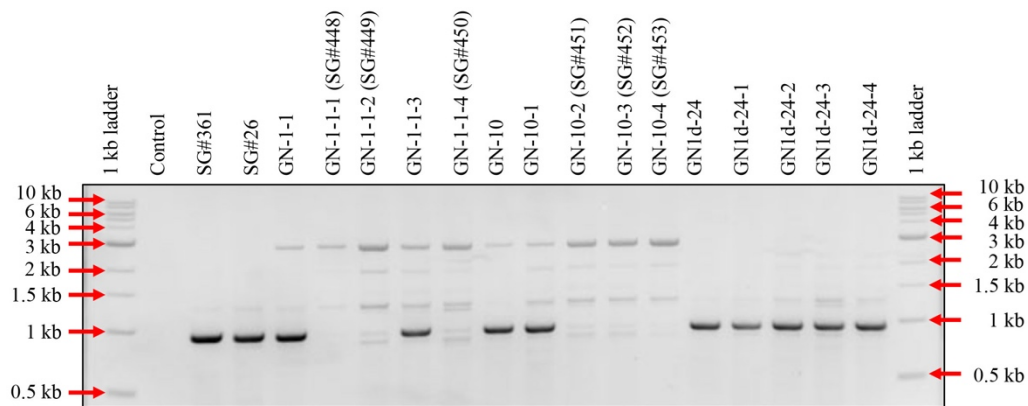
Appendix XX

Construction of strains expressing NCU05950 – GFP fusion protein

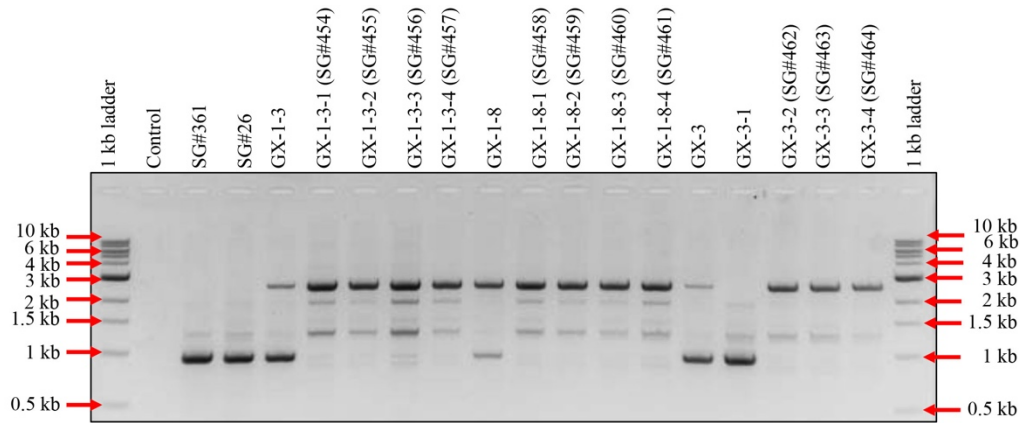
A.

Construction of strains expressing GFP fusion protein from the native NCU05950 promoter (GN strains with genotype; *csp-1 his-3⁺:: Native promoter -NCU05950::Gly::GFP*; *chol-1 ras^{bd}*; *NCU05950^{KO} mat a*) and strains expressing GFP fusion protein from the high-expression *ccg-1* promoter (GX strains with the genotype; *csp-1 his-3⁺:: pccg-1::NCU05950::Gly::GFP*; *chol-1 ras^{bd}*; *NCU05950^{KO} mat a*). PCR using primers targeting *his-3* locus (pBM61F2 & pBM61R2) to select homokaryons after microconidia purification of; A. GN strains and B. GX strains. Transformed nuclei of GN strains show a band of 2313 bp size and transformed nuclei of GX strains show a band of 2773 bp size. Untransformed nuclei show a band of 964 bp size.

A.



B.



B.

Rescue of the phenotype of the NCU05950 deletion mutant by the NCU05950-GFP. All strains, including control SG#26, have the *csp-1*; *ras^{bd}* *chol-1* genetic background. All strains except the control also carry the NCU05950 deletion allele (NCU05950^{KO}). GFP-tagged fusion genes were inserted at the *his-3* locus of a NCU05950^{KO} strain. The fusion genes were expressed either from the native NCU05950 promoter or *ccg-1* promoter. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Three representative replicate tubes for each condition are shown. Growth is from left to right. Blue and black marks indicate position of growth front every 24 hours. Red marks indicate band centers. Periods and growth rates are reported in Appendix XX – C.

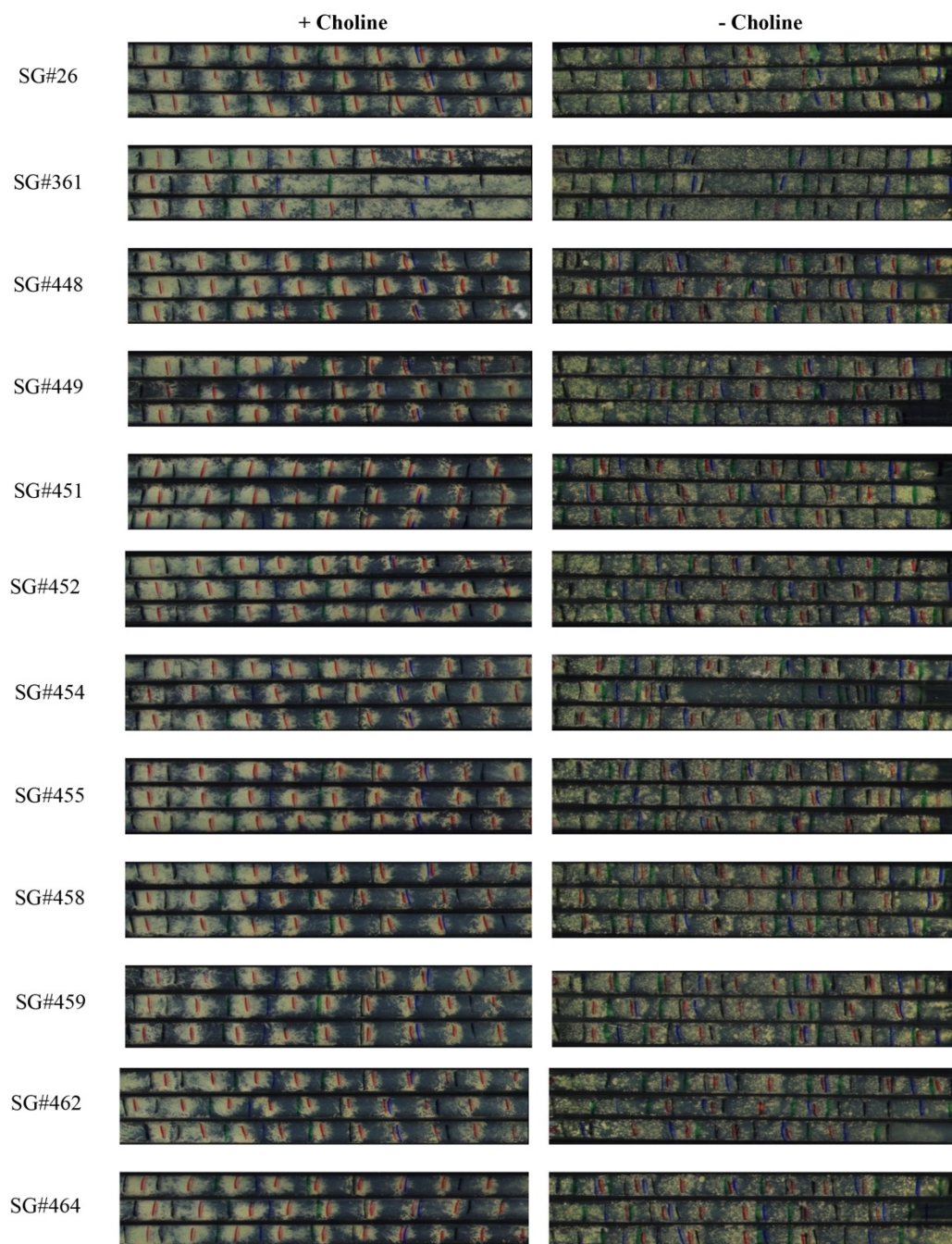
Genotypes of the strains are;

SG#26 – *csp-1*; *chol-1* *ras-1^{bd}* *mat a*

SG#361 – *csp-1*; *chol-1* *ras^{bd}*; NCU05950^{KO}

SG#448, SG#449, SG#451 and SG#452 – *csp-1* *his-3⁺*:: Native promoter - NCU05950::C-Gly::GFP; *chol-1* *ras^{bd}*; NCU05950^{KO} *mat a*

SG#454, SG#455, SG#458, SG#459, SG#462 and SG#464 - *csp-1* *his-3⁺*:: *pccg-1*:: NCU05950::C-Gly::GFP; *chol-1* *ras^{bd}*; NCU05950^{KO} *mat a*



C.

Periods and growth rates of GFP-tagged strains.

Genotype	+ Choline		- Choline	
	Period (h)	Growth Rate (mm/h)	Period (h)	Growth Rate (mm/h)
SG#26	22.00	1.25	47.95	0.56
	21.20	1.28	62.77	0.49
	21.10	1.30	53.51	0.50
SG#361	21.43	1.26	NR	0.52
	24.02	1.33	NR	0.44
	22.26	1.29	NR	0.49
SG#448 (GN-1-1-1)	21.35	1.20	67.30	0.45
	21.37	1.23	57.36	0.41
	21.61	1.23	55.12	0.46
SG#449 (GN-1-1-2)	21.02	1.15	52.69	0.45
	21.41	1.21	42.46	0.47
	21.70	1.19	51.66	0.53
SG#451 (GN-10-2)	21.31	1.25	59.02	0.41
	21.36	1.24	50.88	0.47
	21.12	1.22	38.95	0.50
SG#452 (GN-10-3)	21.77	1.13	46.48	0.52
	21.60	1.25	56.06	0.44
	21.69	1.20	72.02	0.39
SG#454 (GX-1-3-1)	21.20	1.20	65.91	0.46
	21.56	1.20	68.78	0.45

	21.86	1.23	49.45	0.48
SG#455 (GX-1-3-2)	21.59	1.25	62.41	0.40
	21.09	1.24	65.44	0.52
	20.97	1.21	51.47	0.52
SG#458 (GX-1-8-1)	22.01	1.25	48.68	0.45
	21.71	1.19	49.22	0.48
	21.35	1.37	54.87	0.48
SG#459 (GX-1-8-2)	21.44	1.29	48.83	0.45
	21.00	1.26	62.92	0.44
	21.33	1.29	61.28	0.45
SG#462 (GX-3-2)	21.69	1.19	70.87	0.42
	22.22	1.22	50.95	0.50
	21.66	1.16	60.93	0.48
SG#464 (GX-3-4)	21.41	1.22	44.96	0.46
	21.74	1.23	52.07	0.49
	21.37	1.24	69.62	0.45

Genotypes as for Appendix XX - B.

All strains are also *csp-1*; *ras^{bd}* *chol-1*.

Strains were grown with (+) or without (-) 100 μ M choline in the medium.

N.R.: Not rhythmic.

Appendix XXI

Restriction enzyme digestion of recombinant plasmids

A.

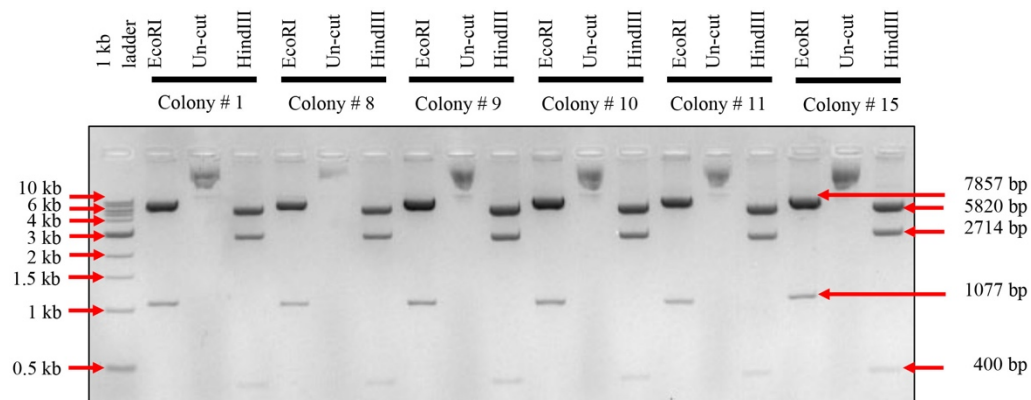
Expected fragment sizes after restriction enzyme digestion of pFN and pFX plasmids to confirm cloning.

Plasmid	Restriction enzyme	Expected fragment sizes
pFN	EcoRI	882 bp 7857 bp
pFN	HindIII	400 bp 2714 bp 5625 bp
pFX	HindIII	400 bp 580 bp 2714 bp 4815 bp
pFX	EcoRI-XbaI	782 bp 7727 bp

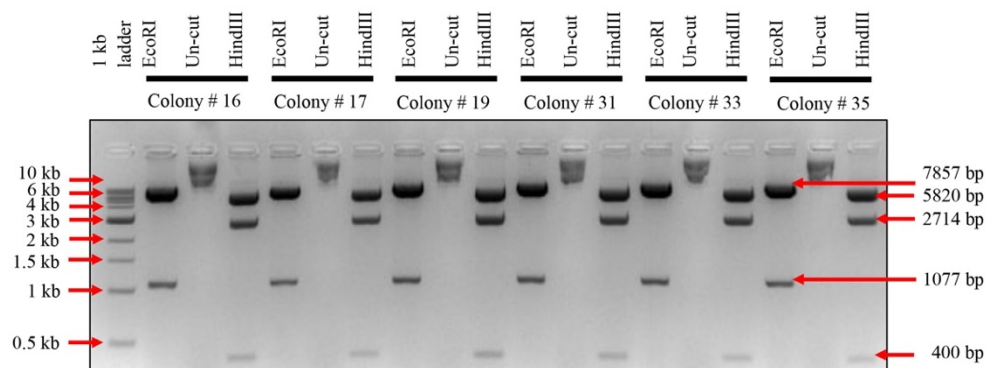
B.

Construction of recombinant plasmids with NCU05950 promoter along with NCU05950 gene coding sequence (pFN) and recombinant plasmids with *ccg-1* promoter followed by NCU05950 gene coding sequence (pFX) to introduce FLAG tag. A. Restriction enzyme digestion of pFN (1,8,9,10,11 & 15) plasmids with EcoRI and HindIII. B. Restriction enzyme digestion of pFN (16,17,19,31,33 & 35) plasmids with EcoRI and HindIII. C. Restriction enzyme digestion of pFX (1-5) plasmids with EcoRI - XbaI and HindIII. D. Restriction enzyme digestion of pFX (6-10) plasmids with EcoRI - XbaI and HindIII.

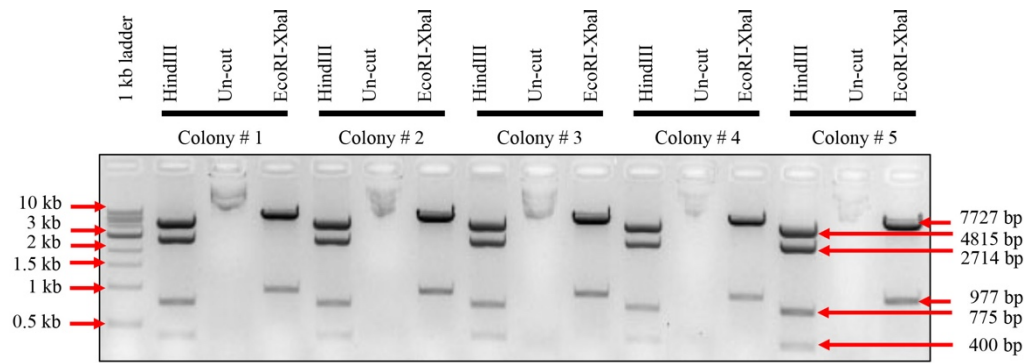
A.



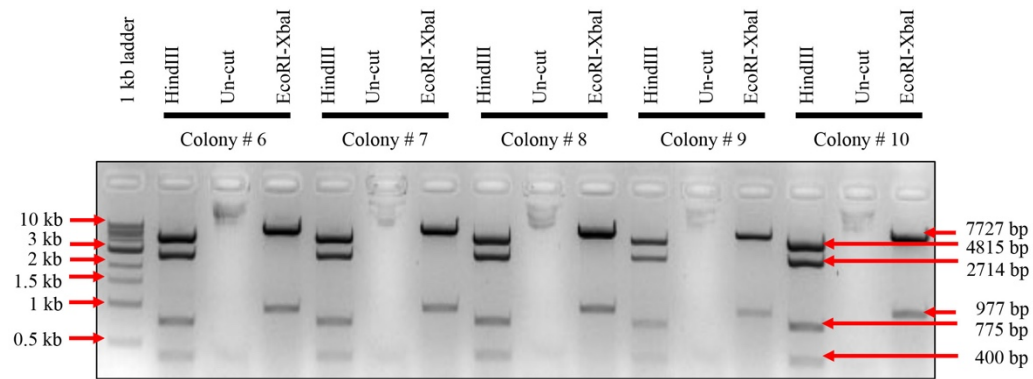
B.



C.



D.



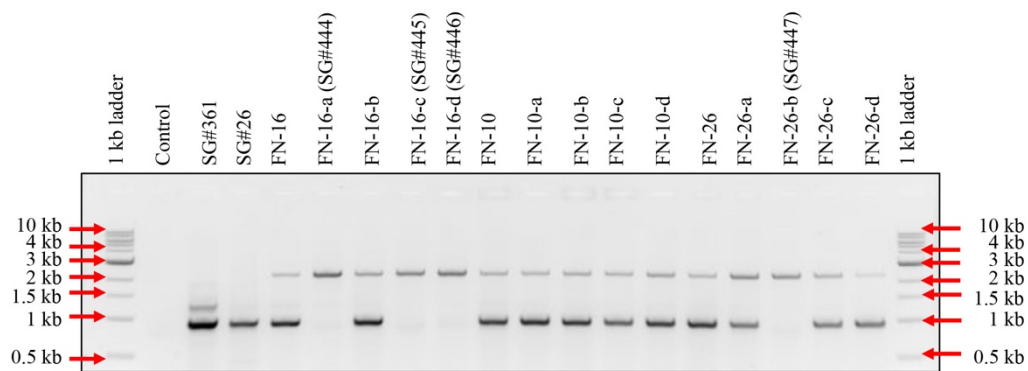
Appendix XXII

Construction of strains expressing NCU05950 – 3XFLAG fusion protein

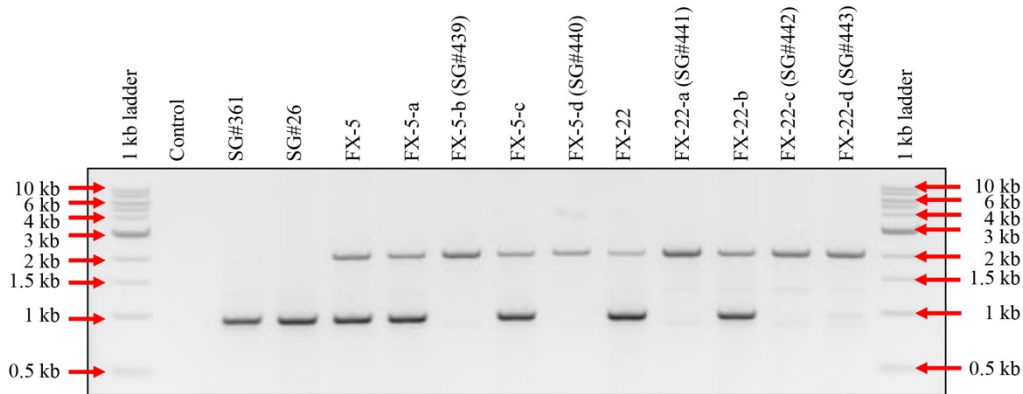
A.

Construction of strains expressing 3xFLAG fusion protein from the native NCU05950 promoter (FN strains with genotype; *csp-1 his-3⁺:: Native promoter -NCU05950::C-Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*) and strains expressing 3xFLAG fusion protein from the high-expression *ccg-1* promoter (FX strains with the genotype; *csp-1 his-3⁺:: pccg-1::NCU05950::C-Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*). PCR using primers targeting *his-3* locus (pBM61F2 & pBM61R2) to select homokaryons after microconidia purification of; A. FN strains and B. FX strains. Transformed nuclei of FN strains show a band of 2543 bp size and transformed nuclei of FX strains show a band of 2083 bp size. Untransformed nuclei show a band of 964 bp size.

A.



B.



B.

Rescue of the phenotype of the NCU05950 deletion mutant by the NCU05950-3xFLAG. All strains, including control SG#26, have the *csp-1; ras^{bd} chol-1* genetic background. All strains except the control also carry the NCU05950 deletion allele (NCU05950^{KO}). 3xFLAG-tagged fusion genes were inserted at the *his-3* locus of a NCU05950^{KO} strain. The fusion genes were expressed either from the native NCU05950 promoter or *cpg-1* promoter. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Three representative replicate tubes for each condition are shown. Growth is from left to right. Periods and growth rates are reported in Appendix XXII - C.

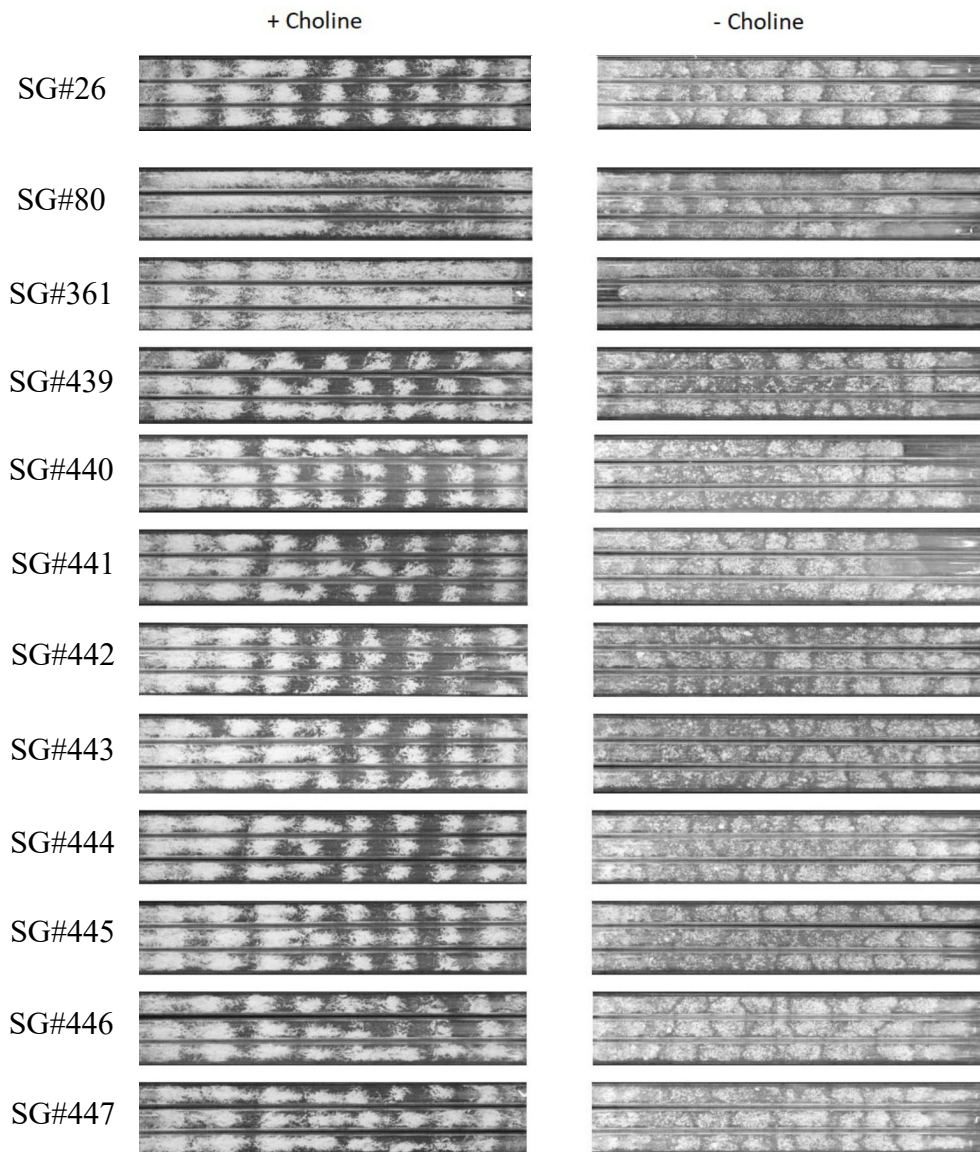
Genotypes of the strains are;

SG#26 – *csp-1; chol-1 ras^{bd} mat a*

SG#361 – *csp-1; chol-1 ras^{bd}; NCU05950^{KO}*

SG#439, SG#440, SG#441, SG#442 and SG#443 – *csp-1 his-3⁺:: pccg-1::NCU05950::C-Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*

SG#444, SG#445, SG#446, SG#447 and SG#448 – *csp-1 his-3⁺:: Native promoter -NCU05950::Gly::3xFLAG; chol-1 ras^{bd}; NCU05950^{KO} mat a*



C.

Periods and growth rates of 3xFLAG-tagged strains.

Genotype	- Choline		+ Choline	
	Growth Rate (mm/h)	Period (h)	Growth Rate (mm/h)	Period (h)
SG#26	0.46 ± 0.03	47.16 ± 2.58	1.41 ± 0.01	21.26 ± 0.08
SG#361	0.52 ± 0.02	NR	1.21 ± 0.01	22.68 ± 0.18
SG#80	0.48 ± 0.02	53.30 ± 2.03	1.40 ± 0.00	NR
SG#439	0.52 ± 0.04	42.07 ± 3.12	1.24 ± 0.03	22.20 ± 0.19
SG#440	0.50 ± 0.02	46.30 ± 3.58	1.22 ± 0.01	22.14 ± 0.09
SG#441	0.42 ± 0.04	52.07 ± 3.91	1.25 ± 0.02	22.02 ± 0.09
SG#442	0.53 ± 0.01	43.37 ± 1.36	1.28 ± 0.01	21.93 ± 0.05
SG#443	0.54 ± 0.02	37.04 ± 1.96	1.29 ± 0.01	21.75 ± 0.10
SG#444	0.55 ± 0.03	42.56 ± 5.26	1.33 ± 0.01	21.92 ± 0.08
SG#445	0.51 ± 0.00	42.08 ± 1.87	1.30 ± 0.00	21.51 ± 0.08
SG#446	0.50 ± 0.01	48.88 ± 2.32	1.24 ± 0.00	21.95 ± 0.20
SG#447	0.47 ± 0.01	49.23 ± 2.64	1.28 ± 0.01	22.51 ± 0.04

Genotypes as for Appendix XXII - B. All strains are also *csp-1*; *ras^{bd} chol-1*.

Strains were grown with (+) or without (-) 100 µM choline in the medium.

Data reported as mean ± S.E.M. and n = 3.

N.R.: Not rhythmic.

Appendix XXIII

**Periods and growth rates of progeny strains from cross between
SG#59 (*ras-1^{bd}; frq¹⁰ mat A*) and SG#444 (*csp-1 his-3⁺:: Native
promoter -NCU05950::Gly::3xFLAG; chol-1 ras^{bd};
NCU05950^{KO} mat a*)**

Genotype	- Choline		+ Choline	
	Growth Rate (mm/h)	Period (h)	Growth Rate (mm/h)	Period (h)
SG#26	0.53	45.18	1.35	19.80
SG#59	1.27	NR	1.38	NR
SG#80	0.48	53.30	1.37	NR
SG#444	0.45	46.94	1.27	20.59
Pg#2	0.98	20.79	1.00	20.24
Pg#3	0.87	19.75	1.07	19.94
Pg#6	1.36	19.69	1.31	20.50
Pg#10	0.51	51.55	1.27	NR
Pg#17	0.55	NR	1.40	NR
Pg#19	0.48	45.52	1.37	20.72
Pg#21	0.33	47.55	1.04	20.16
Pg#24	0.47	67.49	1.34	NR
Pg#27	0.40	64.49	1.34	NR
Pg#28	0.42	46.70	1.37	NR
Pg#29	0.53	42.38	1.38	NR
Pg#31	0.49	44.48	1.31	20.89
Pg#33	1.03	21.03	1.01	20.01
Pg#34	0.49	39.97	1.35	19.21
Pg#40	0.45	NR	1.31	NR
Pg#42	0.54	NR	1.40	NR
Pg#43	0.98	NR	0.94	NR
Pg#44	0.52	40.14	1.36	20.87
Pg#46	0.44	67.12	1.31	NR
Pg#50	0.47	59.33	1.34	NR

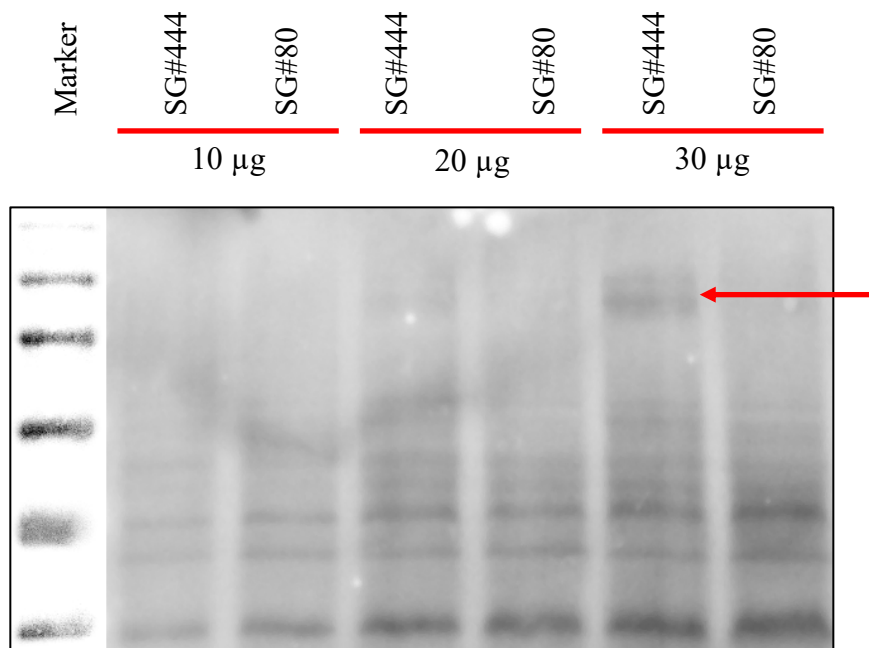
Pg#51	0.45	56.37	1.29	NR
Pg#60	0.53	37.99	1.28	20.93
Pg#68	0.62	34.66	1.28	21.16
Pg#72	0.95	NR	1.00	NR
Pg#75	1.02	24.82	0.96	21.24
Pg#76	0.46	59.42	1.38	NR
Pg#84	0.54	47.85	1.34	NR
Pg#88	1.06	NR	1.09	NR
Pg#92	0.45	58.40	1.30	NR
Pg#97	0.47	44.68	1.34	21.24
Pg#100	1.01	21.54	0.89	20.48
Pg#105	0.48	41.46	1.27	21.00

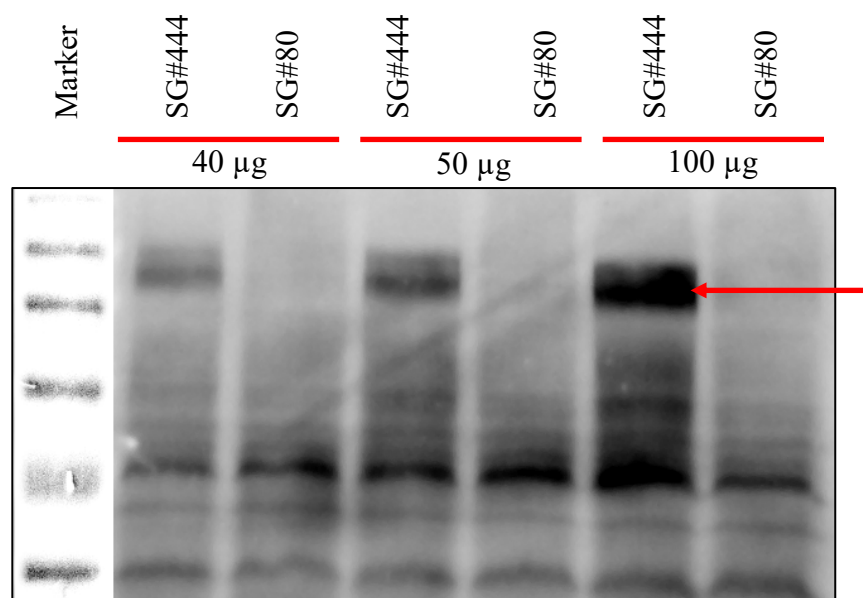
Appendix XXIV

Optimizing Western blotting protocols for quantitation of FRQ protein

Optimizing total protein quantity required for immunodetection with anti-FRQ monoclonal primary antibody and HRP-conjugated goat anti-mouse secondary antibody for quantitation of FRQ protein (108.25 kDa).

Samples were collected from a strain expressing the NCU05950-3xFLAG fusion protein from the native promoter (SG#444) with the genotype *csp-1 his-3⁺::NCU05950::FLAG; ras^{bd} chol-1; NCU05950^{KO}* and SG#80 with the genotype *csp-1; ras^{bd} chol-1; frq¹⁰* was used as the negative control. Different amounts of total protein from each sample were loaded on to a 7.5% SDS-PAGE. Arrow indicates the FRQ protein.

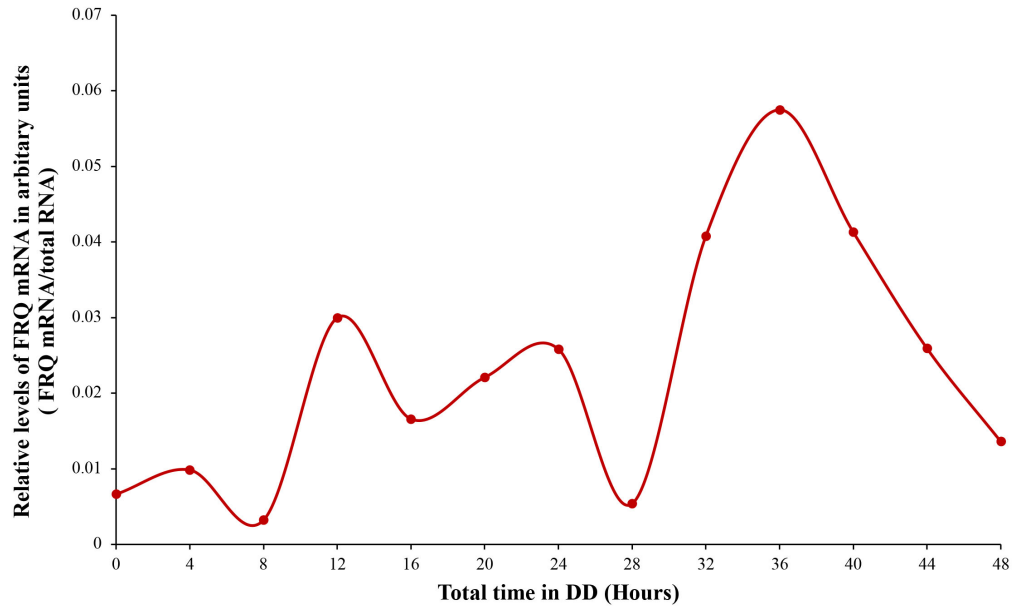




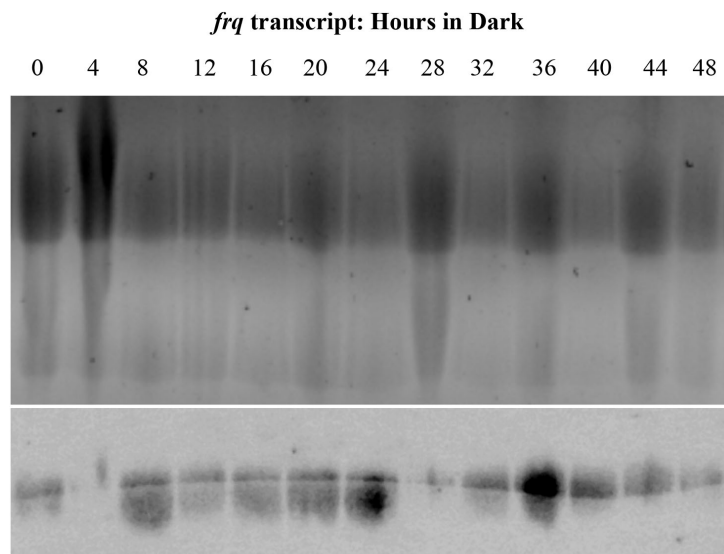
Appendix XXV

Analysis of FRQ transcript by Northern blotting

A.



B.



SG#26 (*csp-1*; *ras^{bd}* *chol-1*) was grown on high choline to repair the *chol-1* defect. Cultures were grown in DD for varying lengths of time. Digoxigenin-labeled probe (484 bp long fragment from the *frq* gene) was used for hybridization.

A. Relative level of *frq* transcript levels normalized to total RNA by ethidium bromide staining of the gel. Points are ratios from a single experiment.

B. Upper panel: Ethidium bromide staining of total RNA at different time points (0–48 h in DD) corresponding to the time-series in A. Lower panel: Immunodetected Northern blot of samples in upper panel. (RNA extraction, Northern blotting, immunodetection and data analysis were done by Alexandra Pana and Aryan Lajevardi).

Appendix XXVI

The strategy used to make deletion mutants under the control of *ccg-1* promoter

Wild type - Coding sequence (including introns): 890 nt:

gcgacggtcttttctccaactttacttcgacgacgtctggcttcaagacgagacttttcttgcgcttcgcgga
attccaattgacaaacgtcatccgacgacttgataccgaccgaccgaccaccatccgactggcgacacg
gcacataaagaaggaggacaagataacaagATGGGCAACTTTTGCTCAACCTGCTTCGGCGG
TAGGAGGAGCGATGACTACGATGAGtaggtttcgcatagcggccacagtccgatgacatggcc
atcaaaagaggcattcgacggaacaagagaactgacgtggactgttcggtgacacagGAGGACGAGGC
CCAGCATCTCTTCGACGAGAACAACATGCACTACGGGAGCTTTGATCAGCAACACAT
GATGAACCAGGAAGATCCCCAAGAGACAGAAAGGGAAATTGCGGCTCTGCAGGGC
GTGGTGGAAACGCACATCAAAGtatgccctcccacctgaaatgtctcccatccaagatctagtccgaga
cccaagtccacggagccggtagagcgaataactgacttggaaaccgcgagTAACATGGTTCGACATCTA
TGATATGGTCCCGCATGATAAACCCATGCAGGACGCTCCTGCTCCCTACGGCTTTGCC
AACCAGCGCTACAATGCCCTGCTTTCCAAGCTCTCAACCCACGATGACATGGCCGCC
GTTGCACGGGTTGACTGGGGCACCCCTGAGGACGACAGCATGGAGATGCTGCGCAA
AGCTTCCTTGCCAACCATCCCCATCAAGGCTGAAGGAGGCGAGGCCCTCGTCGGTAA
CTTACCGATGCCGCTGCCGCCATGCGCTGAaaccataccacgacaccaaaaccatgacttgctg
tct

Wild type - Translated sequence: 159 aa

MGNFCSTCFG	GRRSDDYDEE	DEAQHLFDEN	NMHYGSFDQQ
HMMNQEDPQE	TEREIAALQG	VVERTSNNMV	DIYDMVPHDK
PMQDAPAPYG	FANQRYNALL	SKLSTHDDMA	AVARVDWGTP
EDDSMEMLRK	ASLPTIPIKA	EGGEALVGNF	
TDAAAAMR*			

Deletion of first glycine:

Coding sequence

TCT AGA ATG GGC AAC TTT TGC TCA ACC TGC TTC GGC
GGT.....

Translated sequence

MGNFCSTCFG GRRSDDYDEE DEAQHLFDEN

XbaI site: TCT AGA

UV90Δ1XbaIF-

5' GC TCT AGA ATG AAC TTT TGC TCA ACC TGC TTC GGC 3'

Deletion of seven amino acids:

Coding sequence

TCT AGA ATG GGC AAC TTT TGC TCA ACC TGC TTC GGC GGT.....

Translated sequence

MGNFCSTCFG GRRSDDYDEE DEAQHLFDEN

XbaI site: TCT AGA

UV90Δ7XbaIF-

5' GC TCT AGA ATG TTC GGC GGT AGG AGG AGC GAT GAC 3'

Deletion ten amino acids (all 3 glycines)

Coding sequence

TCT AGA ATG GGC AAC TTT TGC TCA ACC TGC TTC GGC GGT AGG
AGG AGC GAT GAC TAC GAT GAG GAG GAC GAG GCC.....

Translated sequence

MGNFCSTCFG GRRSDDYDEE DEAQHLFDEN

XbaI site: TCT AGA

UV90Δ10XbaIF-

5' GC TCT AGA ATG AGG AGG AGC GAT GAC TAC GAT GAG 3'

Appendix XXVII

Restriction enzyme digestion of recombinant plasmids

A.

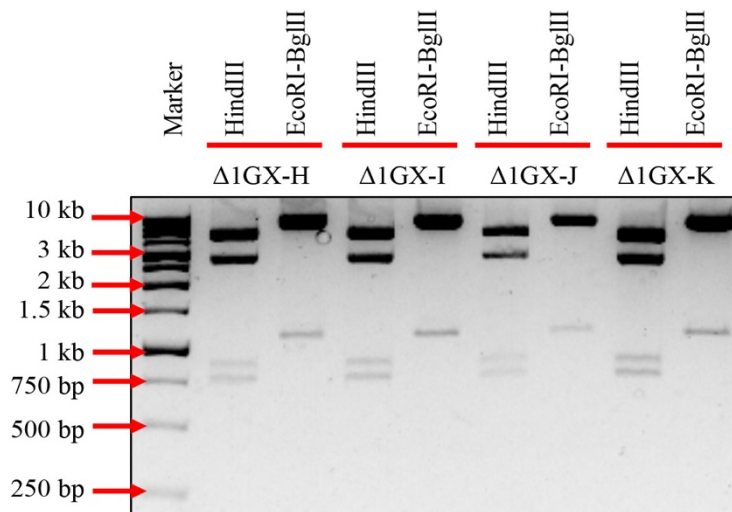
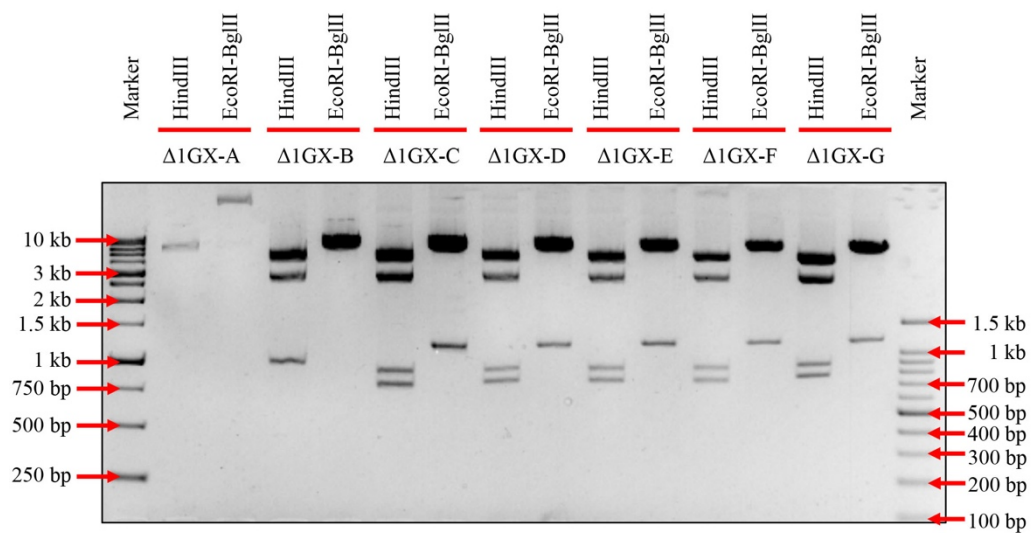
Digestion of *pccg-I-Δ1/Δ7/Δ10*NCU05950::10XGly::GFP/3XFLAG plasmids.

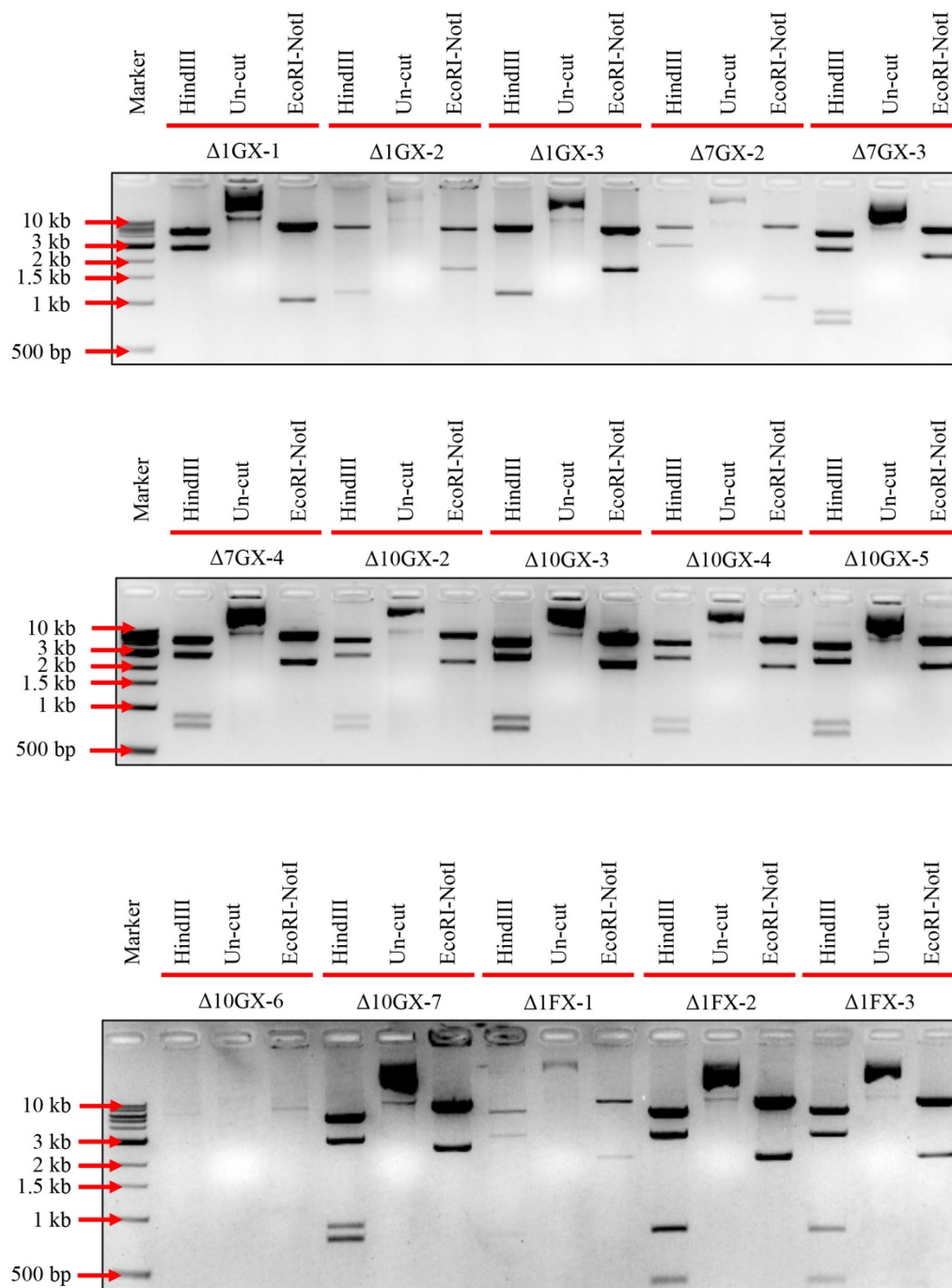
Plasmid	Restriction enzyme	Expected fragment sizes
Δ1NCU05950::10XGly::GFP (pΔ1GX)	HindIII	4815 bp 2714 bp 860 bp 772 bp
	EcoRI-BglII	8060 bp 1101 bp
	EcoRI-NotI	6797 bp 2364 bp
Δ7NCU05950::10XGly::GFP (pΔ7GX)	HindIII	4815 bp 2714 bp 860 bp 754 bp
	EcoRI-NotI	6797 bp 2346 bp
Δ10NCU05950::10XGly::GFP (pΔ10GX)	HindIII	4815 bp 2714 bp 860 bp 745 bp

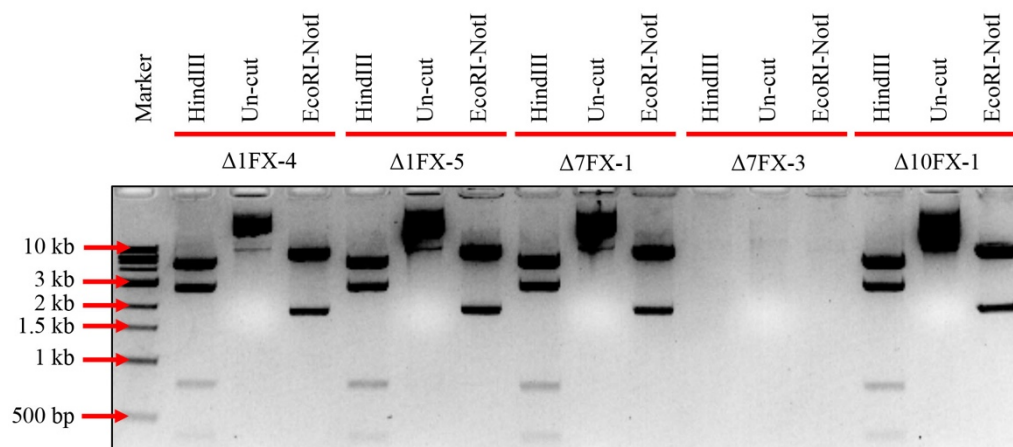
	EcoRI-NotI	6797 bp 2337 bp
Δ1NCU05950::10XGly::3xFLAG (pΔ1FX)	HindIII	4815 bp 2714 bp 772 bp 400 bp
	EcoRI-NotI	6797 bp 1904 bp
Δ7NCU05950::10XGly::3xFLAG (pΔ7FX)	HindIII	4815 bp 2714 bp 754 bp 400 bp
	EcoRI-NotI	6797 bp 1886 bp
Δ10NCU05950::10XGly::3xFLAG (pΔ10FX)	HindIII	4815 bp 2714 bp 745 bp 400 bp
	EcoRI-NotI	6797 bp 1877 bp

B.

Agarose gel electrophoresis of un-digested recombinant plasmids and recombinant plasmids digested with different restriction enzymes to confirm the cloning as well as the presence of the insert. Details of different band sizes expected from each type of plasmid are given on Appendix XXVII - A.







Appendix XXVIII

The strategy used to make deletion mutants under the control of native promoter

Wild type NCU05950

CATTCTTCCTTTGCCTGATGAACCGACCTCGCTAGCAGTCCCCTCCCCGCGGGCAC
CCGACAGAAGCCGAAGCAACTCGACTAAACCAGCACCACAAGCCATTTTCACGACGG
GACCCAACTTTTTTTTTTTTTTTTTTTGGTTTTTTTTTGTCTGGTCTGATCTCATTTTCGT
CATCGATCGAGTTTCATCTCTTCgcgacggtcttttctcccaactttacttcgacgacgtctggcttcaa
gacgagactttttctgtcgcttcgcgcaattccaattgacaaacgtcatccgacgacttgataccgaccgacc
gaccgaccaccatccgactggcgacacggcacataaagaaggaggacaagataacaagATGGCAAC
TTTTGCTCAACCTGCTTCGGCGGTAGGAGGAGCGATGACTACGATGAGgtaggtttcggc
atagcggccacagtccgatgacatggccatcaaaagaggcattcgacggaacaagagaactgacgtggactg
ttgcgtgacacagGAGGACGAGGCCAGCATCTCTTCGACGAGAACACATGCACTACG
GGAGCTTTGATCAGCAACACATGATGAACCAGGAAGATCCCCAAGAGACAGAAAGG
GAAATTGCGGCTCTGCAGGGCGTGGTGGAACGCACATCAAAgtatgccctccacctgaaa
tgtctcccatccaagatctagtccgagaccaagtccacggagccggtagagcgaataactgacttgaaccg
cgagAACATGGTCGACATCTATGATATGGTCCCGCATGATAAACCCATGCAGGACGC
TCCTGCTCCCTACGGCTTTGCCAACCAGCGCTACAATGCCCTGCTTTCCAAGCTCTCA
ACCCACGATGACATGGCCGCCGTTGCACGGGTTGACTGGGGCACCCCTGAGGACGA
CAGCATGGAGATGCTGCGCAAAGCTTCCTTGCCAACCATCCCCATCAAGGCTGAAGG
AGGCGAGGCCCTCGTCGGTAACCTTACCGATGCCGCTGCCGCCATGCGCTGA

GGGACCC – SanDI site

AGATCT - BglII

Following are the commercially synthesized fragments:

Native promoter - ^1 UV90

SanDI - BglII fragment (611bp)

GGGACCCAACTTTTTTTTTTTTTTTTTTGGTTTTTTTTTGTCTGGTCTGATCTCATTTCGTCATCGATCG
AGTTTCATCTCTTCGCGACGGTCTTTCTCCCAACTTTACTTCGACGACGTCTGGCTTCAAGACGAGACT
TTTTCTTGTCGCTTCGCGCGAATTCCAATTGACAAACGTCATCCGACGACTTGATACCGACCGACCGACC
GACCACCATCCGACTGGCGACACGGCACATAAAGAAGGGAGGACAAGATAACAAGATGAACTTTTGC
TCAACCTGCTTCGGCGGTAGGAGGAGCGATGACTACGATGAGGTAGGTTTCGGCATAGCGGCCACAG
TCCGATGACATGGCCATCAAAAGAGGCATTGACGGAACAAGAGAACTGACGTGGACTGTTGCGTGA
CACAGGAGGACGAGGCCAGCATCTCTCGACGAGAACAACATGCACTACGGGAGCTTTGATCAGCAA
CACATGATGAACCAGGAAGATCCCCAAGAGACAGAAAGGGAAATTGCGGCTCTGCAGGGCGTGTTGG
AACGCACATCAAAATGATGCCCTCCACCTGAAATGTCTCCCATCCAAGATCT

Native promoter - ^7 UV90

SanDI - BglII fragment (593bp)

GGGACCCAACTTTTTTTTTTTTTTTTTTGGTTTTTTTTTGTCTGGTCTGATCTCATTTCGTCATCGATCG
AGTTTCATCTCTTCGCGACGGTCTTTCTCCCAACTTTACTTCGACGACGTCTGGCTTCAAGACGAGACT
TTTTCTTGTCGCTTCGCGCGAATTCCAATTGACAAACGTCATCCGACGACTTGATACCGACCGACCGACC
GACCACCATCCGACTGGCGACACGGCACATAAAGAAGGGAGGACAAGATAACAAGATGTTTCGGCGGT
AGGAGGAGCGATGACTACGATGAGGTAGGTTTCGGCATAGCGGCCACAGTCCGATGACATGGCCATC
AAAAGAGGCATTGACGGAACAAGAGAACTGACGTGGACTGTTGCGTGACACAGGAGGACGAGGCC
CAGCATCTCTTCGACGAGAACAACATGCACTACGGGAGCTTTGATCAGCAACACATGATGAACCAGGA
AGATCCCCAAGAGACAGAAAGGGAAATTGCGGCTCTGCAGGGCGTGTTGGAACGCACATCAAAATGAT
GCCCTCCACCTGAAATGTCTCCCATCCAAGATCT

Native promoter - ^10 UV90

SanDI - BglII fragment (584bp)

GGGACCCAACTTTTTTTTTTTTTTTTTTGGTTTTTTTTTGTCTGGTCTGATCTCATTTCGTCATCGATCG
AGTTTCATCTCTTCGCGACGGTCTTTCTCCCAACTTTACTTCGACGACGTCTGGCTTCAAGACGAGACT
TTTTCTTGTCGCTTCGCGCGAATTCCAATTGACAAACGTCATCCGACGACTTGATACCGACCGACCGACC
GACCACCATCCGACTGGCGACACGGCACATAAAGAAGGGAGGACAAGATAACAAGATGAGGAGGAG
CGATGACTACGATGAGGTAGGTTTCGGCATAGCGGCCACAGTCCGATGACATGGCCATCAAAAGAGG
CATTGACGGAACAAGAGAACTGACGTGGACTGTTGCGTGACACAGGAGGACGAGGCCAGCATCTC
TTCGACGAGAACAACATGCACTACGGGAGCTTTGATCAGCAACACATGATGAACCAGGAAGATCCCCA
AGAGACAGAAAGGGAAATTGCGGCTCTGCAGGGCGTGTTGGAACGCACATCAAAATGATGCCCTCCCA
CTGAAATGTCTCCCATCCAAGATCT

Appendix XXIX

Restriction enzyme digestion of recombinant plasmids

A.

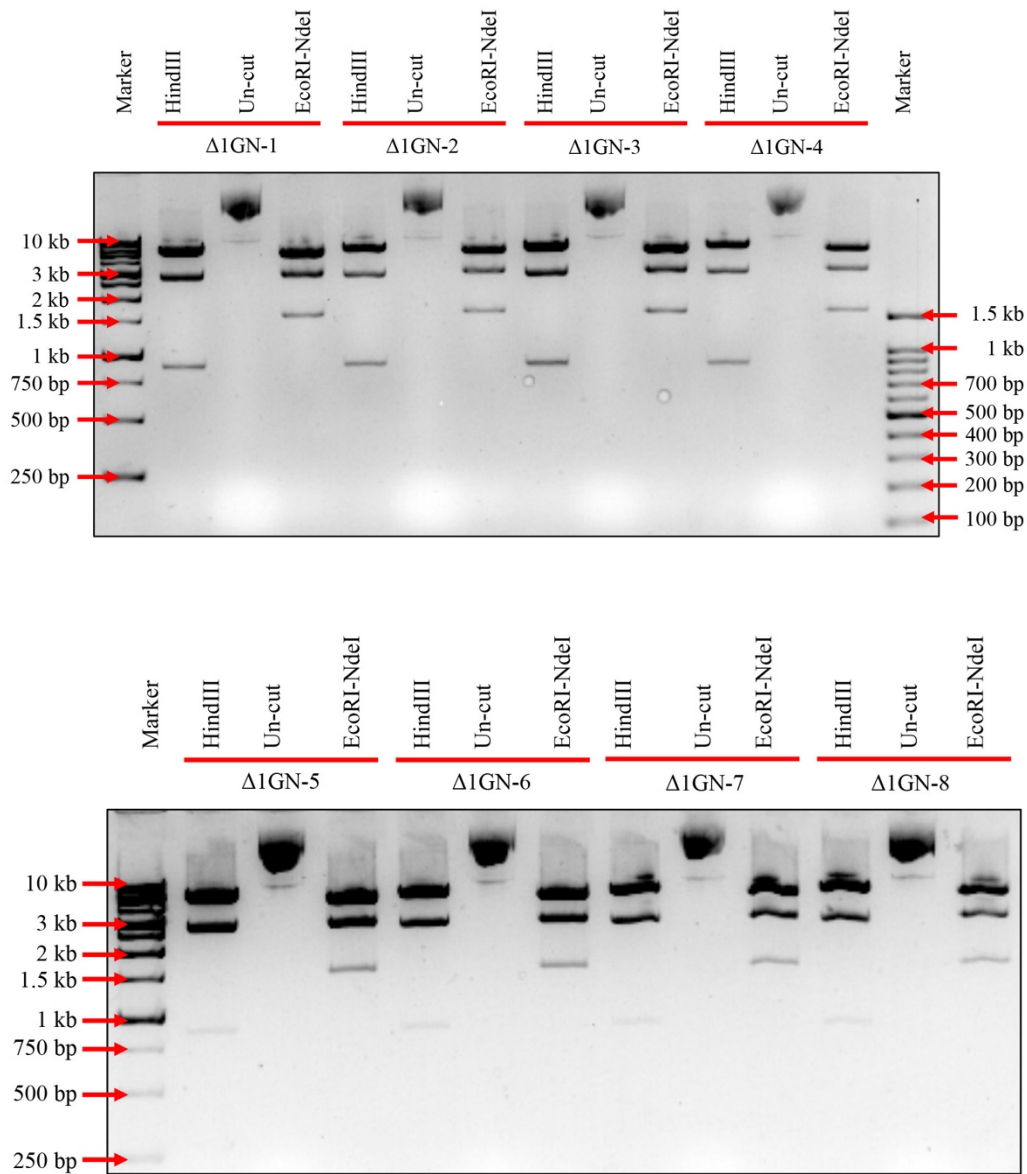
Digestion of *NCU05950 promoter*: $\Delta 1$ / $\Delta 7$ / $\Delta 10$ NCU05950::10XGly::GFP/3XFLAG plasmids.

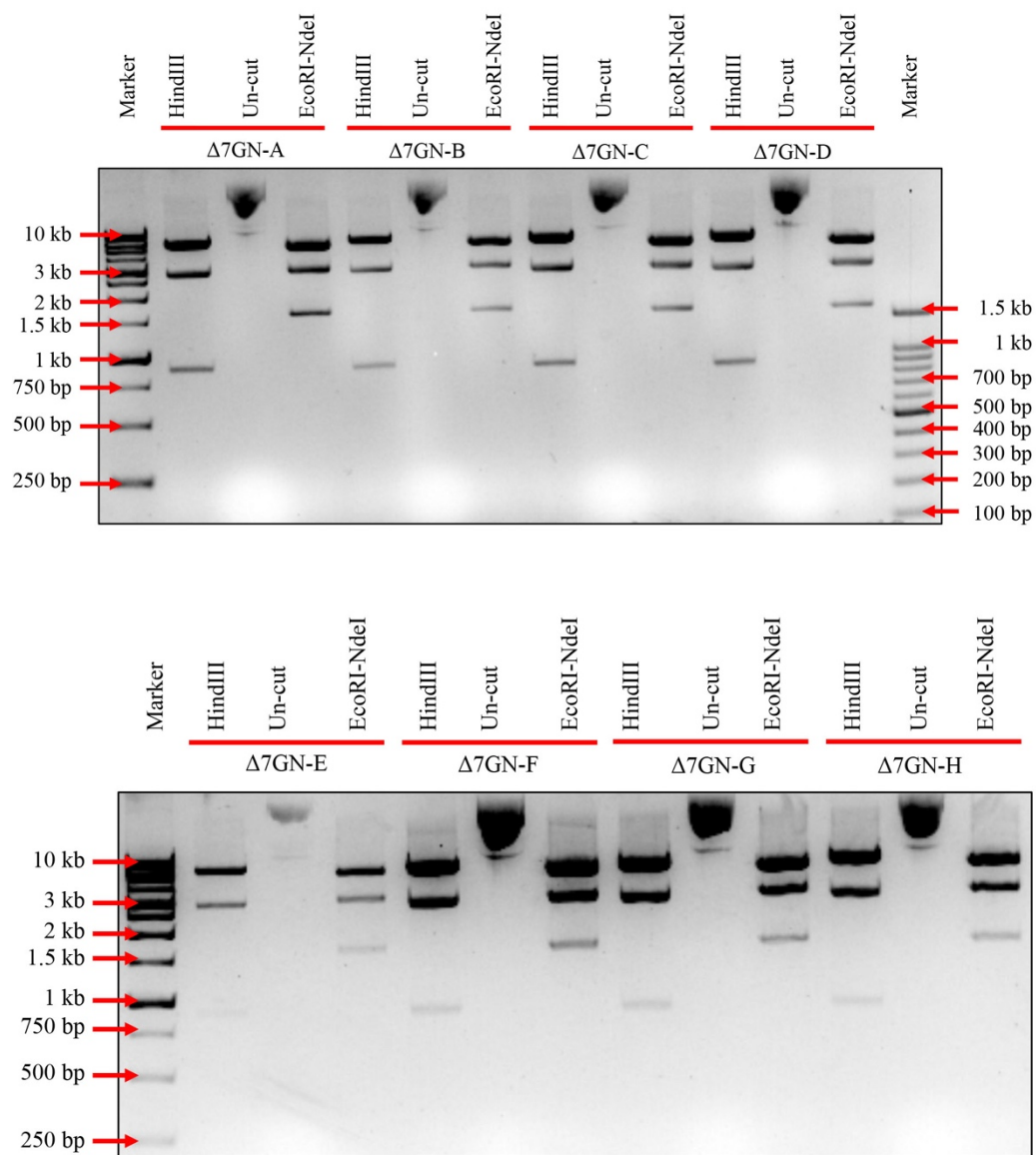
Plasmid	Restriction enzyme	Expected fragment sizes
NCU05950 promoter:: $\Delta 1$ NCU05950 :: 10XGly::GFP (p $\Delta 1$ GN)	HindIII	5817 bp 2714 bp 860 bp
	EcoRI-NdeI	5031 bp 2826 bp 1534 bp
NCU05950 promoter:: $\Delta 7$ NCU05950 :: 10XGly::GFP (p $\Delta 7$ GN)	HindIII	5799 bp 2714 bp 860 bp
	EcoRI-NdeI	5031 bp 2826 bp 1516 bp
NCU05950 promoter:: $\Delta 10$ NCU05950 :: 10XGly::GFP (p $\Delta 10$ GN)	HindIII	5790 bp 2714 bp 860 bp
	EcoRI-NdeI	5031 bp 2826 bp 1507 bp

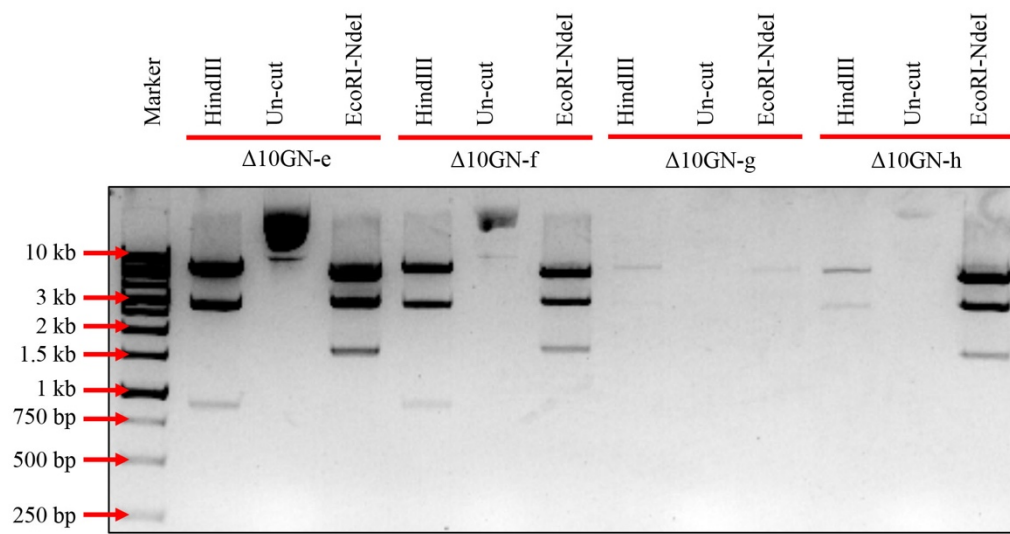
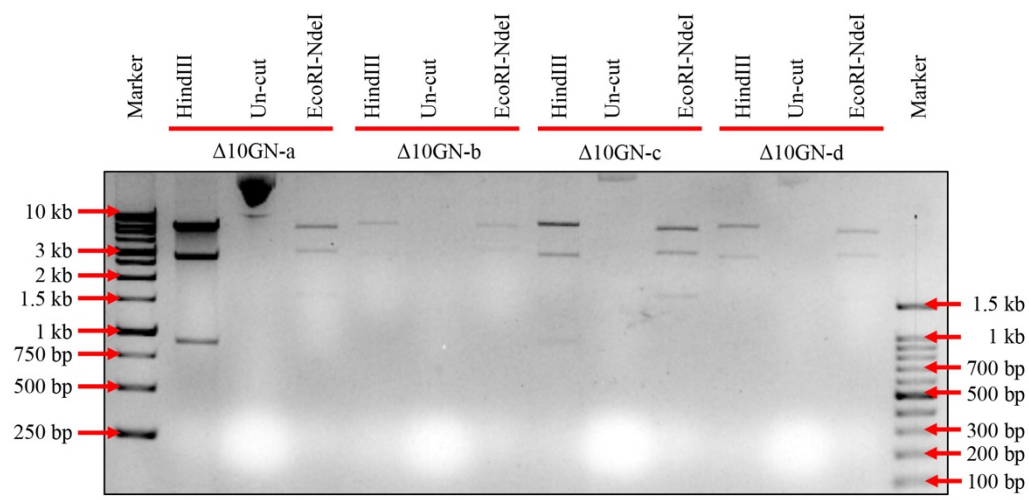
NCU05950 promoter:: $\Delta 1$ NCU05950 :: 10XGly::3xFLAG (p $\Delta 1$ FN)	HindIII	5817 bp 2714 bp 400 bp
	EcoRI-NotI	6797 bp 1074 bp 1060 bp
NCU05950 promoter:: $\Delta 7$ NCU05950 :: 10XGly::3xFLAG (p $\Delta 7$ FN)	HindIII	5799 bp 2714 bp 400 bp
	EcoRI-NotI	6797 bp 1060 bp 1056 bp
NCU05950 promoter:: $\Delta 10$ NCU05950 :: 10XGly::3xFLAG (p $\Delta 10$ FN)	HindIII	5790 bp 2714 bp 400 bp
	EcoRI-NotI	6797 bp 1060 bp 1047 bp

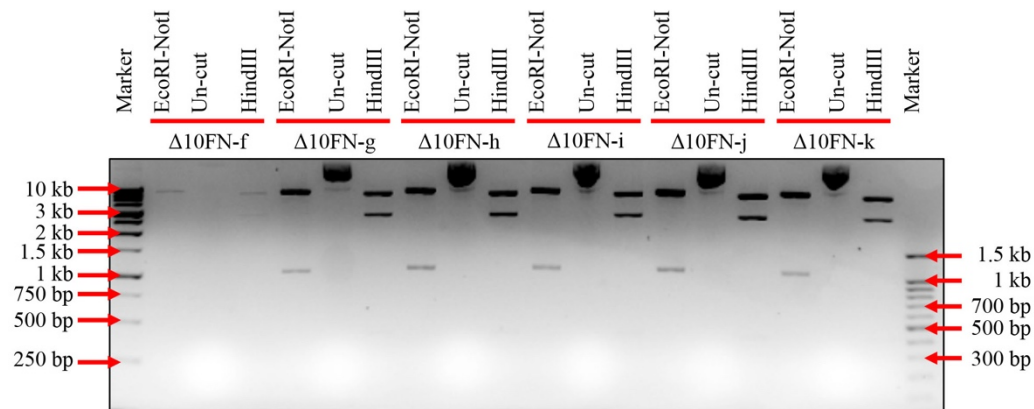
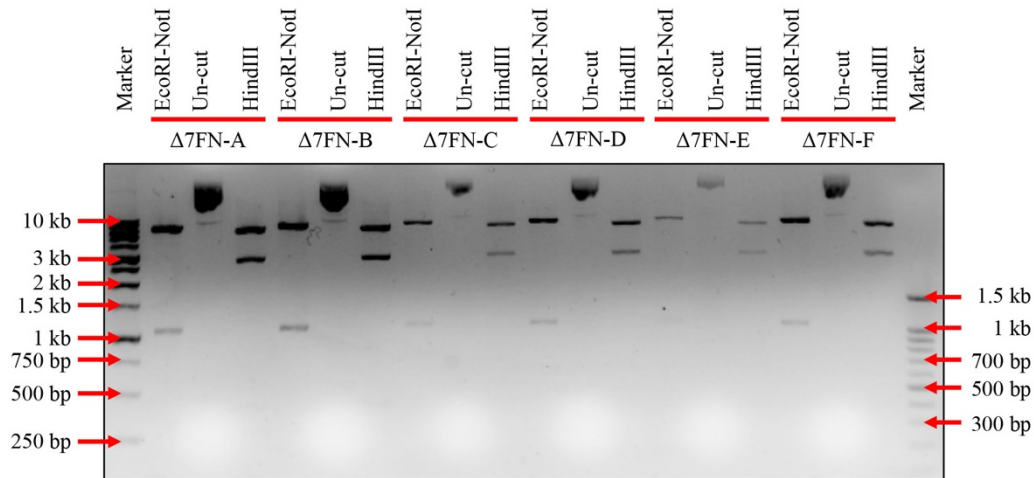
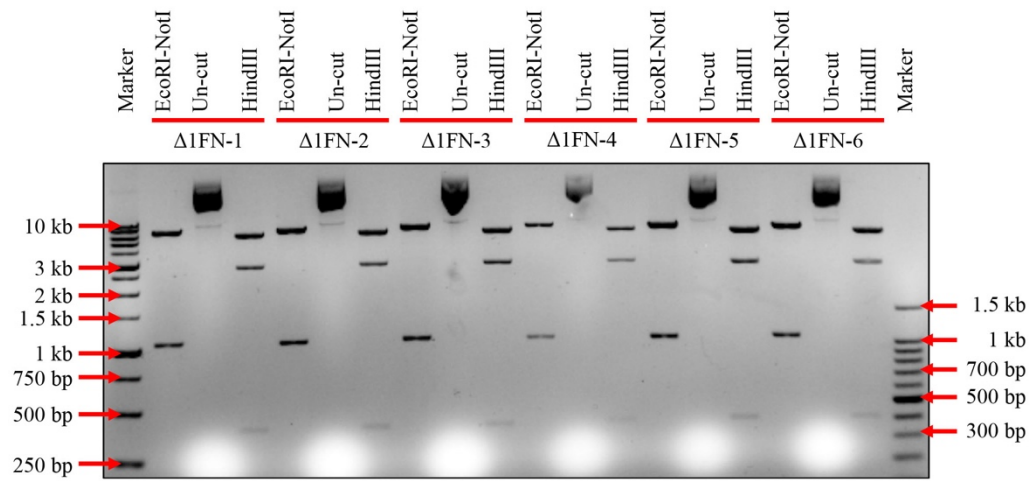
B.

Agarose gel electrophoresis of un-digested recombinant plasmids and recombinant plasmids digested with different restriction enzymes to confirm the cloning as well as the presence of the insert. Details of different band sizes expected from each type of plasmid are given on Appendix XXIX - A.



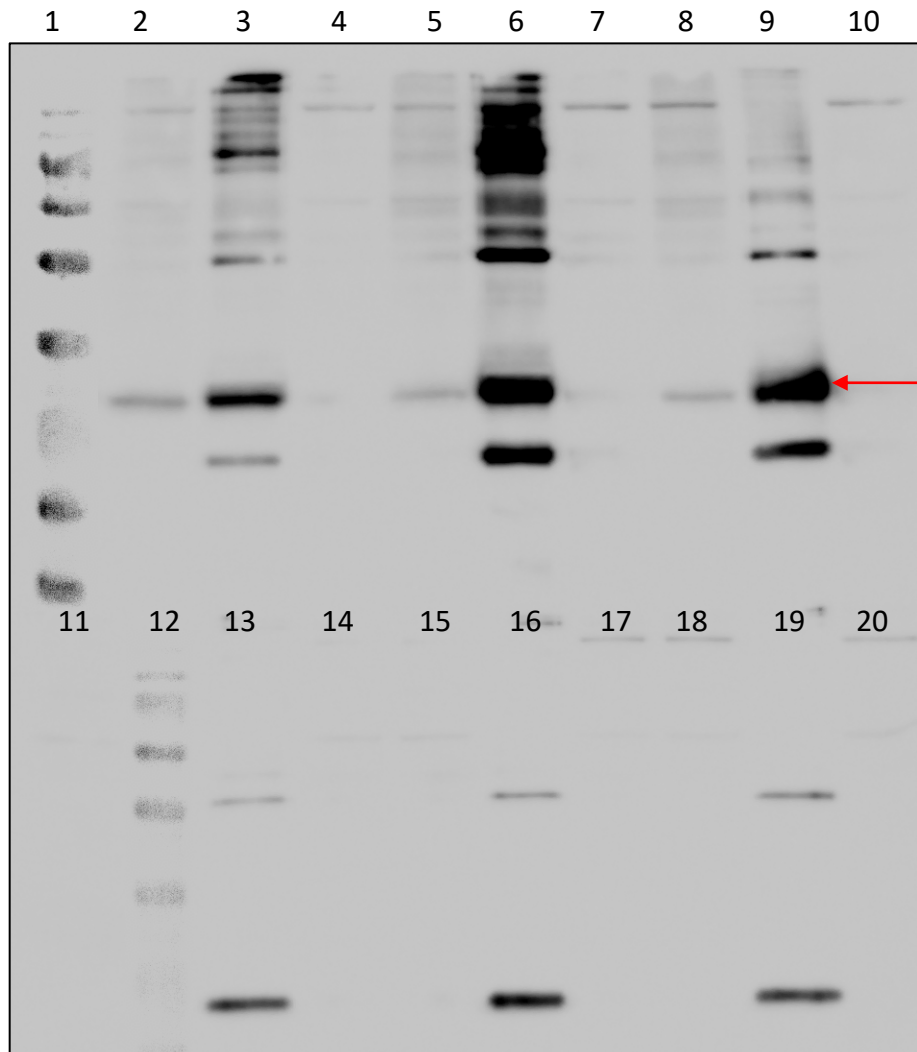






Appendix XXX

Optimizing the conditions for immunoprecipitation



Western blot with anti-FLAG antibodies. The arrow indicates the 20.71 kDa size VTA::3XFLAG protein.

Experiment A: No pre-clearing with Sepharose beads and overnight incubation with Anti-FLAG M2 beads.

Experiment B: Sepharose beads for 1hr and overnight incubation with Anti - FLAG M2 beads.

Experiment C: Sepharose beads - 1hr and Anti-FLAG M2 beads - 3hrs.

Lane No. 1: Colored protein marker

Lane No. 2: SG#444 - total cell extract 20 μ g

Lane No. 3: SG#444 – Exp. A - Immunoprecipitate with 1 mg of total protein

Lane No. 4: SG#444 – Exp. A - Supernatant after incubating with Anti-FLAG M2 beads - 20 μ g

Lane No. 5: SG#444 - Exp. B - Pre-cleared lysate - 20 μ g

Lane No. 6: SG#444 - Exp. B - Immunoprecipitate with 1 mg of total protein

Lane No. 7: SG#444 - Exp. B - Supernatant after incubating with Anti-FLAG M2 beads - 20 μ g

Lane No. 8: SG#444 - Exp. C - Pre-cleared lysate - 20 μ g

Lane No. 9: SG#444 - Exp. C - Immunoprecipitate with 1 mg of total protein

Lane No. 10: SG#444 - Exp. C - Supernatant after incubating with Anti-FLAG M2 beads - 20 μ g

Lane No. 11: SG#26 - total cell extract 20 μ g

Lane No. 12: Colored protein marker

Lane No. 13: SG#26 – Exp. A - Immunoprecipitate with 1 mg of total protein

Lane No. 14: SG#26 – Exp. A - Supernatant after incubating with Anti-FLAG M2 beads - 20 μ g

Lane No. 15: SG#26 - Exp. B - Pre-cleared lysate - 20 μ g

Lane No. 16: SG#26 - Exp. B - Immunoprecipitate with 1 mg of total protein

Lane No. 17: SG#26 - Exp. B - Supernatant after incubating with Anti-FLAG M2 beads - 20 μ g

Lane No. 18: SG#26 - Exp. C - Pre-cleared lysate - 20 μ g

Lane No. 19: SG#26 - Exp. C - Immunoprecipitate with 1 mg of total protein

Lane No. 20: SG#26 - Exp. C - Supernatant after incubating with Anti-FLAG M2 beads - 20 μ g.

Appendix XXXI

Conditions used to optimize IP and MS analysis

Trial No.	Culture condition	Amount of Anti-FLAG M2 beads used	Reaction volume	Incubation time with Anti-FLAG M2 beads	Strains used	MS results
1.	30°C LL 48 hours	30 µL	5 mL	Overnight	SG#26 SG#444	Total - 243 Only on SG#444 - 88
2.	30°C LL 48 hours	10 µL	5 mL	Overnight	SG#26 SG#444 SG#588	Total - 263 Only on SG#444 - 68 Only on SG#588-7
3.	25°C LL 24 hours	30 µL	1.5 mL	Overnight	SG#26 SG#444 SG#588	Total - 371 Only on SG#444 - 129 Only on SG#588-25
4.	25°C LL 24 hours	30 µL	5 mL	Overnight	SG#26 SG#444 SG#588	Total - 192 Only on SG#444 - 32 Only on SG#588-60
5.	25°C	10 µL	5 mL	Overnight	SG#26	Total -59

	LL 24 hours				SG#444 SG#588	Only on SG#444 – 27 Only on SG#588- 16
6.	25°C LL 24 hours	10 µL *	5 mL	2 hours	SG#26 SG#444 SG#588	Total -75 Only on SG#444 – 17 Only on SG#588- 26
7.	25°C LL 24 hours	10 µL*	5 mL	2 hours	SG#26 SG#444 SG#588	Total - 153 Only on SG#444 – 45 Only on SG#588- 39
8.	25°C LL 24 hours	10 µL*	5 mL	2 hours	SG#26 SG#444 SG#588	Total - 148 Only on SG#444 – 13 Only on SG#588- 75
9.	25°C LL 24 hours	10 µL*	5 mL	Overnight	SG#26 SG#444 SG#588	Total - 222 Only on SG#444 – 33 Only on SG#588- 103
10	25°C LL 24 hours	10 µL*	5 mL	Overnight	SG#26 SG#444 SG#588	Total - 149

					SG#572 SG#564	Only on SG#444 – 7 On all three NCU059 50::FLA G tagged strains - 40 Only on SG#588- 16
11.	25°C LL 24 hours	10 µL*	5 mL	Overnight	SG#26 SG#444 SG#588 SG#572 SG#564	Total - 116 Only on SG#444 – 13 On all three NCU059 50::FLA G tagged strains- 30 Only on SG#588- 13

* Anti-FLAG M2 beads were incubated with 1 mL of 0.1 M glycine-HCl at pH 3.5 for 10 minutes at room temperature.

Note: MS results are the number of different proteins identified.

NCU05950::3XFLAG tagged strains:

- SG#444 (NCU05950::3XFLAG)

*csp-1 his-3⁺:: Native promoter -NCU05950:: Gly:: 3xFLAG; chol-1
ras^{bd}; NCU05950^{KO} mat a*

- SG#572 (NCU05950::3XFLAG in *frq^{l0}* background)
csp-1 his-3⁺:: Native promoter -NCU05950::Gly::3xFLAG; chol-1
ras^{bd}; frq^{l0}; NCU05950^{KO}
- SG#564 (Native promoter – Δ 7-NCU05950::3xFLAG)
csp-1 his-3⁺:: Native promoter – Δ 7-NCU05950::Gly::3xFLAG; chol-1
ras^{bd}; NCU05950^{KO} mat a

Appendix XXXII

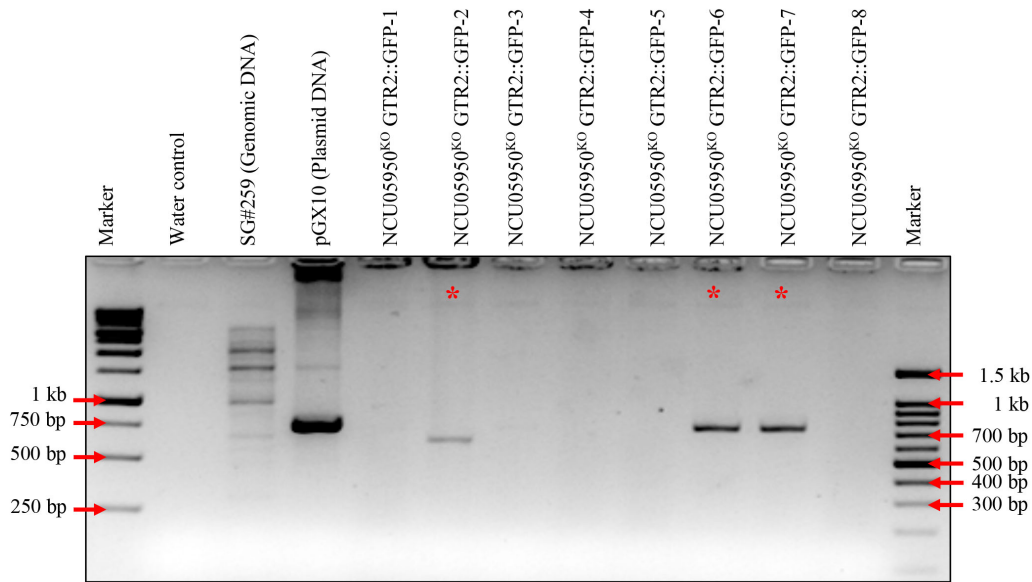
Construction of strains expressing GTR2::GFP fusion protein

A.

Spore PCR with XbaIGFP-F and PacIGFP-R primers to identify *csp-1 his-3⁺::*

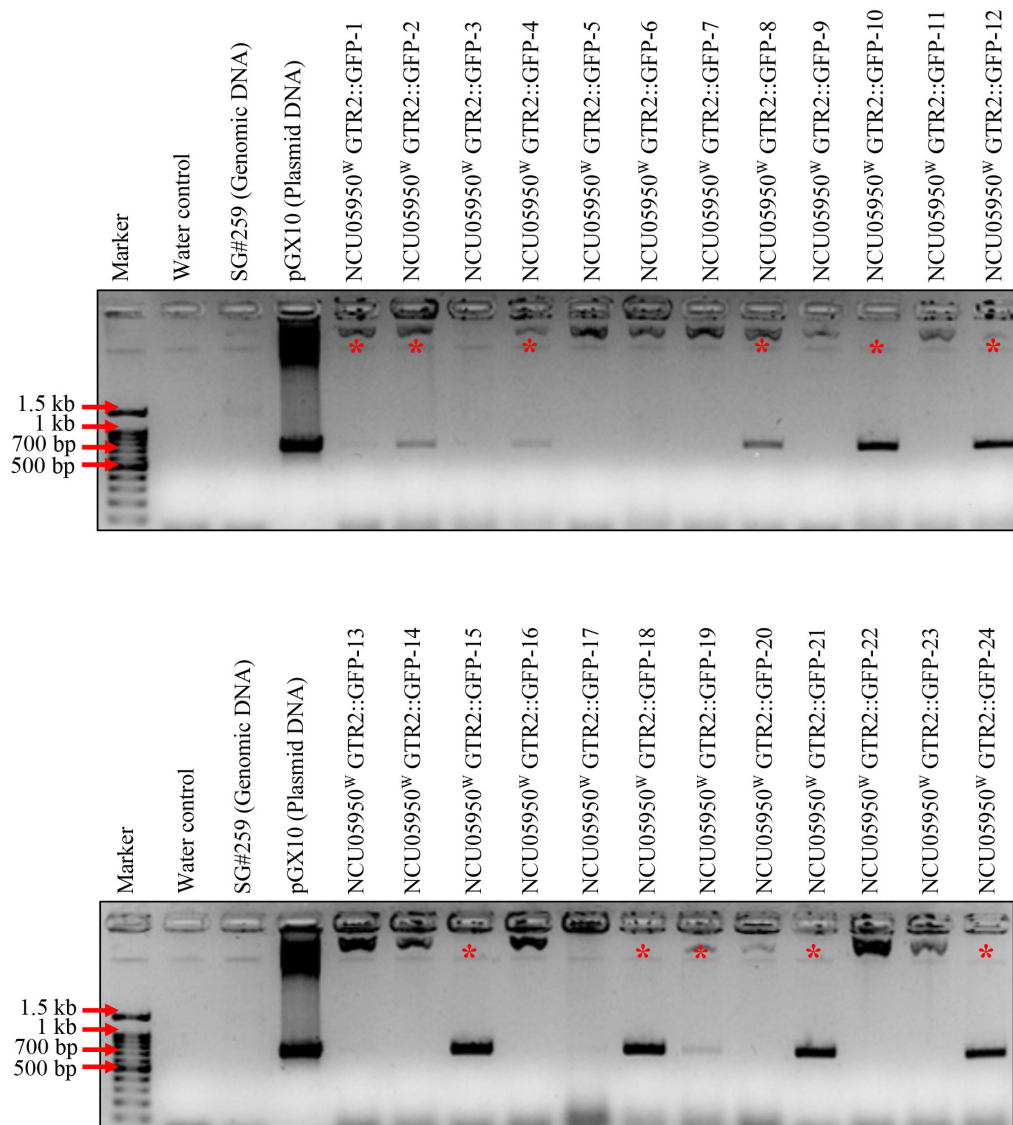
pccg-1::NCU00376::Gly::GFP; *chol-1 ras^{bd} :: NCU05950^{KO} mat 'a'* strains.

Strains carrying the wild type GTR2 gene tagged with GFP were recognized by a PCR product of 734 bp and those were marked with (*).



B.

Spore PCR with XbaIGFP-F and PacIGFP-R primers to identify *csp-1 his-3⁺::pccg-1::NCU00376::Gly::GFP*; *chol-1 ras^{bd} mat 'a'* strains. Strains carrying the wild type GTR2 gene tagged with GFP were recognized by a PCR product of 734 bp and those were marked with (*).



Appendix XXXIII

TOR signaling pathway components in yeast used to find the homologous genes and proteins in *Neurospora crassa*.

1. EGO2

Name Description: Exit from rapamycin-induced GrOwth arrest

Paralog: EGO4

2. EGO3/SLM4/ NIR1/GSE1

Name Description: Synthetic Lethal with Mss4

3. GTR1

Name Description: GTP binding protein Resemblance

4. GTR2

Name Description: GTP binding protein Resemblance

5. TOR1

Name Description: Target Of Rapamycin 1

Paralog: TOR2

6. TOR2

Name Description: Target Of Rapamycin 2

Paralog: TOR1

7. KOG1

Name Description: Kontroller Of Growth

8. LST8

Name Description: Lethal with Sec Thirteen

9. TCO89

Name Description: Tor Complex One

10. AVO1

Name Description: Adheres VOraciously (to TOR2)

11. AVO2

Name Description: Adheres VOraciously (to TOR2)

12. TSC11 / AVO3

Name Description: Temperature-sensitive Suppressors of Csg2 mutants

13. LST8

Name Description: Lethal with Sec Thirteen

14. BIT61

Name Description: Binding partner of Tor2p 1

Paralog: BIT2

15. SCH9

16. TAP42

Name Description: Two A phosphatase Associated Protein

17. SIT4

Name Description: Suppressor of Initiation of Transcription

18. VAM6

Name Description: VAcuolar Morphogenesis

19. FPR1

Name Description: Fk 506-sensitive Proline Rotamase

20. FPR2

Name Description: FKBP Proline Rotamase

21. FPR3

Name Description: Fk 506-sensitive Proline Rotamase 3

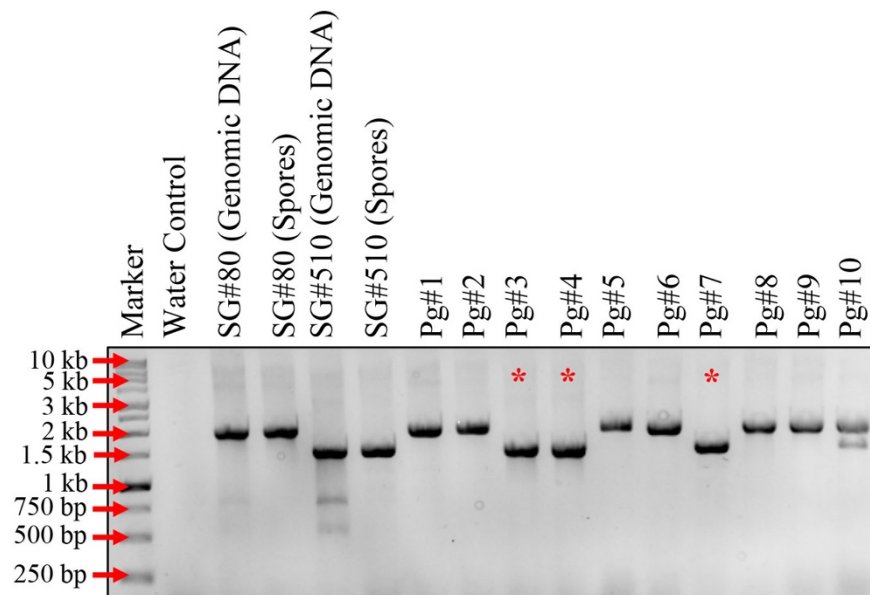
Paralog: FPR4

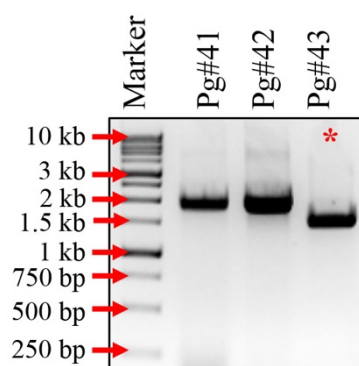
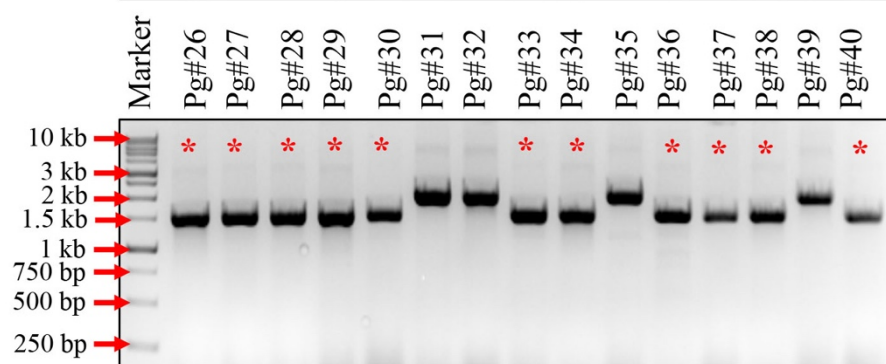
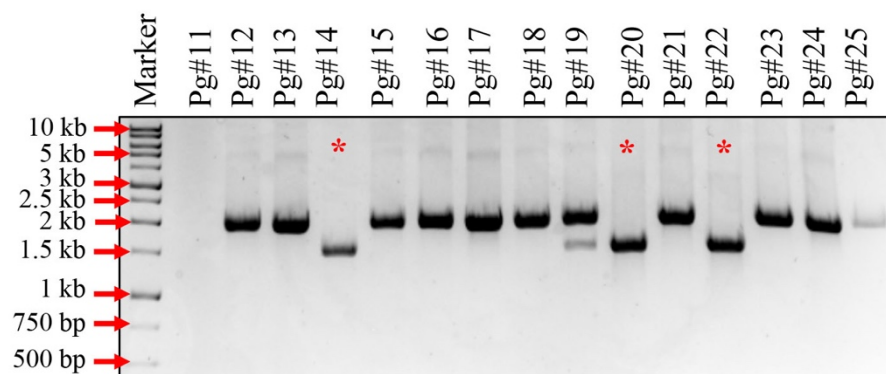
Appendix XXXIV

Spore PCR analysis of *csp-1* progeny strains obtained from genetic cross between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and GTR2^{KO} strains.

A.

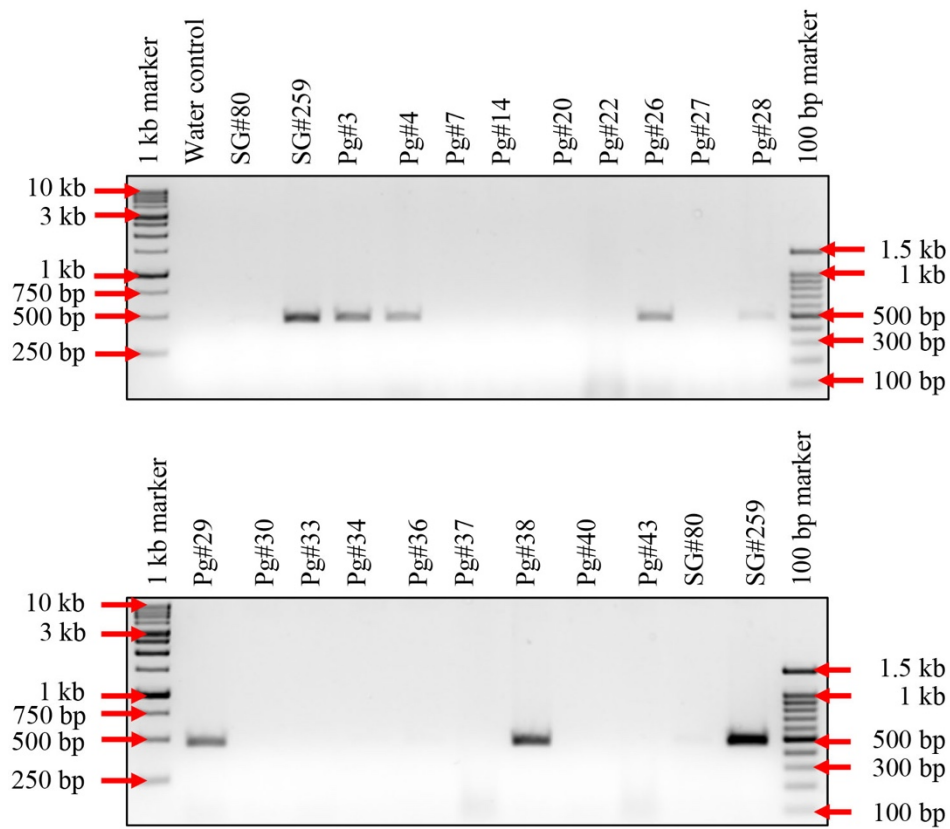
Spore PCR with GTR2F and GTR2R primers to identify GTR2^{KO} mutant strains. Strains carrying the wild type GTR2 gene were recognized by a PCR product of 1935 bp and GTR2^{KO} mutant strains were identified by a PCR product of ~1500 bp. GTR2^{KO} mutant strains used for further testing and experiments were marked with (*).





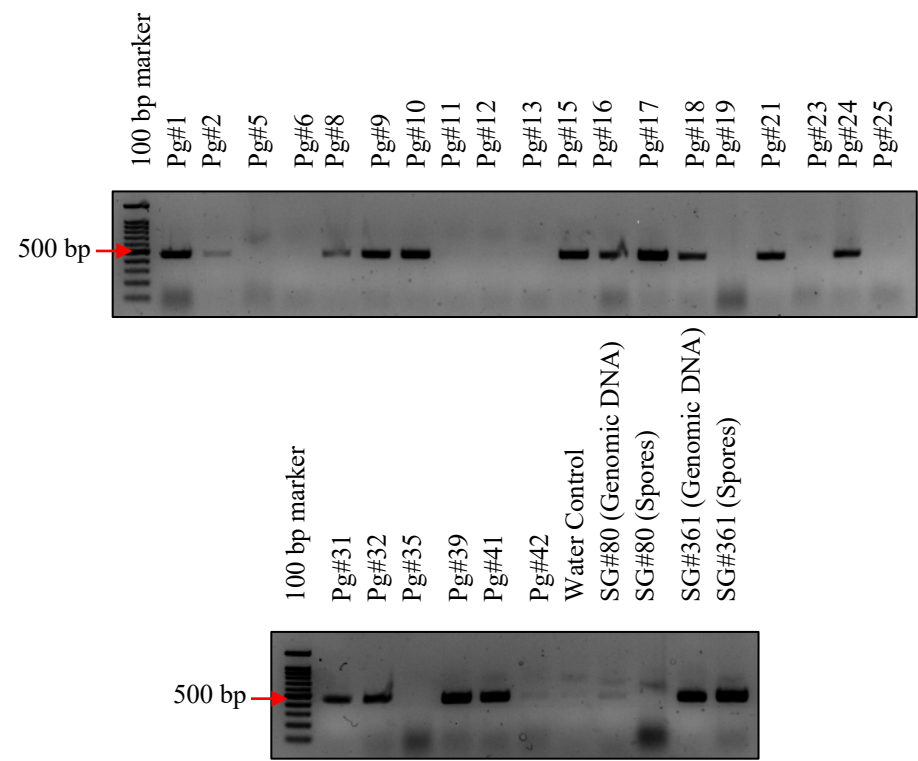
B.

Spore PCR with FrqF and FrqR primers to identify *frq*¹⁰ mutant strains in GTR2^{KO} background. Strains carrying the wild type *frq* gene were recognized by a PCR product of 484 bp.



C.

Spore PCR with FrqF and FrqR primers to identify *frq*¹⁰ mutant strains in GTR2 wild type background. Strains carrying the wild type *frq* gene were recognized by a PCR product of 484 bp.

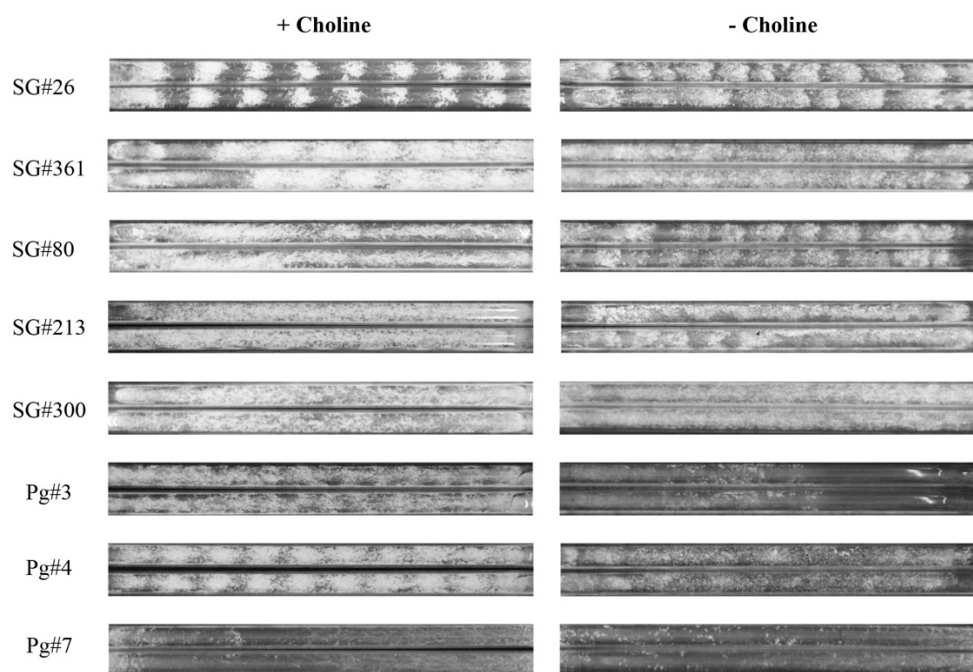


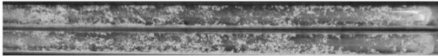
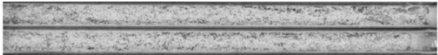
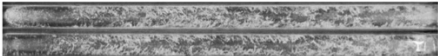
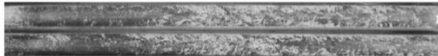
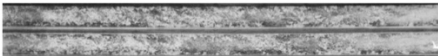
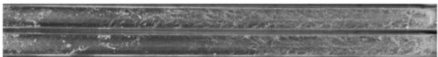
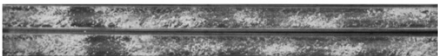
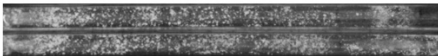
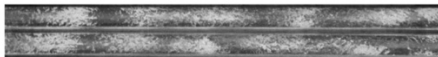
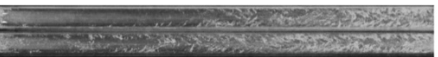
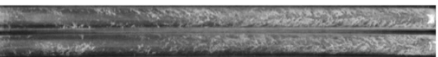
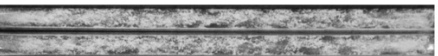
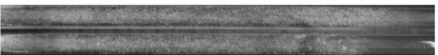
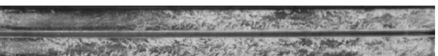
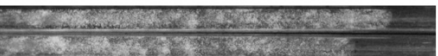
Appendix XXXV

Race tube analysis of *csp-1* progeny strains obtained from genetic cross between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and GTR2^{KO} strains.

A.

Conidiation phenotypes of GTR2 knockout mutant progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and FGSC#16041 (GTR2^{KO}) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Two representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Genotypes, periods and growth rates are listed in Table 9.5 & Appendix XXXV - B.



	+ Choline	- Choline
Pg#14		
Pg#20		
Pg#22		
Pg#26		
Pg#27		
Pg#28		
Pg#29		
Pg#30		
Pg#33		
Pg#34		
Pg#36		
Pg#37		
Pg#38		
Pg#40		
Pg#43		

B.

Periods, growth rates and summary of *csp-1* progeny obtained from cross between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq^{l0} mat A*) and GTR2^{KO} strains.

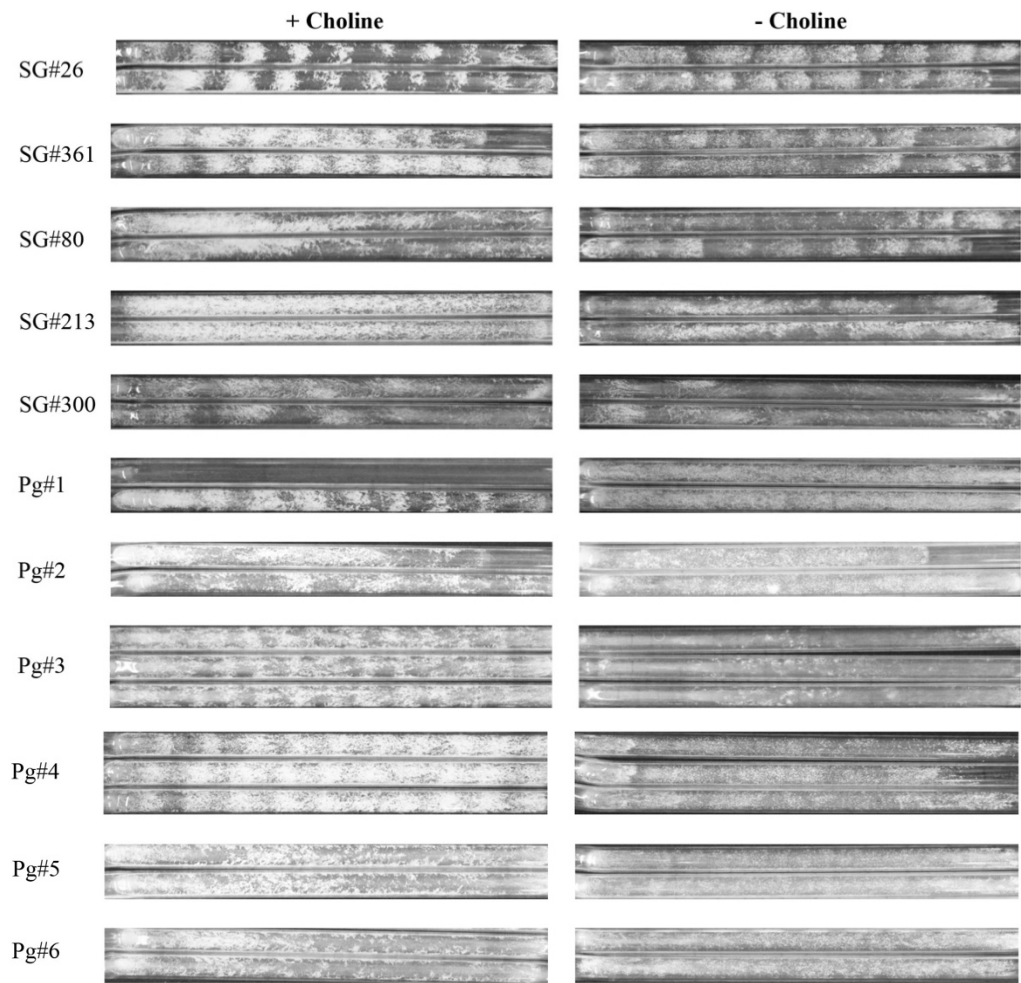
Strain No.	Without Choline		With 100 μ M Choline		GTR2	Ras- 1 ^{bd}	chol	frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
3	0.30	-	0.92	20.83	<i>gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.31	-	0.90	23.51				
4	0.51	45.97	1.09	20.70	<i>gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.44	46.79	1.09	21.09				
7	2.05	-	2.07	-	<i>gtr2^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>chol+</i>	<i>frq^{l0}</i>
	2.16	-	1.98	-				
14	0.53	-	1.18	-	<i>gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.48	-	1.15	-				
20	0.38	-	0.98	-	<i>gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.38	-	0.97	-				
22	1.71	-	1.62	-	<i>gtr2^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>chol+</i>	<i>frq^{l0}</i>
	1.63	-	1.65	-				
26	0.47	-	1.04	-	<i>gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.50	-	1.04	-				

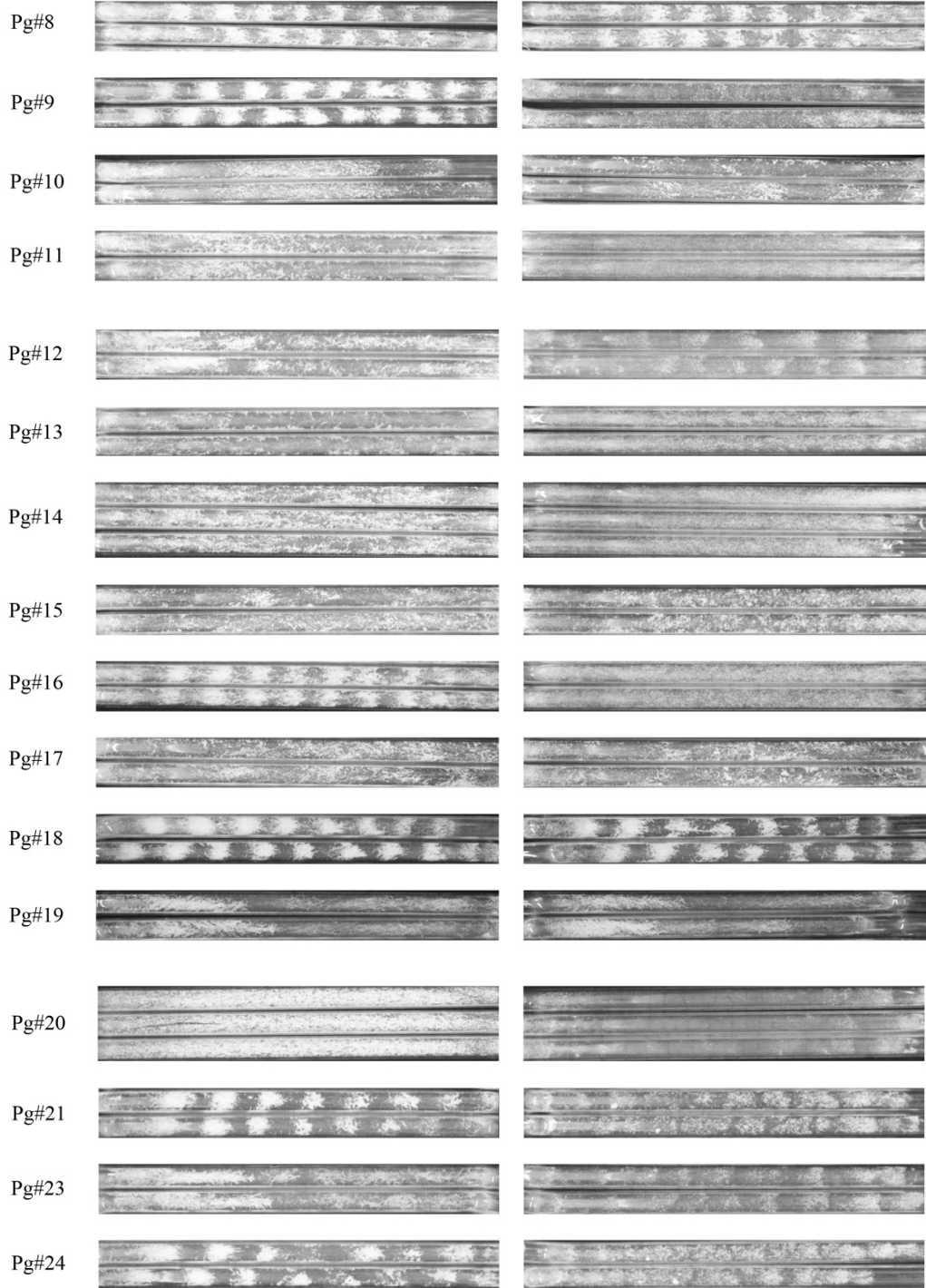
27	0.77	-	2.04	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.82	-	2.09	-				
28	0.61	38.29	1.99	19.60	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.59	73.30	2.01	19.81				
29	2.09	19.88	1.97	20.15	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol-1</i>	<i>frq⁺</i>
	2.07	19.16	1.98	19.84				
30	0.46	-	1.20	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.51	-	1.22	-				
33	1.87	-	1.80	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol+</i>	<i>frq^{l0}</i>
	1.90	-	1.82	-				
34	0.34	-	0.90	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.35	-	0.90	-				
36	1.97	-	2.02	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol+</i>	<i>frq^{l0}</i>
	2.13	-	1.97	-				
37	0.40	-	0.98	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.35	-	0.97	-				
38	0.55	-	1.71	-	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.56	-	1.57	-				
40	2.11	16.66	1.97	14.71	<i>gtr2^{KO}</i>	<i>Ras- l^{bd+}</i>	<i>chol+</i>	<i>frq^{l0}</i>
	2.15	14.70	1.96	15.56				

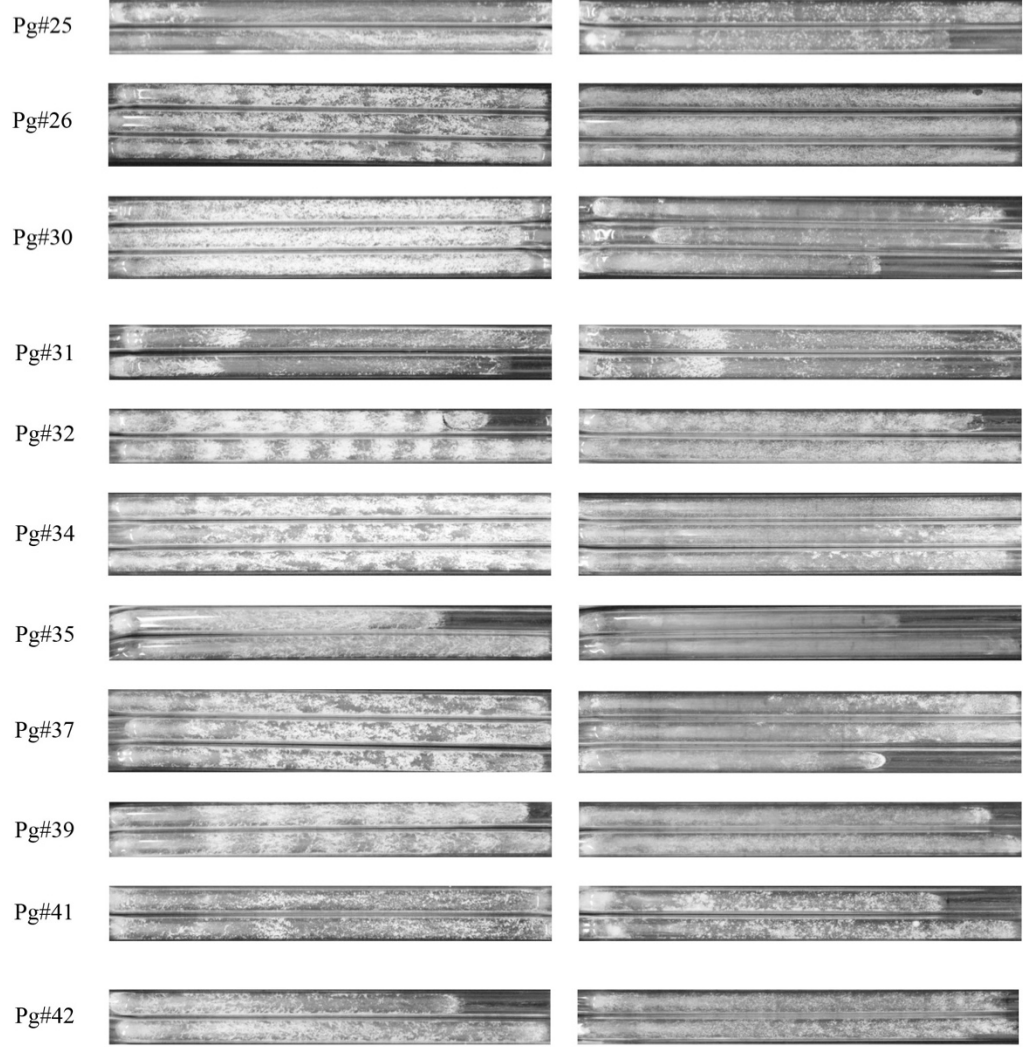
43	1.16	-	1.09	-	<i>gtr2^{KO}</i>	<i>Ras-_{l^{bd}}</i>	<i>chol⁺</i>	<i>frq^{l0}</i>
	1.17	-	1.08	-				
SG#80 control	0.51	48.13	1.33	-	<i>gtr2⁺</i>	<i>Ras-_{l^{bd}}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.48	55.15	1.27	-				
SG#213 control	0.63	37.18	1.28	-	<i>gtr2⁺</i>	<i>Ras-_{l^{bd}}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.67	35.65	1.18	-				
SG#26 control	0.55	37.89	1.27	21.53	<i>gtr2⁺</i>	<i>Ras-_{l^{bd}}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.55	43.50	1.29	21.64				
SG#361 control	0.47	-	1.37	18.99	<i>gtr2⁺</i>	<i>Ras-_{l^{bd}}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.48	-	1.34	20.84				
SG#300 control	0.53	-	1.23	-	<i>gtr2⁺</i>	<i>Ras-_{l^{bd}}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.51	-	1.26	-				

C.

Conidiation phenotypes of GTR2 wild type gene carrying progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and FGSC#16041 (GTR2^{KO}) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Two representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Genotypes, periods and growth rates are listed in Table 9.6 & Appendix XXXV - D.







D.

Summary of *csp-1* progeny obtained from cross between SG#300 (*csp-1: chol-1 ras-1^{bd}, frq¹⁰ mat A*) and GTR2^{KO} strains.

Strain No.	Without Choline		With 100 μ M Choline		GTR2	Ras- <i>l</i> ^{bd}	chol	frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
1	0.5	-	1.21	19.36	<i>Gtr2</i> ⁺	<i>Ras-l^{bd}</i>	<i>Chol-l</i>	<i>Frq</i> ⁺
	0.55	-	NA	NA				
2	0.84	-	2.49	13.07	<i>Gtr2</i> ⁺	<i>Ras-l^{bd+}</i>	<i>Chol</i> ⁺	<i>Frq</i> ⁺
	1.14	-	2.74	14.06				
3	0.33	-	0.98	20.46	<i>Gtr2</i> ^{KO}	<i>Ras-l^{bd}</i>	<i>Chol-l</i>	<i>Frq</i> ⁺
	0.33	-	0.96	20.39				
	0.39	-	0.94	20.91				
4	0.41	-	1.12	21.4	<i>Gtr2</i> ^{KO}	<i>Ras-l^{bd}</i>	<i>Chol-l</i>	<i>Frq</i> ⁺
	0.47	-	1.11	21.6				
	0.46	-	1.11	21.27				
5	0.55	-	1.18	-	<i>Gtr2</i> ⁺	<i>Ras-l^{bd+}</i>	<i>Chol-l</i>	<i>Frq</i> ¹⁰
	0.51	-	1.18	-				
6	0.64	-	1.41	-	<i>Gtr2</i> ⁺	<i>Ras-l^{bd+}</i>	<i>Chol-l</i>	<i>Frq</i> ¹⁰
	0.63	-	1.41	-				
8	1.3	19.92	1.26	19.94	<i>Gtr2</i> ⁺	<i>Ras-l^{bd}</i>	<i>Chol</i> ⁺	<i>Frq</i> ⁺
	1.26	20.6	1.24	19.61				
9	0.58	-	1.17	20.81	<i>Gtr2</i> ⁺	<i>Ras-l^{bd}</i>	<i>Chol-l</i>	<i>Frq</i> ⁺
	0.51	-	1.23	20.98				
10	3.07	-	3.07	-	<i>Gtr2</i> ⁺	<i>Ras-l^{bd+}</i>	<i>Chol</i> ⁺	<i>Frq</i> ⁺
	2.85	-	2.75	-				
11	0.56	-	1.18	-	<i>Gtr2</i> ⁺	<i>Ras-l^{bd+}</i>	<i>Chol-l</i>	<i>Frq</i> ¹⁰
	0.59	-	1.18	-				
12	0.63	32.31	1.37	-	<i>Gtr2</i> ⁺	<i>Ras-l^{bd}</i>	<i>Chol-l</i>	<i>Frq</i> ¹⁰
	0.51	55.27	1.39	-				
13	0.6	-	1.26	-	<i>Gtr2</i> ⁺	<i>Ras-l^{bd+}</i>	<i>Chol-l</i>	<i>Frq</i> ¹⁰
	0.58	-	1.25	-				
14	0.48	-	1.17	-	<i>Gtr2</i> ^{KO}			<i>Frq</i> ¹⁰

	0.54	-	1.18	-		<i>Ras- I^{bd}</i>	<i>Chol- I</i>	
	0.5	-	1.17	-				
15	1.02	-	2.66	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd+}</i>	<i>Chol⁺</i>	<i>Frq⁺</i>
	1.1	-	2.75	-				
16	0.51	-	1.13	21.19	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq⁺</i>
	0.57	-	1.18	20.4				
17	2.52	-	2.4	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd+}</i>	<i>Chol⁺</i>	<i>Frq⁺</i>
	2.53	-	2.63	-				
18	1.44	19.56	1.33	20.39	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>Frq⁺</i>
	1.43	18.97	1.25	20.35				
19	2.26	-	2.15	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd+}</i>	<i>Chol⁺</i>	<i>Frq¹⁰</i>
	2.26	-	2.02	-				
20	0.46	-	1.01	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq¹⁰</i>
	0.41	-	1	-				
	0.36	-	1	-				
21	0.61	38.79	1.5	19.87	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq⁺</i>
	0.45	53.13	1.52	20.16				
23	0.47	52.74	1.52	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq¹⁰</i>
	0.49	52.43	1.53	-				
24	0.51	-	1.46	20.32	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq⁺</i>
	0.51	-	1.48	20.1				
25	0.54	-	3.04	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd+}</i>	<i>Chol- I</i>	<i>Frq¹⁰</i>
	0.66	-	3.01	-				
26	0.47	-	1.08	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq⁺</i>
	0.5	-	1.08	-				
	0.51	-	1.07	-				
30	0.57	-	1.22	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq¹⁰</i>
	0.56	-	1.22	-				
	0.46	-	1.22	-				
31	2.66	-	2.88	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd+}</i>	<i>Chol⁺</i>	<i>Frq⁺</i>
	2.78	-	3.04	-				
32	0.54	-	1.19	21.28	<i>Gtr2⁺</i>	<i>Ras- I^{bd+}</i>	<i>Chol- I</i>	<i>Frq⁺</i>
	0.53	-	1.2	20.46				
34	0.35	-	0.96	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- I</i>	<i>Frq¹⁰</i>
	0.37	-	0.95	-				
	0.36	-	0.93	-				

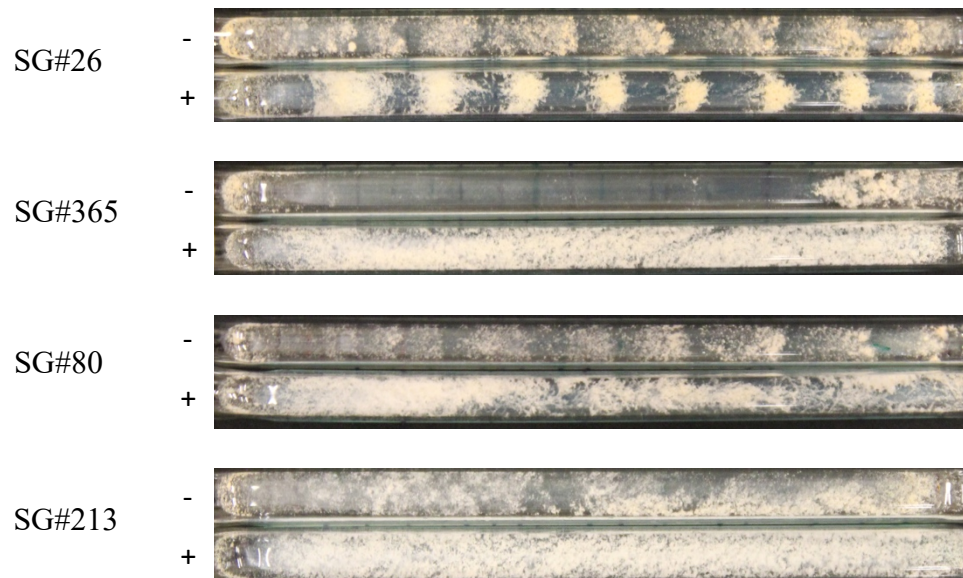
35	0.55	-	1.38	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd+}	<i>Chol-I</i>	<i>Frq</i> ¹⁰
	0.65	-	1.4	-				
37	0.59	-	0.99	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ¹⁰
	0.4	-	1.04	-				
	0.39	-	1	-				
39	0.72	-	1.35	22.65	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ⁺
	0.54	-	1.29	22.51				
41	1.16	-	2.95	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd+}	<i>Chol</i> ⁺	<i>Frq</i> ⁺
	1.23	-	2.91	-				
42	0.62	-	1.37	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd+}	<i>Chol-I</i>	<i>Frq</i> ¹⁰
	0.53	-	1.36	-				
SG#26 control	0.48	51.28	1.33	20.73	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ⁺
	0.44	61.56	1.38	19.01				
SG#80 control	0.54	50.54	1.35	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ¹⁰
	0.54	44.87	1.41	-				
SG#213 control	0.67	-	1.17	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ¹⁰
	0.55	-	1.24	-				
SG#300 control	1.93	-	1.96	22.93	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ¹⁰
	2.01	-	1.88	21.97				
SG#361 control	0.5	-	1.19	21.51	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>	<i>Frq</i> ⁺
	0.48	-	1.19	21.7				

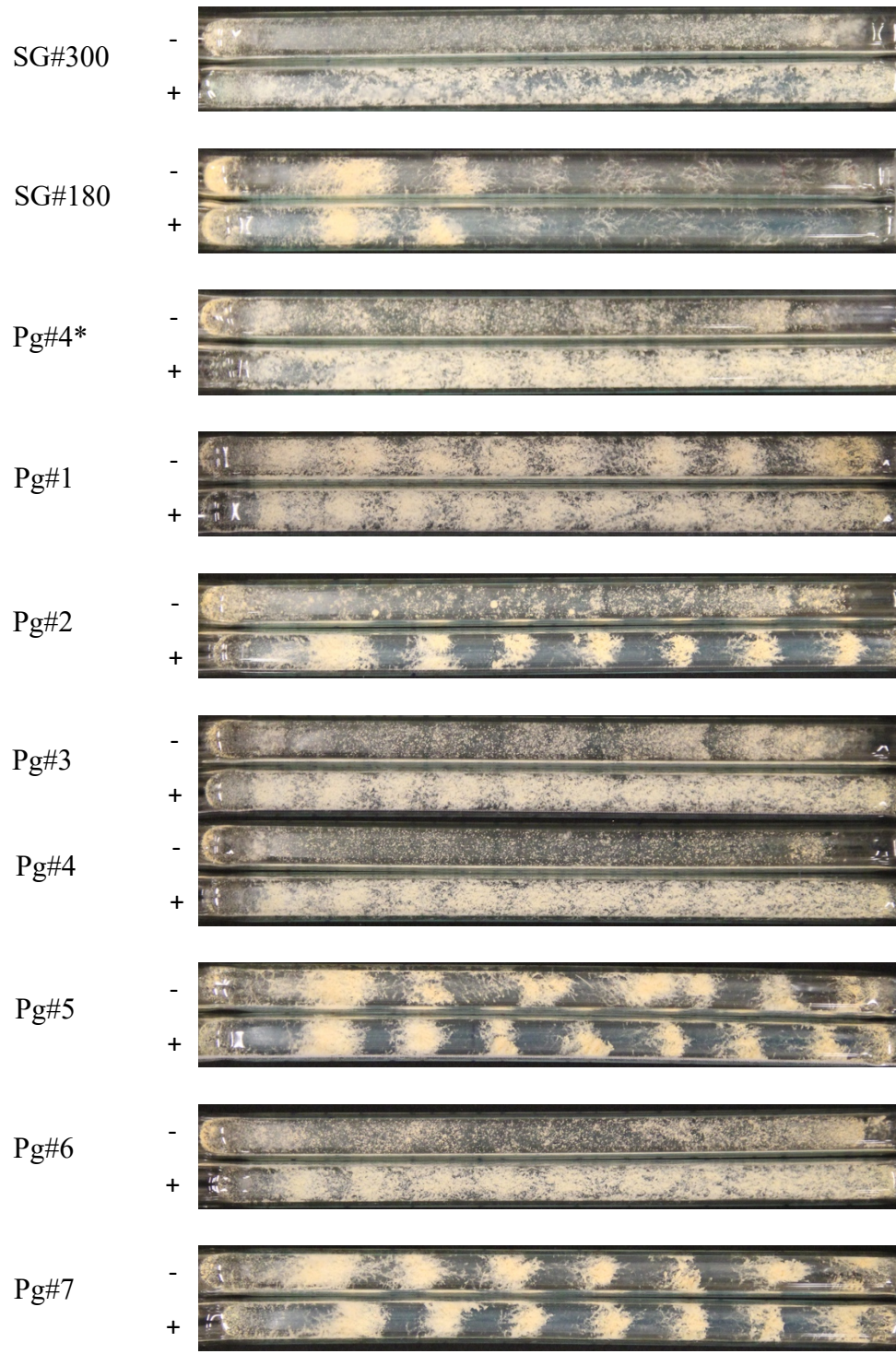
Appendix XXXVI

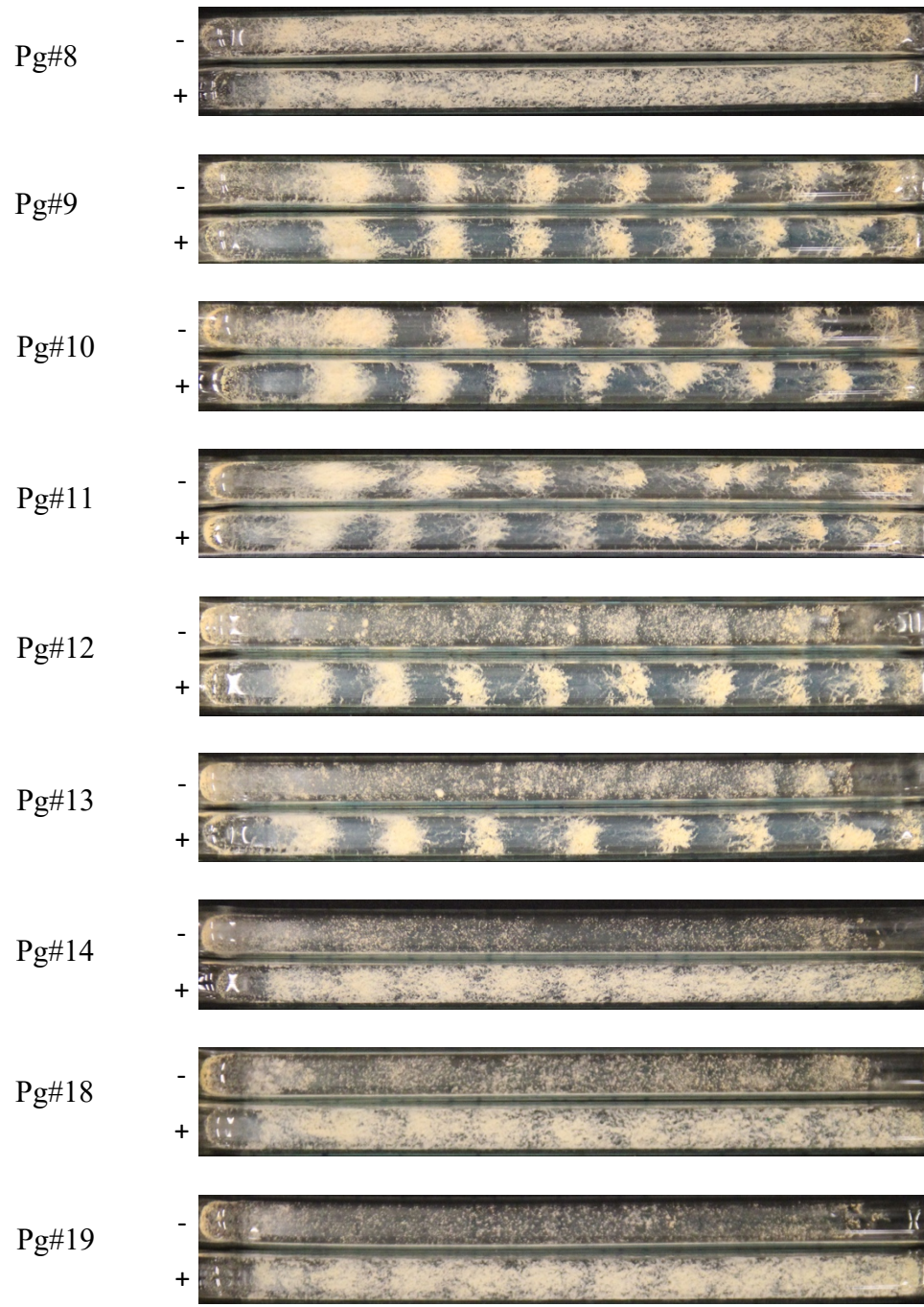
Race tube analysis of *csp-1* progeny strains obtained from genetic backcross with selected *GTR2^{KO}* strains.

A.

Conidiation phenotypes of progeny strains obtained from the backcross performed between SG#180 (*ras-1^{bd} mat a*) and pg#4* (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; mat A*) strains. Strains were grown with (+, bottom tube) or without (-, top tube) 100 μ M choline in the medium. One representative replicate tube for each condition and three representative strains for each genotype are shown. Growth is from left to right. Genotypes, periods and growth rates are listed in Table 9.7 & Appendix XXXVI – B.



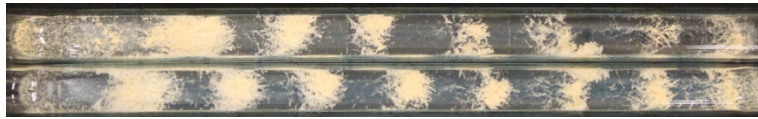




Pg#20

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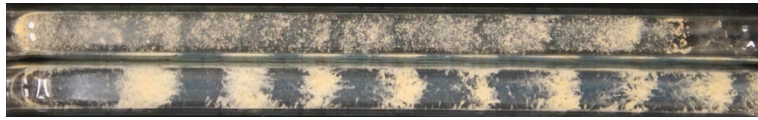
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Pg#21

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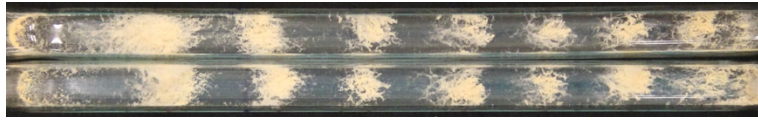
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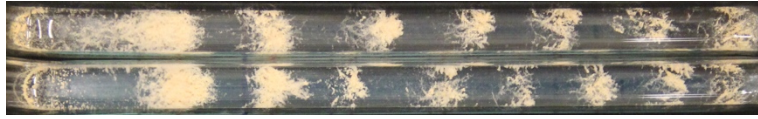
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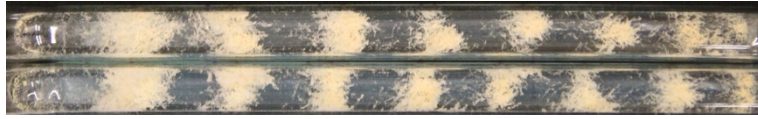
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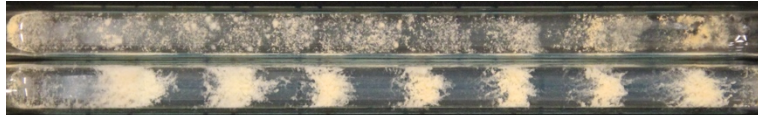
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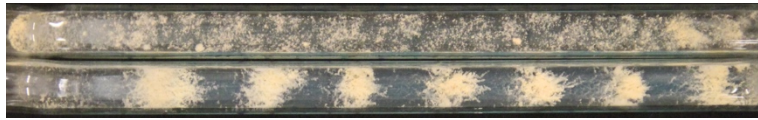
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Pg#26

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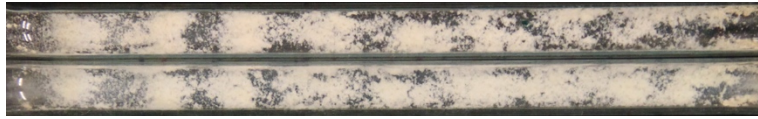
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Pg#27

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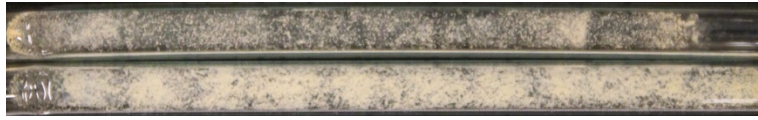
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Pg#28

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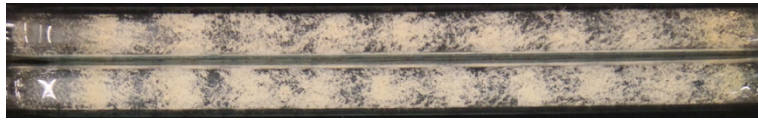
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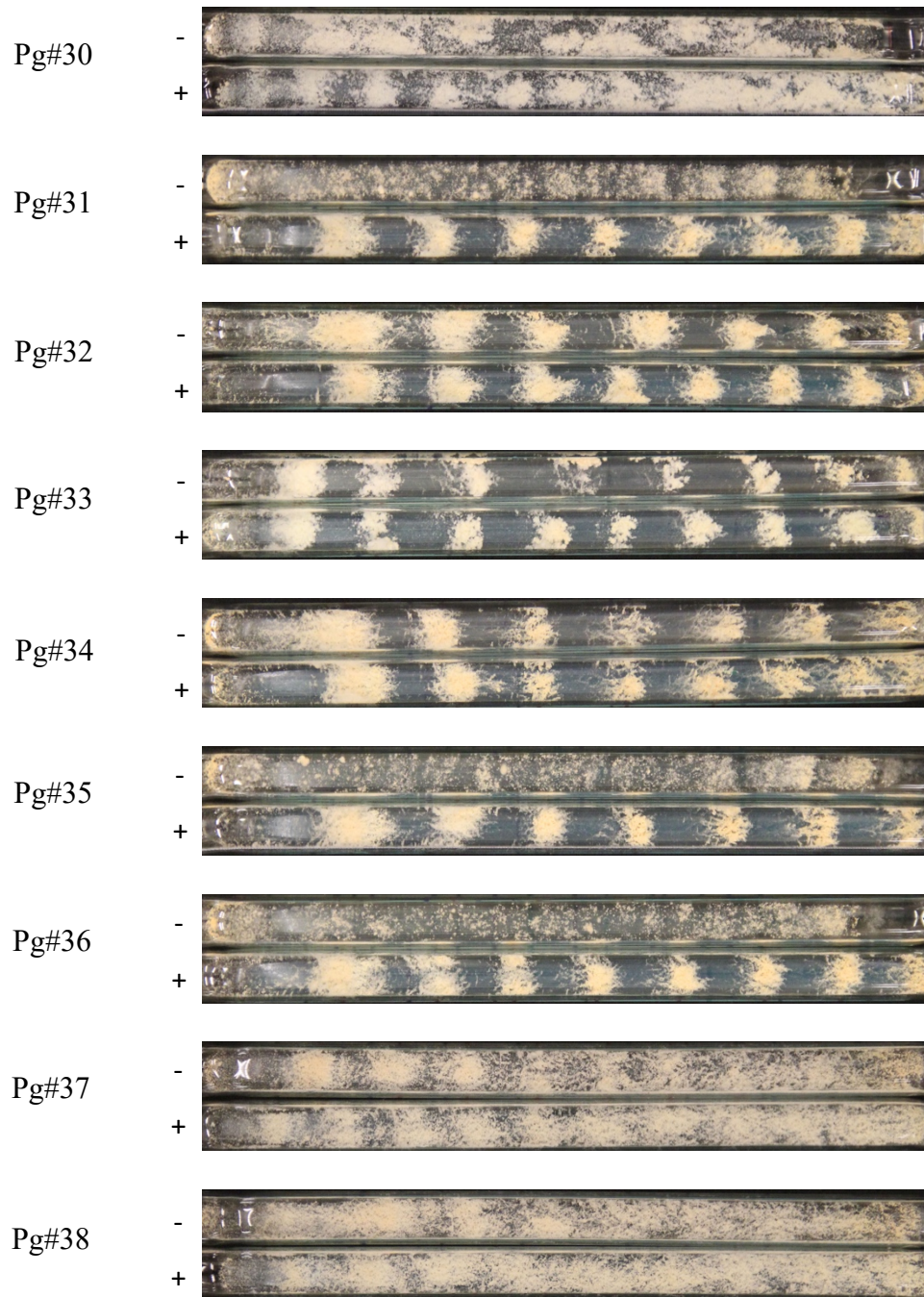


Pg#29

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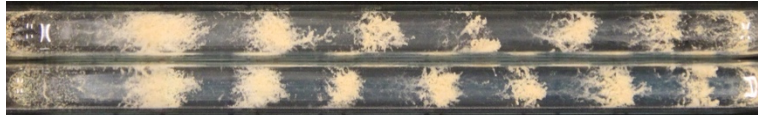
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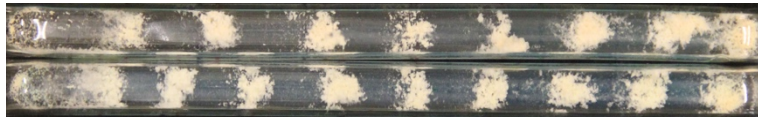
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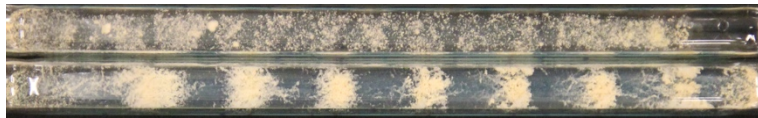
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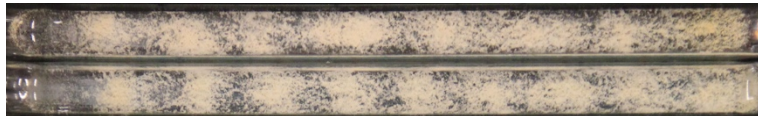
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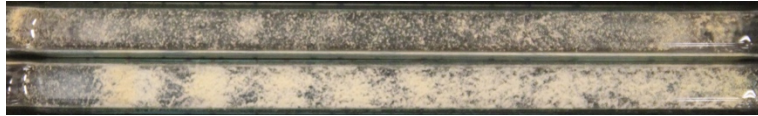
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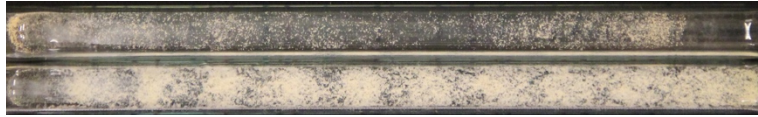
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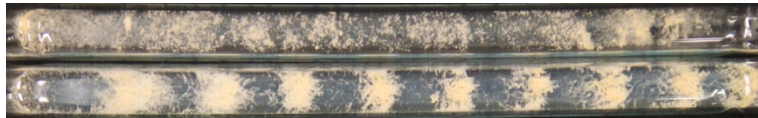
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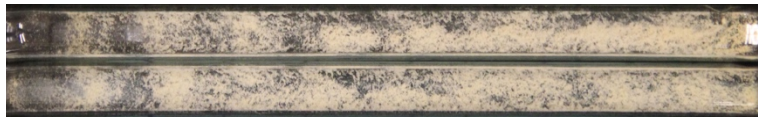
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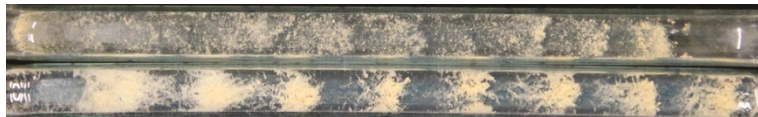
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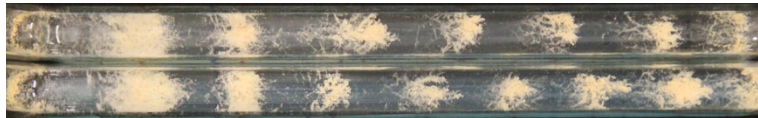
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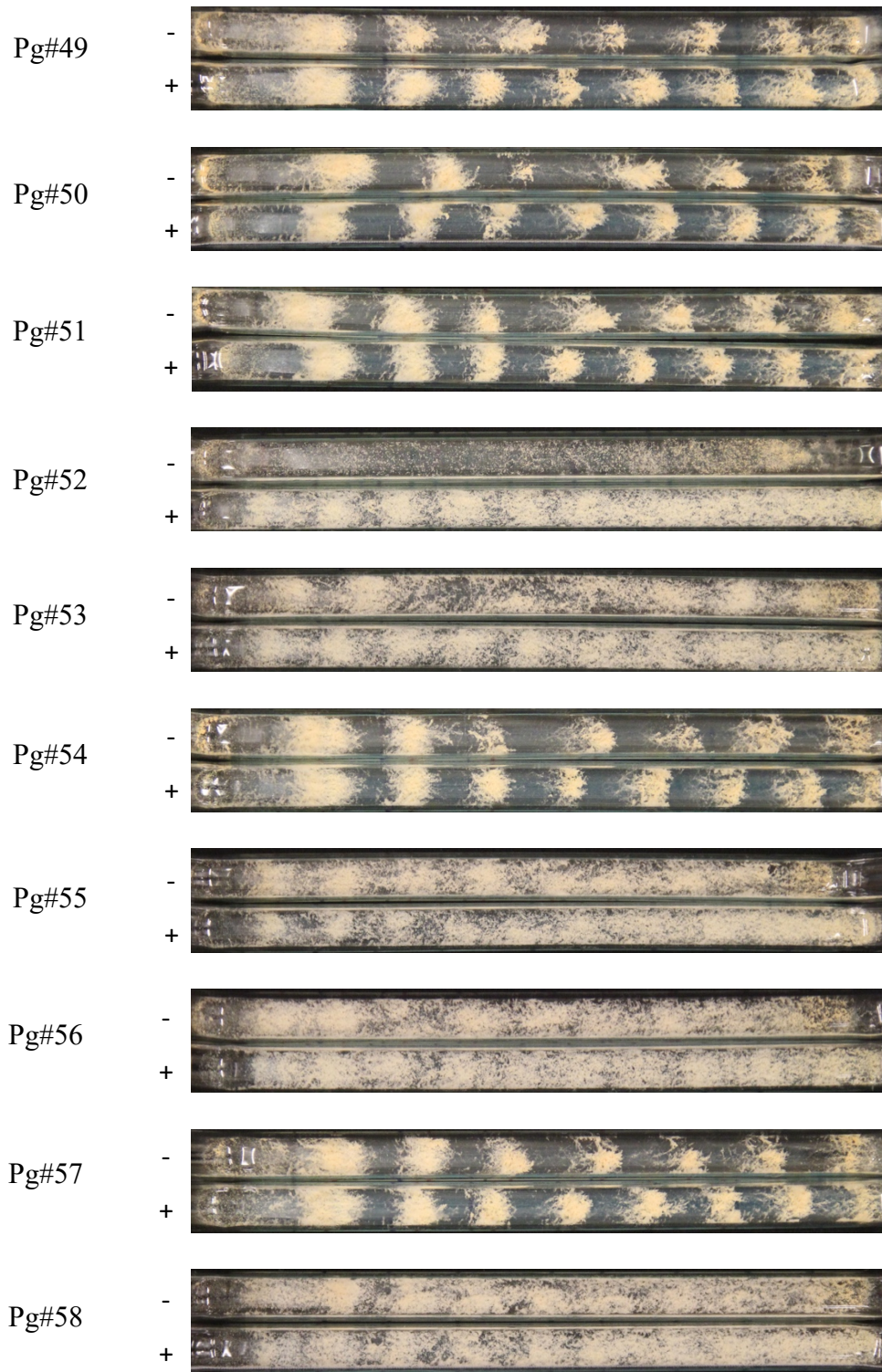
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Pg#59

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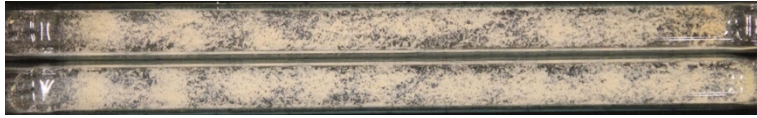
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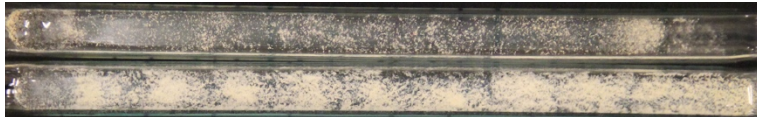
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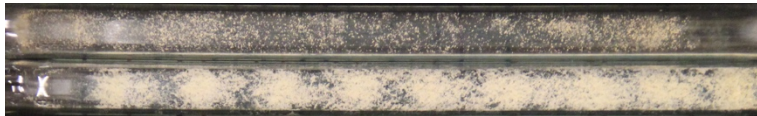
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Pg#62

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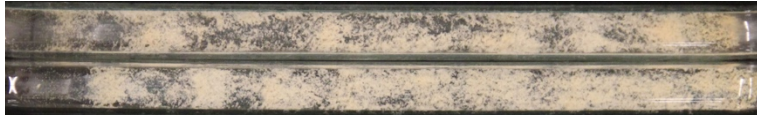
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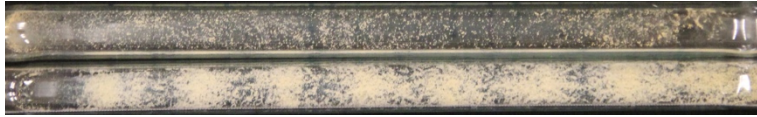
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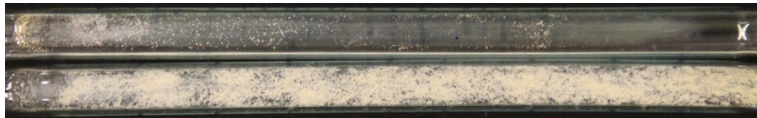
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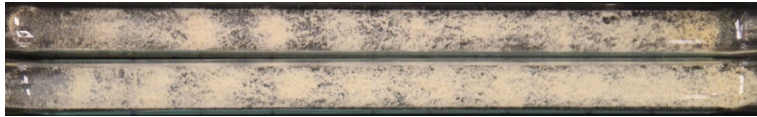
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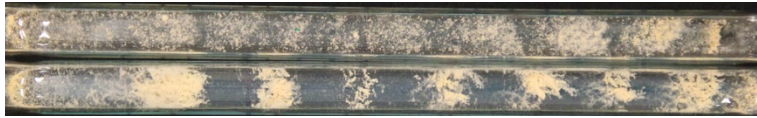
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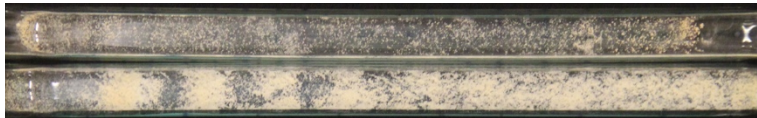
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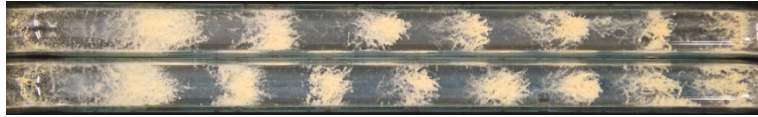
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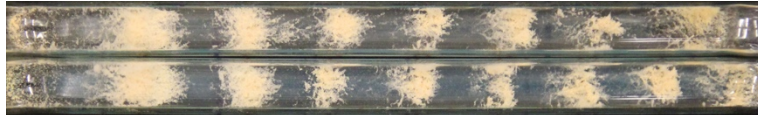
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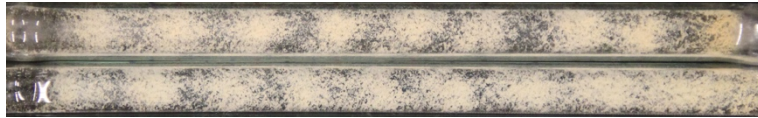
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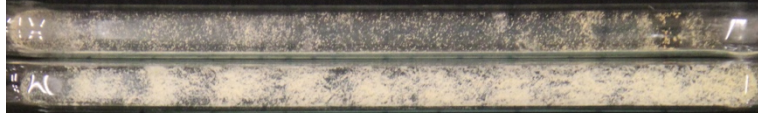
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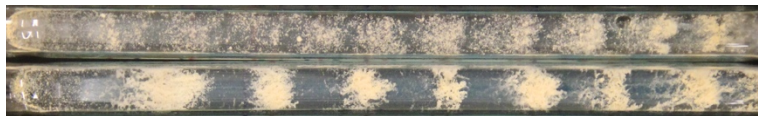
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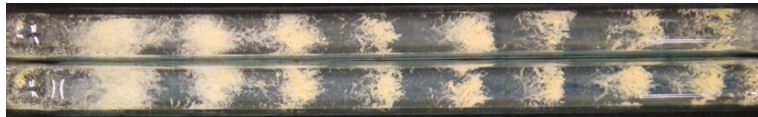
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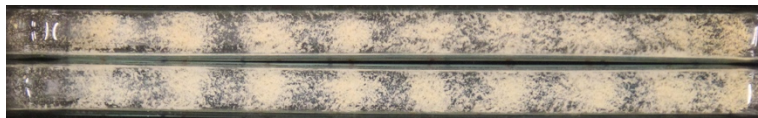
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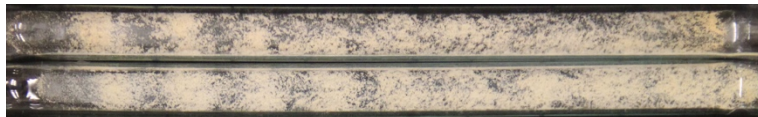
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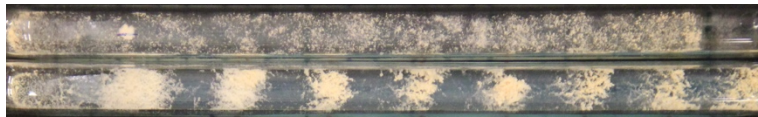
Pg#76

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Pg#77

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B.

Summary of *csp-1* progeny obtained from back cross between SG#180 (*ras-1^{bd}* *mat a*) and pg#4 (*csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}* *mat A*) strains.

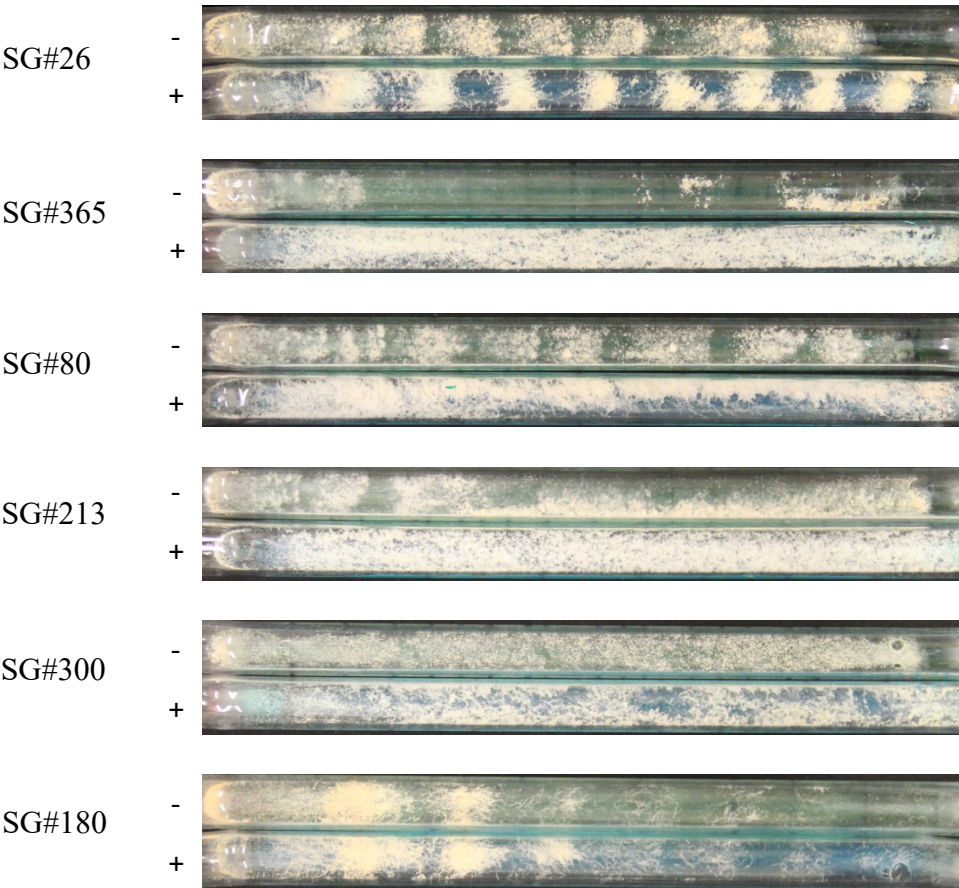
Strain No.	Without Choline		With 100 μ M Choline		GTR2	Ras-1 ^{bd}	Chol
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)			
SG#80 (control)	0.48	66.37	1.37	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
SG#213 (control)	0.66	-	1.34	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
SG#26 (control)	0.53	54.78	1.39	21.67	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
SG#365 (control)	0.57	-	1.29	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
SG#300 (control)	0.52	-	1.21	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
SG#180 (control)	1.49	22.51	1.37	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
Pg#4 (control)	0.43	-	1.17	21.66	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
1	1.32	22.39	1.16	21.85	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
2	0.47	-	1.48	21.44	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
3	0.38	-	1.12	21.22	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
4	0.46	-	1.11	21.81	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
5	1.80	18.69	1.54	21.42	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
6	0.49	-	1.18	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
7	1.59	21.70	1.51	20.90	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
8	1.24	-	1.21	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
9	1.58	21.62	1.48	20.94	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
10	1.54	21.36	1.44	20.81	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
11	1.58	21.92	1.46	21.05	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>
12	0.49	-	1.41	21.18	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
13	0.55	-	1.54	21.57	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
14	0.44	-	1.20	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
18	0.43	-	1.19	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>
19	0.43	-	1.15	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>

20	1.51	21.44	1.44	21.13	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
21	0.50	38.35	1.37	20.91	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
22	1.62	19.42	1.52	20.90	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
23	1.67	21.22	1.44	20.57	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
24	1.58	21.08	1.48	21.56	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
25	0.45	-	1.57	21.42	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
26	0.54	-	1.52	21.09	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
27	1.32	20.15	1.21	21.57	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
28	0.42	-	1.07	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
29	1.22	22.03	1.13	21.90	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
30	1.35	-	1.15	21.80	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
31	0.46	-	1.52	20.76	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
32	1.71	20.66	1.47	21.15	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
33	1.80	19.03	1.49	20.05	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
34	1.64	21.02	1.49	20.47	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
35	0.47	-	1.51	21.83	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
36	0.50	-	1.42	21.12	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
37	1.25	20.65	1.13	21.27	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
38	1.28	-	1.20	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
39	1.73	20.60	1.50	21.20	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
40	1.61	20.54	1.37	21.00	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
41	0.56	-	1.51	20.69	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
42	1.39	-	1.23	21.24	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
43	0.55	-	1.17	22.01	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
44	0.46	-	1.13	21.66	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
45	0.44	-	1.38	20.83	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
46	1.31	-	1.20	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
47	0.48	-	1.44	20.93	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
48	1.81	20.62	1.55	20.84	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
49	1.81	20.89	1.51	21.23	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
50	1.79	20.81	1.54	21.01	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
51	1.65	20.96	1.39	20.84	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
52	0.43	-	1.17	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol-I</i>
53	1.51	-	1.18	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
54	1.60	21.25	1.44	21.18	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺
55	1.28	21.54	1.17	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺

56	1.21	21.88	1.11	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
57	1.59	21.33	1.44	21.56	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
58	1.16	-	1.14	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
59	1.60	20.90	1.42	20.28	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
60	1.22	-	1.18	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
61	0.46	-	1.18	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
62	0.44	-	1.24	21.13	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
63	1.30	-	1.18	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
64	0.43	-	1.34	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
65	0.33	-	1.17	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
66	1.21	22.34	1.16	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
67	0.52	-	1.48	20.76	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
68	0.51	-	1.22	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
69	1.63	20.52	1.50	20.76	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
70	1.54	21.29	1.46	21.38	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
71	1.29	21.18	1.22	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
72	0.49	-	1.16	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
73	0.50	48.40	1.48	21.00	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>
74	1.48	21.12	1.46	20.57	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
75	1.36	21.87	1.37	21.73	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
76	1.22	-	1.15	-	<i>Gtr2^{KO}</i>	<i>Ras-I^{bd}</i>	<i>Chol⁺</i>
77	0.44	-	1.49	20.66	<i>Gtr2⁺</i>	<i>Ras-I^{bd}</i>	<i>Chol-I</i>

C.

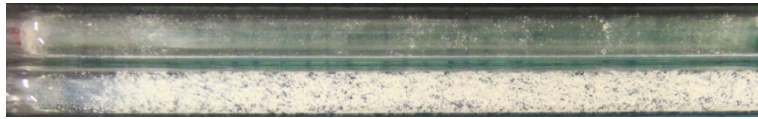
Conidiation phenotypes of progeny strains obtained from the backcross performed between SG#180 (*ras-1^{bd} mat a*) and pg#20* (*csp-1; gtr2^{KO}; chol-1 ras-1^{bd}; frq¹⁰ mat A*) strains. Strains were grown with (+, bottom tube) or without (-, top tube) 100 μ M choline in the medium. One representative replicate tube for each condition are shown. Growth is from left to right. Genotypes, periods and growth rates are listed in Table 9.8 & Appendix XXXVI - D.



Pg#20*

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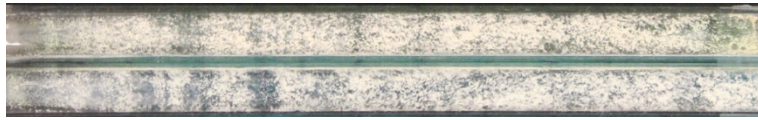
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Pg#1

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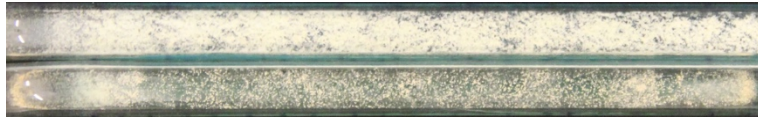
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Pg#2

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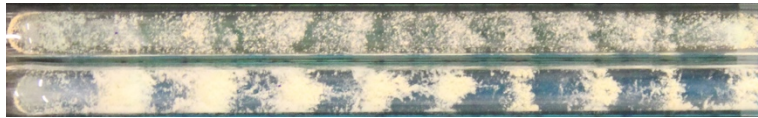
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Pg#3

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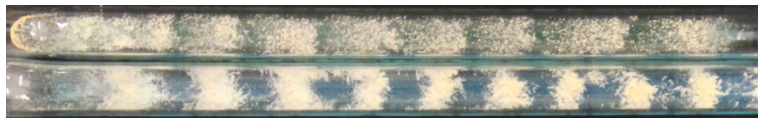
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Pg#4

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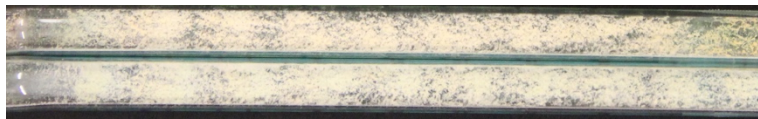
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Pg#5

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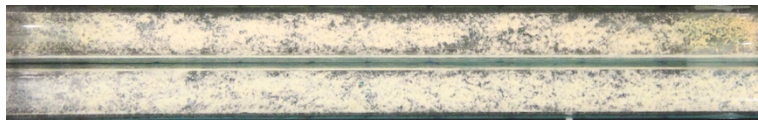
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Pg#6

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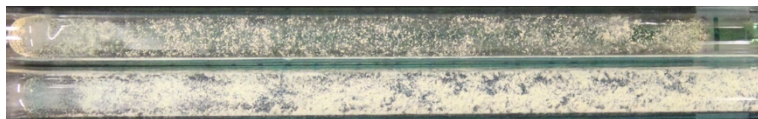
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Pg#7

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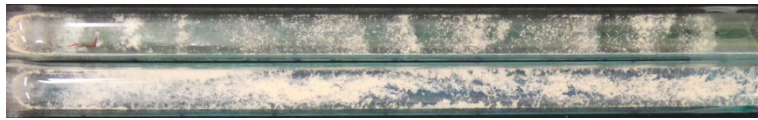
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Pg#8

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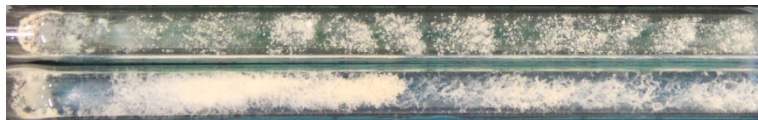
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Pg#9

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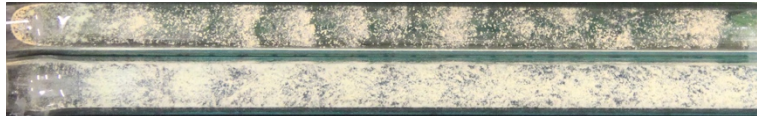
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Pg#10

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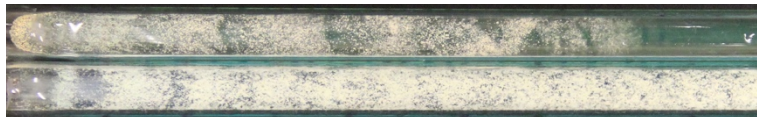
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Pg#11

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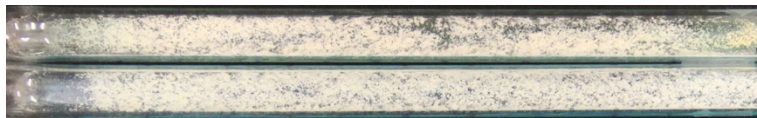
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Pg#12

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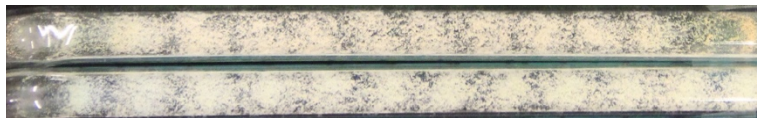
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Pg#13

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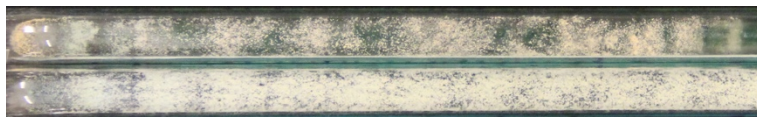
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Pg#14

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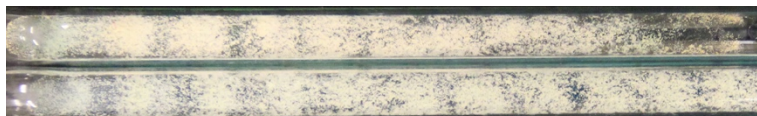
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Pg#15

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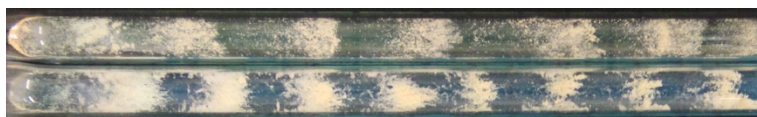
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Pg#16

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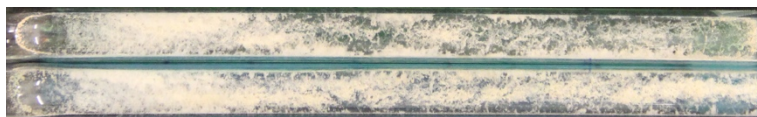
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Pg#17

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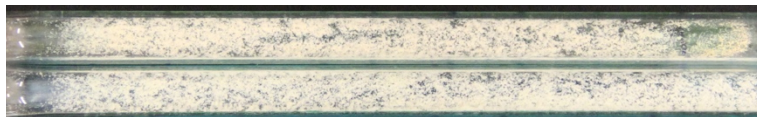
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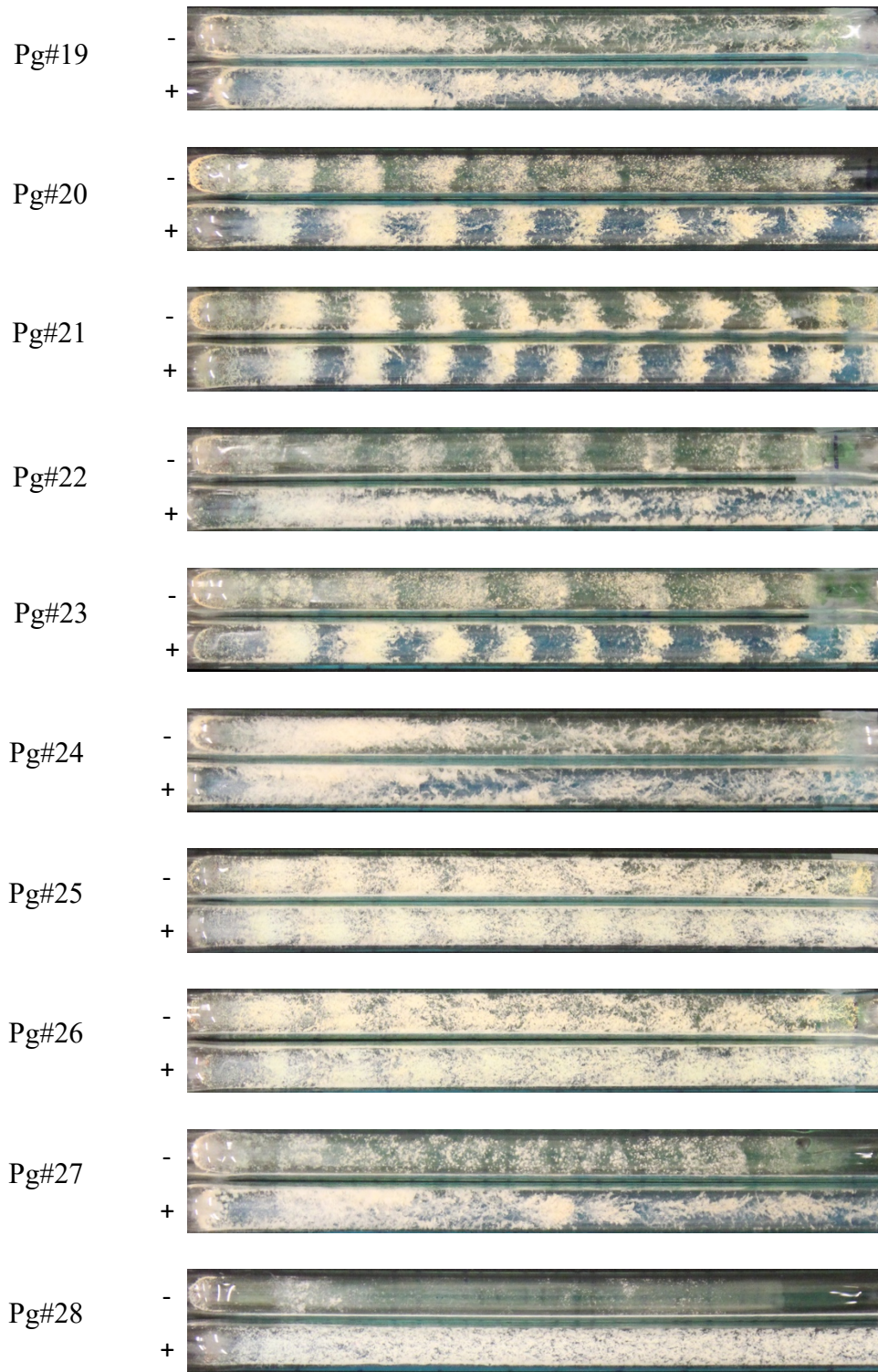


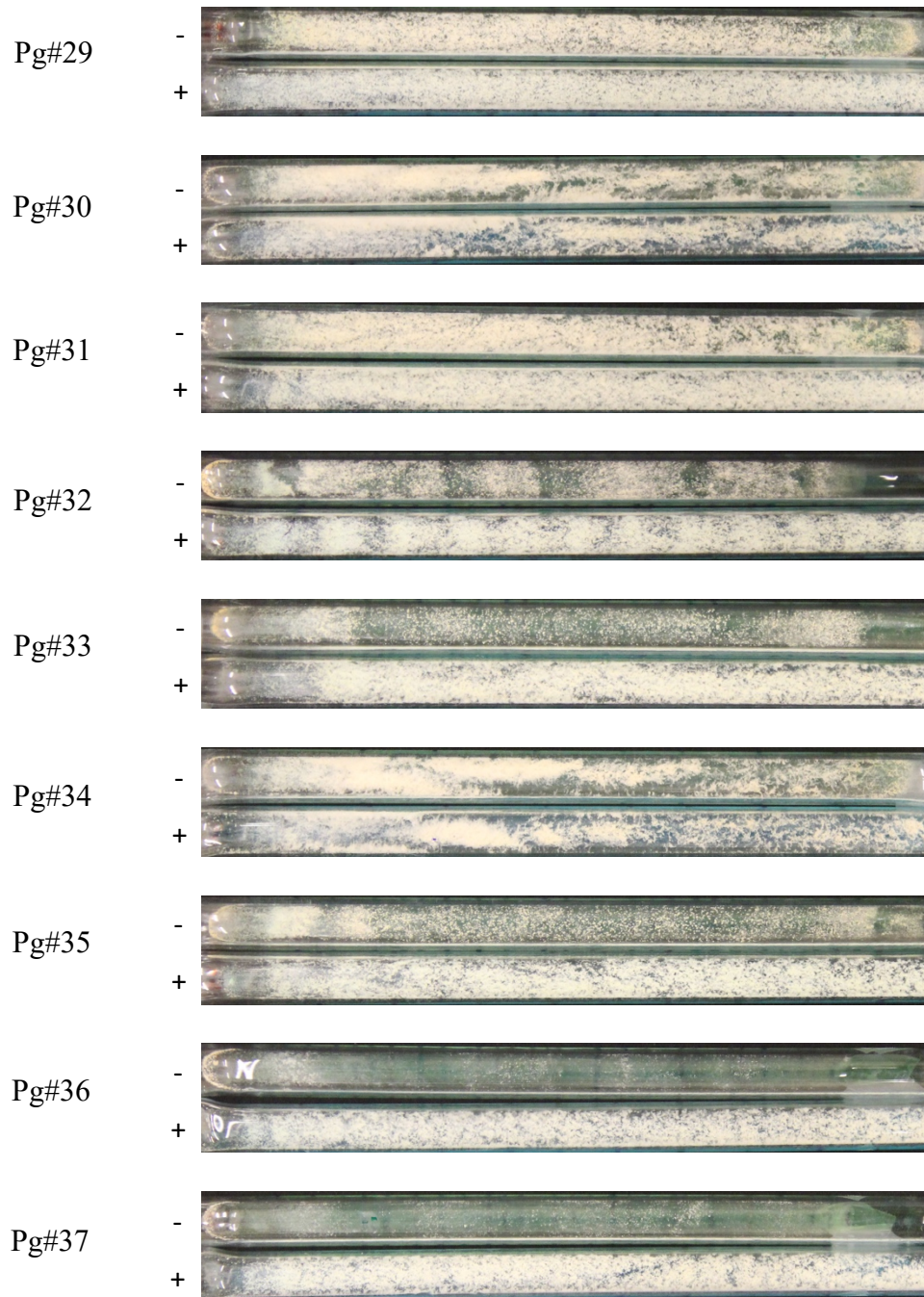
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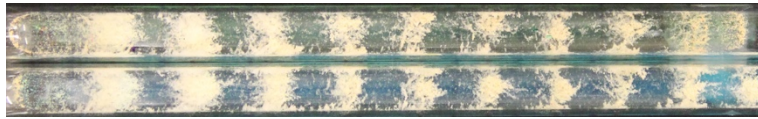




Pg#38

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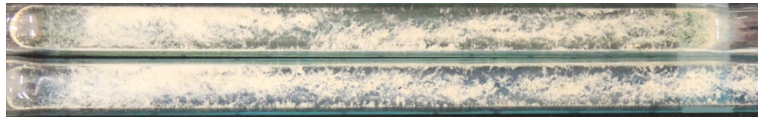
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Pg#39

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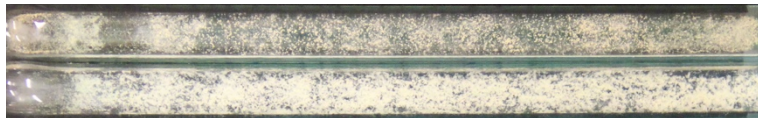
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Pg#40

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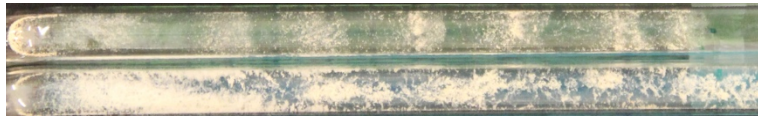
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Pg#41

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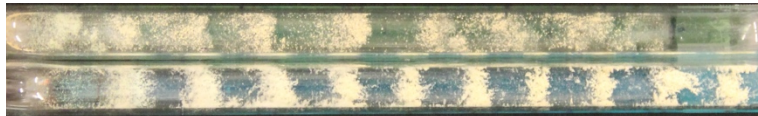
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Pg#42

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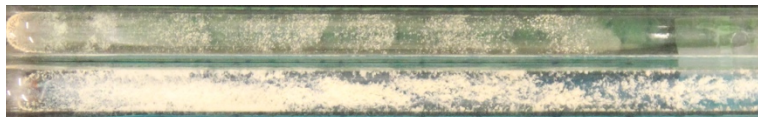
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Pg#43

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Pg#44

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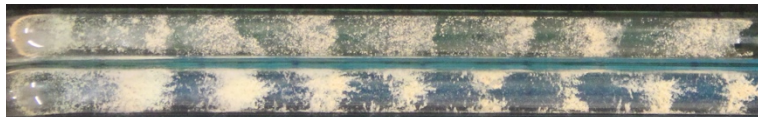
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Pg#45

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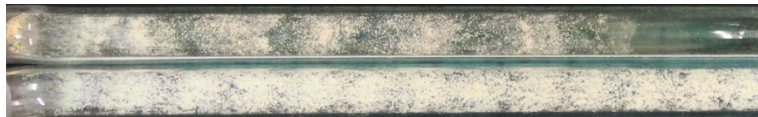
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Pg#46

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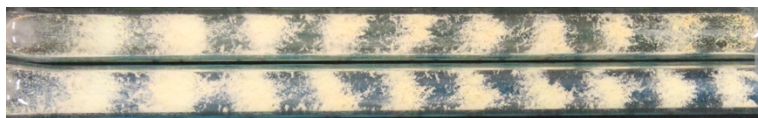
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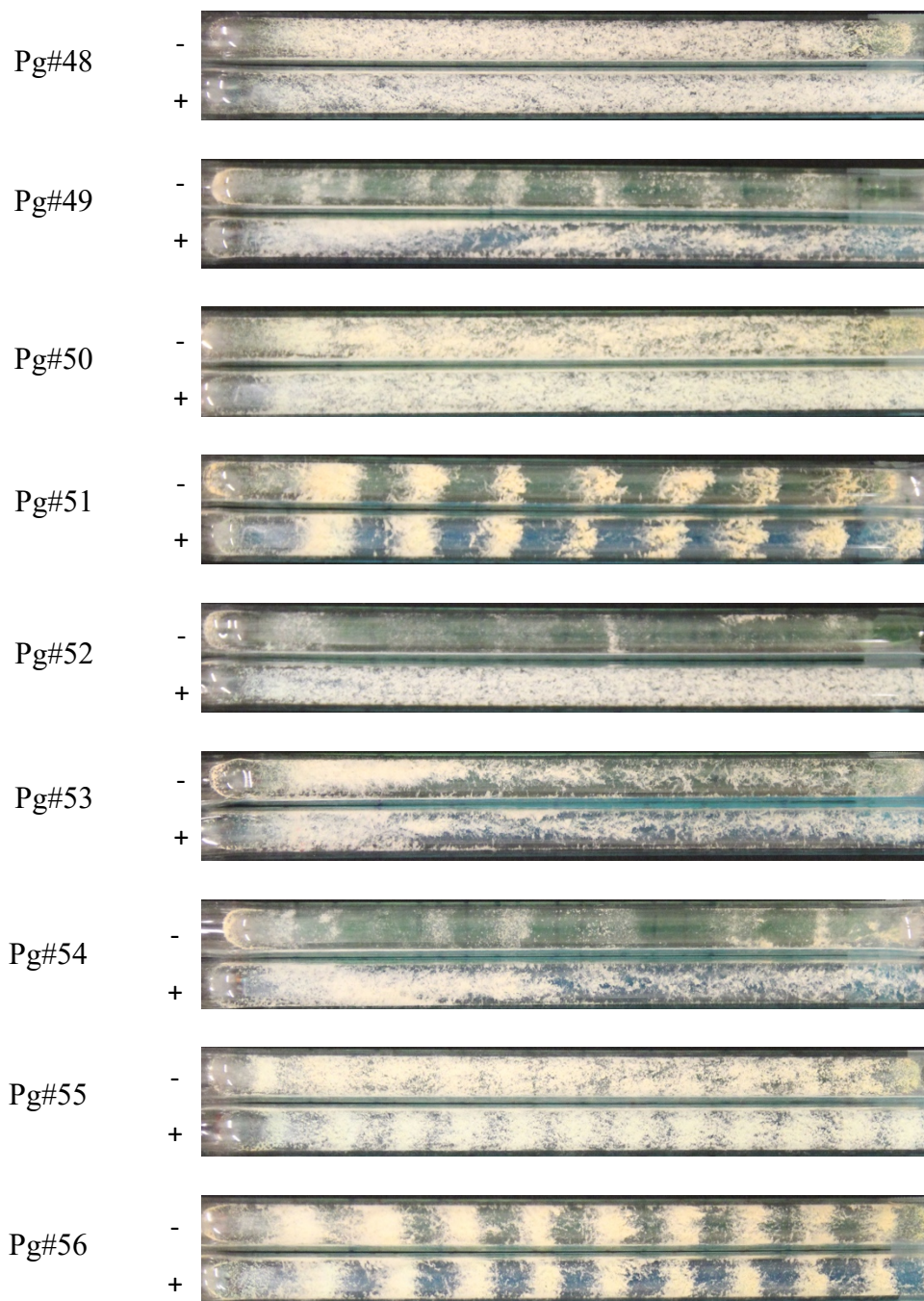


Pg#47

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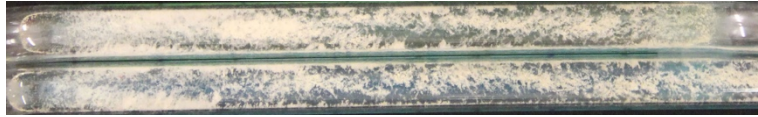
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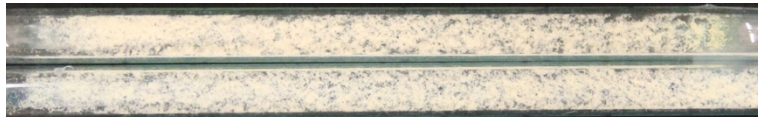
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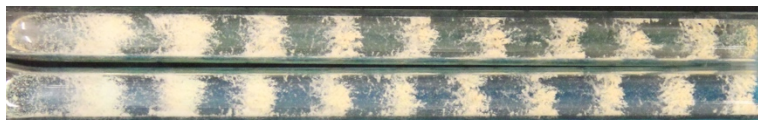
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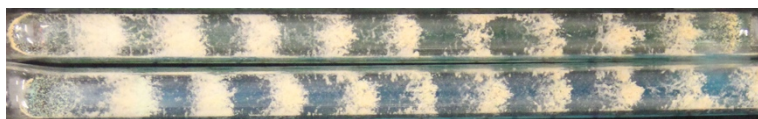
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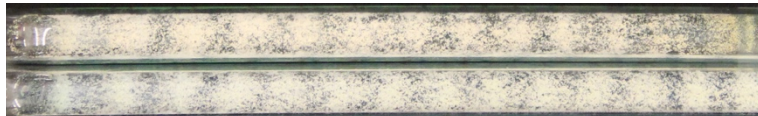
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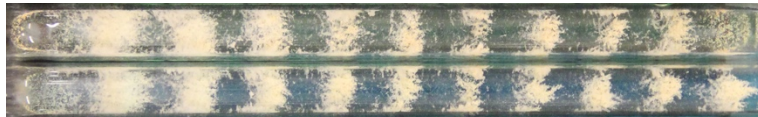
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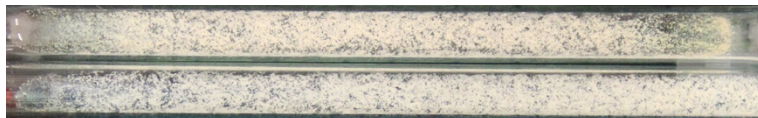
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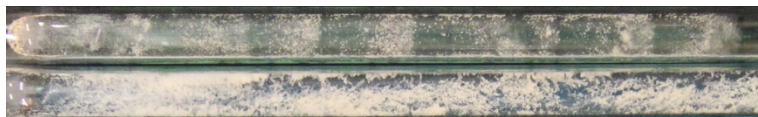
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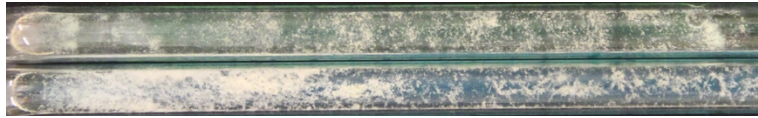
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Pg#65

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Pg#66

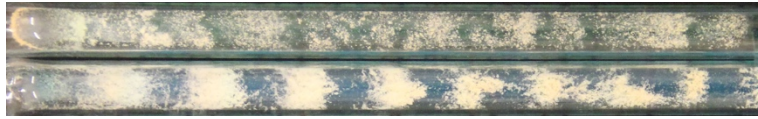
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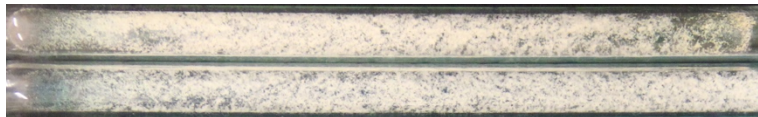
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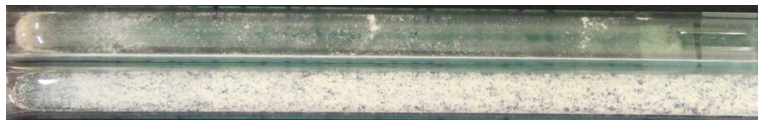
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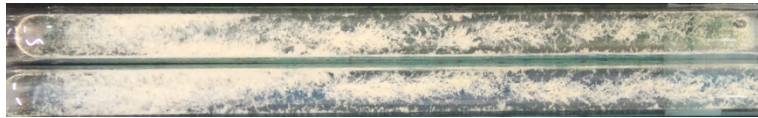
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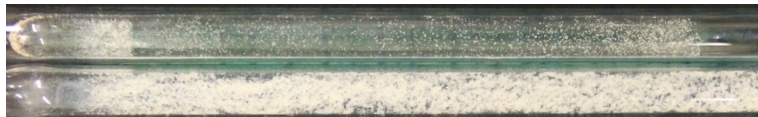
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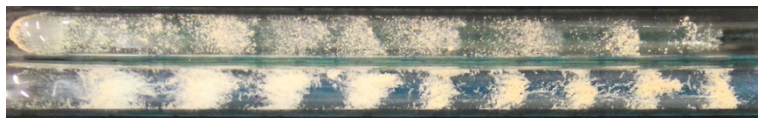
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Pg#72

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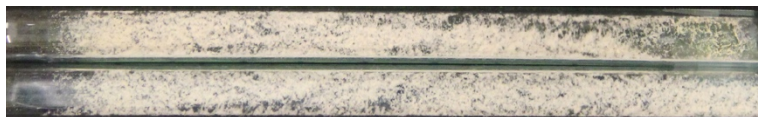
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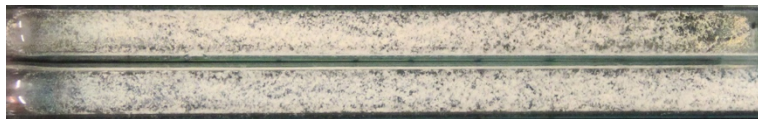
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Pg#74

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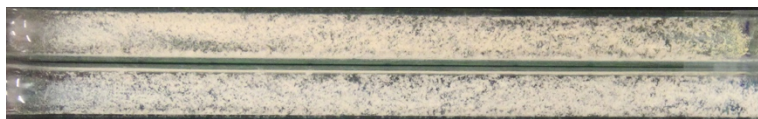
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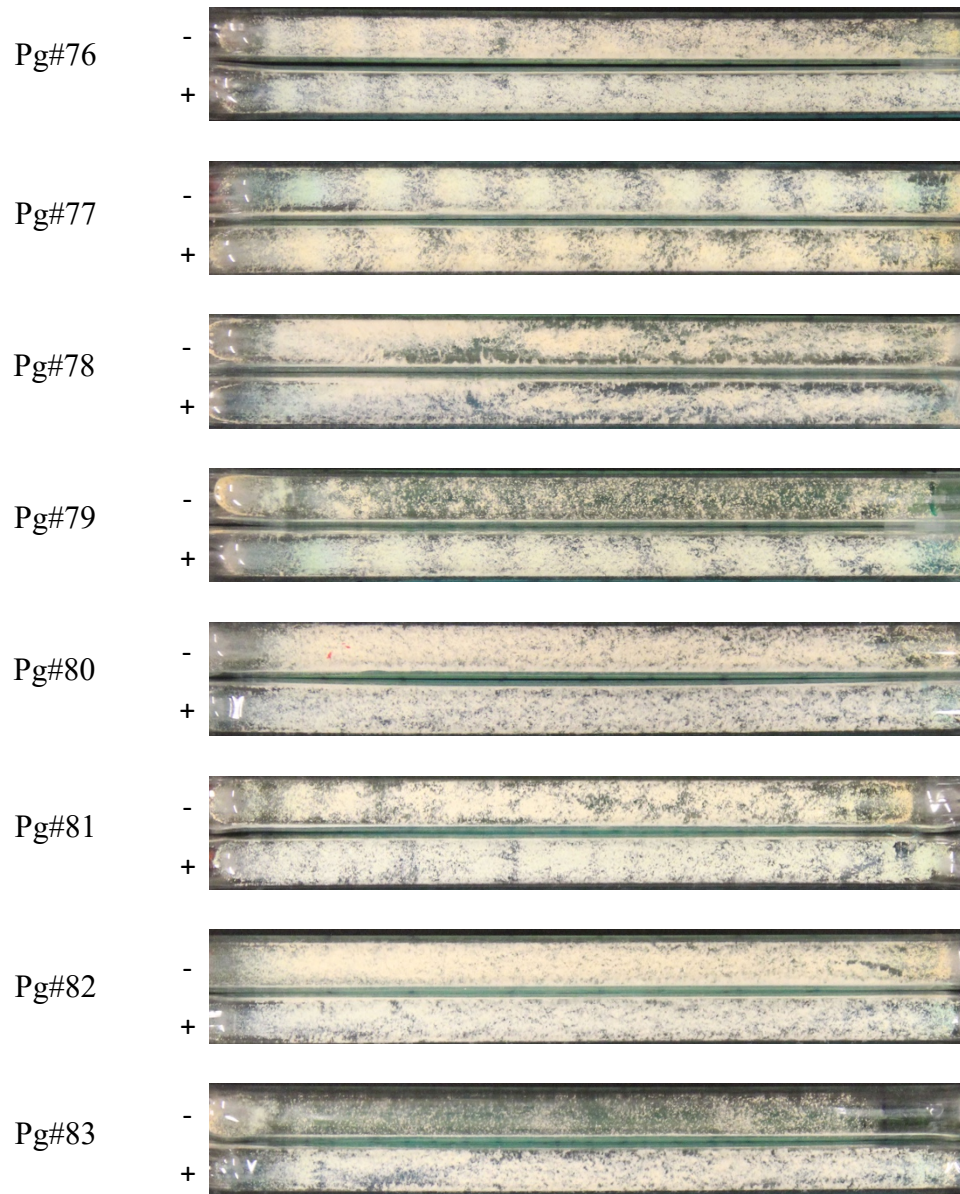


Pg#75

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D.

Summary of *csp-1* progeny obtained from back cross between SG#180 (*ras-1^{bd}* mat a) and pg#20 (*csp-1*; *gtr2^{KO}*; *chol-1 ras-1^{bd}*; *frq¹⁰* mat A) strains.

Strain No.	Without Choline		With 100 μ M Choline		GTR2	Ras-1 ^{bd}	Chol	Frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
SG#80 (control)	0.56	52.68	1.32	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
SG#213 (control)	0.65	-	1.32	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
SG#26 (control)	0.46	60.48	1.33	20.97	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
SG#365 (control)	0.56	-	1.20	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
SG#300 (control)	0.50	-	1.19	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
SG#180 (control)	1.44	21.87	1.31	-	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
Pg#20 (control)	0.33	-	0.98	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
1	0.97	-	0.81	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
2	0.34	-	1.04	-	<i>Gtr2^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
3	0.44	48.78	1.24	20.40	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
4	0.39	64.11	1.20	18.89	<i>Gtr2⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>

5	1.09	-	1.08	22.10	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
6	0.99	-	1.00	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
7	0.45	-	1.12	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
8	0.46	-	1.32	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
9	0.43	-	1.42	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
10	0.41	45.15	1.18	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
11	0.31	98.14	0.93	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
12	0.96	-	0.90	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
13	0.95	-	0.98	21.05	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
14	0.35	77.48	0.94	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
15	0.97	-	0.96	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
16	0.44	80.58	1.31	20.10	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
17	1.26	-	1.28	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
18	0.99	-	0.96	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
19	1.25	-	1.35	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
20	0.48	57.63	1.31	20.00	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>

21	1.27	20.10	1.27	19.75	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ⁺
22	0.46	66.32	1.23	-	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ¹⁰
23	0.36	88.21	1.27	20.31	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ⁺
24	1.42	-	1.43	-	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
25	1.18	21.57	1.17	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ⁺
26	1.18	21.77	1.18	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ⁺
27	0.32	-	1.38	-	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ¹⁰
28	0.31	-	0.97	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ⁺
29	0.91	-	0.93	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
30	1.20	-	1.26	-	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
31	1.16	-	1.19	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
32	0.32	-	0.95	20.71	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ⁺
33	0.37	-	0.88	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ⁺
34	1.17	-	1.16	-	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
35	0.36	-	0.91	-	<i>Gtr2</i> ⁺	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ⁺
36	0.35	-	0.99	-	<i>Gtr2</i> ^{KO}	<i>Ras- l^{bd}</i>	<i>Chol- l</i>	<i>frq</i> ¹⁰

37	0.35	-	0.99	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
38	1.34	19.62	1.28	19.81	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
39	1.33	-	1.28	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
40	0.39	-	0.97	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
41	0.43	76.91	1.28	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
42	0.39	66.34	1.17	19.99	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
43	0.32	-	1.15	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
44	0.34	-	0.97	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
45	0.41	68.60	1.31	20.10	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
46	0.31	91.18	0.88	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
47	1.21	20.30	1.24	18.98	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
48	1.02	-	1.02	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
49	0.41	-	1.20	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
50	1.13	-	1.10	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
51	1.57	20.63	1.51	20.69	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
52	0.36	-	0.94	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol¹</i>	<i>frq¹⁰</i>

53	1.30	-	1.29	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
54	0.44	-	1.34	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁻ <i>l</i>	<i>frq</i> ¹⁰
55	0.99	20.01	0.92	20.82	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ⁺
56	1.23	19.90	1.14	19.95	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ⁺
57	1.23	-	1.19	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
58	1.23	-	1.21	-	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
59	1.28	19.40	1.24	19.83	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ⁺
60	1.28	19.78	1.24	19.78	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ⁺
61	0.90	20.37	0.86	20.83	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ⁺
62	1.24	19.92	1.20	19.82	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ⁺
63	0.96	-	0.93	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
64	0.40	62.57	1.22	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
65	0.46	-	1.25	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁻ <i>l</i>	<i>frq</i> ¹⁰
66	1.02	-	0.94	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰
67	0.37	50.58	1.21	20.35	<i>Gtr2</i> ⁺	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁻ <i>l</i>	<i>frq</i> ⁺
68	1.06	-	1.02	-	<i>Gtr2</i> ^{KO}	<i>Ras-I</i> ^{bd}	<i>Chol</i> ⁺	<i>frq</i> ¹⁰

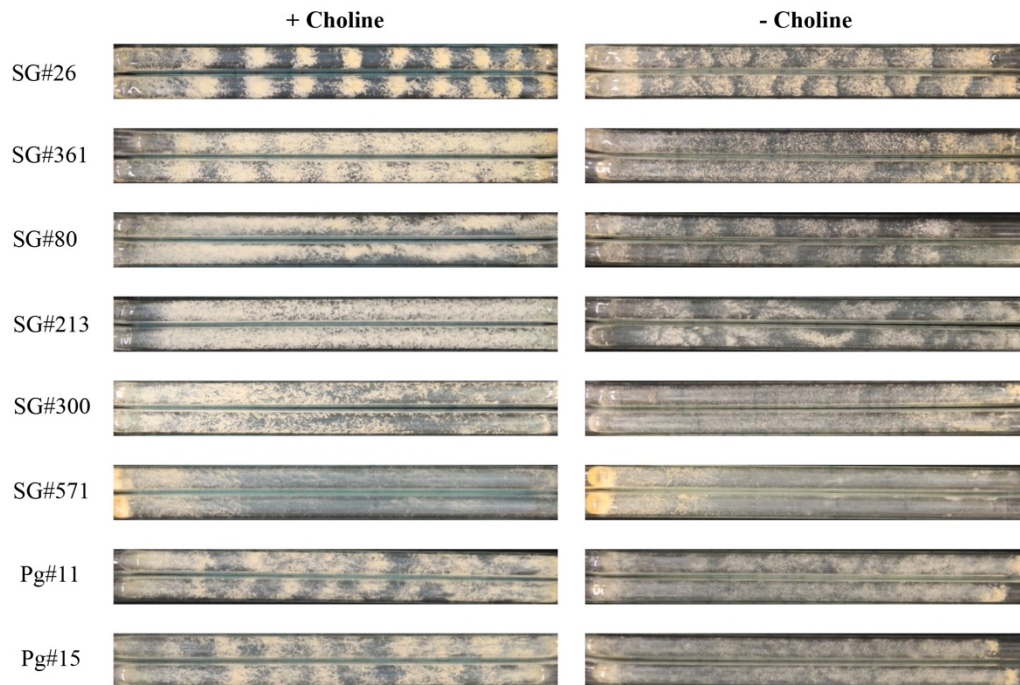
69	0.32	-	0.94	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
70	1.25	-	1.24	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
71	0.38	-	1.01	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>
72	0.37	71.00	1.18	20.33	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
73	0.92	-	0.97	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
74	1.05	-	1.02	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
75	1.10	-	1.09	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
76	0.98	-	0.88	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
77	1.21	21.47	1.12	21.86	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
78	1.23	-	1.19	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
79	0.41	-	1.11	21.45	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq⁺</i>
80	1.15	-	1.17	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
81	0.83	-	0.80	-	<i>Gtr2^{KO}</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
82	1.18	-	1.14	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
83	0.43	-	1.14	-	<i>Gtr2⁺</i>	<i>Ras- I^{bd}</i>	<i>Chol- 1</i>	<i>frq¹⁰</i>

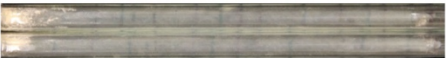
Appendix XXXVII

Race tube analysis of *csp-1* progeny strains obtained from the genetic cross performed between SG#300 and STK-10^{KO} strains.

A.

Conidiation phenotypes of progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and STK-10^{KO} (FGSC#17939 = SG#571) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Two representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Periods and growth rates are listed in Table 9.9 & Appendix XXXVII - B. (Pg#A, Pg#B, Pg#C & Pg#D are from a different batch. Picked dates are different.)



Pg#23		
Pg#34		
Pg#41		
Pg#44		
Pg#46		
Pg#48		
Pg#A		
Pg#B		
Pg#C		
Pg#D		

B.

Summary of *csp-1* progeny obtained from cross between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and Stk-10^{KO} strains.

Strain Number	Without Choline		With Choline		Stk-10	Ras-1 ^{bd}	Chol-1	Frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
11	0.62	-	1.12	22.39	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.65	-	1.15	21.89				
15	0.58	-	1.16	21.85	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.59	-	1.18	21.74				
23	0.55	-	1.18	22.87	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.62	-	1.12	21.28				
34	0.6	-	1.14	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.61	-	1.15	-				
41	0.62	-	1.17	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.53	-	1.17	-				
44	0.62	-	1.13	21.76	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.65	-	1.11	21.85				
46	0.52	-	1.16	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.55	-	1.18	-				
48	0.51	-	1.22	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.67	-	1.18	-				
SG#26 (control)	0.6	37.85	1.33	21.02	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.55	47.63	1.34	20.94				
SG#80 (control)	0.47	61.68	1.34	-	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.51	57.68	1.37	-				
SG#213 (control)	0.57	78.57	1.31	-	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.59	71.05	1.31	-				

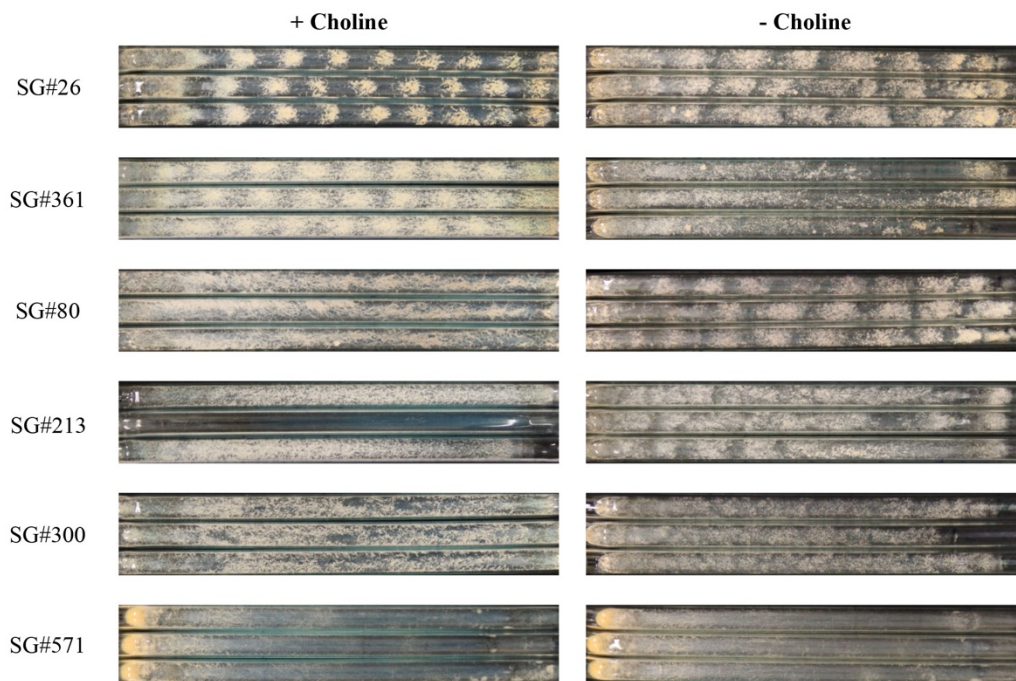
SG#300 (control)	0.5	-	1.3	-	<i>Stk-10</i> ⁺	<i>Ras-1</i> ^{bd}	<i>Chol-1</i>	<i>frq</i> ^{l0}
	0.5	-	1.31	-				
SG#361 (control)	0.51	-	1.25	21.99	<i>Stk-10</i> ⁺	<i>Ras-1</i> ^{bd}	<i>Chol-1</i>	<i>frq</i> ⁺
	0.47	-	1.24	23.01				
SG#552 (control)	1.85	-	2.12	-	<i>Stk-10</i> ^{KO}	<i>Ras-1</i> ^{bd+}	<i>Chol</i> ⁺	<i>frq</i> ⁺
	1.87	-	2.15	-				

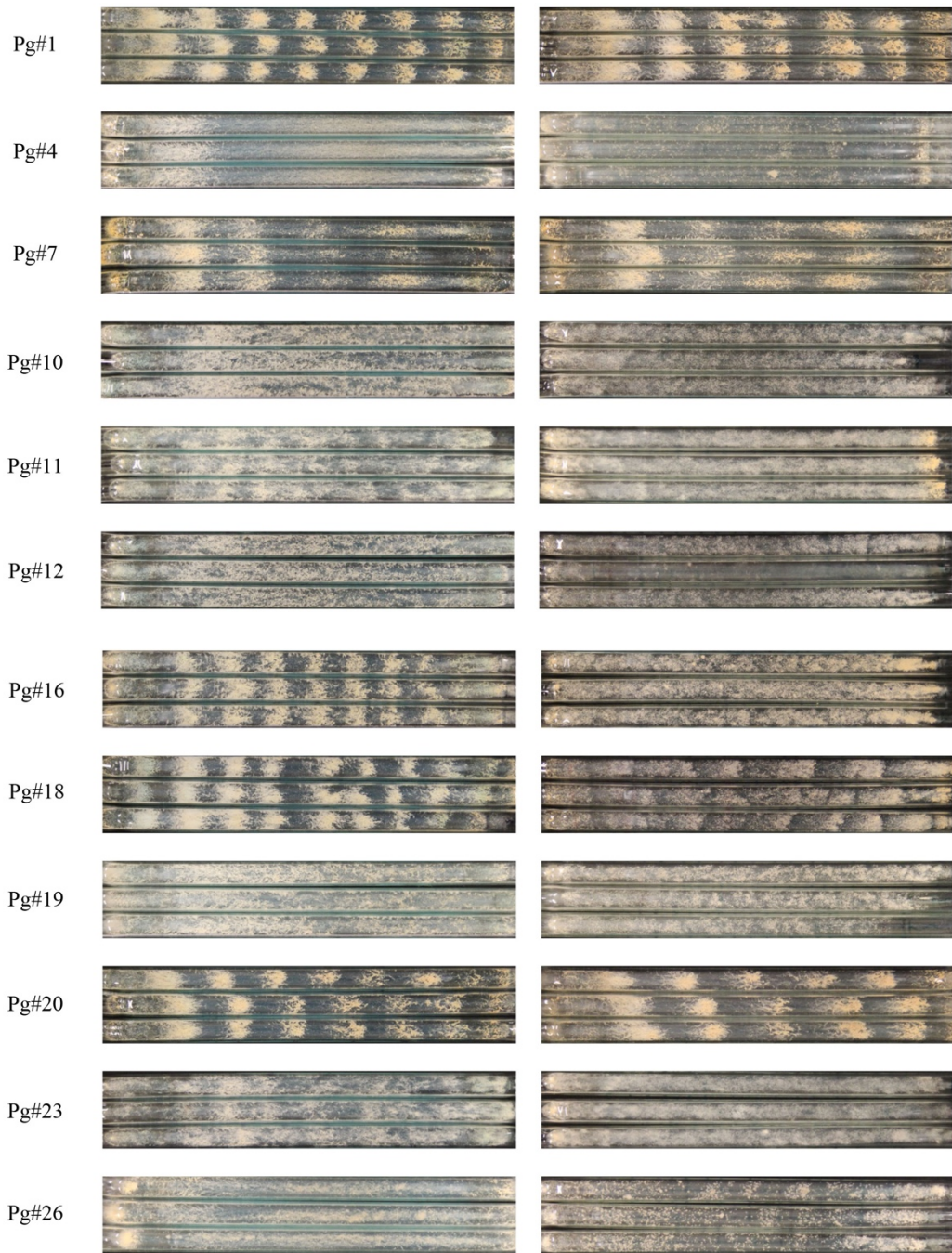
C.

Conidiation phenotypes of progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq^{l0} mat A*) and STK-10^{KO} (FGSC#17939 = SG#571) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Three representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Periods and growth rates are listed in Table 9.10 & Appendix XXXVII - D.

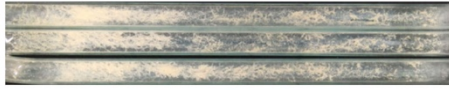
Progeny strains with *csp-1, frq^{l0}, stk-10⁺* genetic background - pg#4, pg#10, pg#12, pg#19, pg#26, pg#29, pg#33, pg#36, pg#40 & pg#47)

Progeny strains with *csp-1, frq⁺, stk-10⁺* genetic background - (pg#1, pg#7, pg#16, pg#18, pg#20, pg#28, pg#37, pg#39, pg#42 & pg#45)





Pg#47



Pg#48



D.

Summary of *csp-1* progeny obtained from cross between SG#300 (*csp-1: chol-1 ras-1^{bd}, frq^{l0} mat A*) and Stk-10^{KO} strains.

Strain No.	Without Choline		With 100 μ M Choline		Stk-10	Ras-1 ^{bd}	chol	frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
SG#26 (control)	0.44	57.32	1.25	21.04	Stk-10 ⁺	Ras-1 ^{bd}	chol-1	frq ⁺
	0.47	63.82	1.27	21.21				
	0.48	61.43	1.25	21.34				
SG#361 (control)	0.38	-	1.18	21.36	Stk-10 ⁺	Ras-1 ^{bd}	chol-1	frq ⁺
	0.45	-	1.18	21.21				
	0.32	-	1.18	21.37				
SG#300 (control)	0.47	-	1.11	-	Stk-10 ⁺	Ras-1 ^{bd}	chol-1	frq ^{l0}
	0.42	-	1.11	-				
	0.45	-	1.09	-				
SG#80 (control)	0.43	71.18	1.28	-	Stk-10 ⁺	Ras-1 ^{bd}	chol-1	frq ^{l0}
	0.44	71.07	1.30	-				
	0.42	64.81	1.29	-				
SG#213 (control)	0.59	-	1.25	-	Stk-10 ⁺	Ras-1 ^{bd}	chol-1	frq ^{l0}
	0.49	-	1.24	-				
	0.54	-	1.14	-				
SG#571 (control)			2.12	-	Stk-10 ⁺	Ras-1 ^{bd+}	chol+	frq ⁺
			1.99	-				
			2.13	-				
11	0.55	-	1.04	21.89	Stk-10 ^{KO}	Ras-1 ^{bd}	chol-1	frq ⁺
	0.53	-	1.12	22.39				
	0.53	-	1.12	22.28				

23	0.52	-	1.21	22.97	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.51	-	1.24	22.28				
	0.51	-	1.26	23.77				
34	0.49	-	1.11	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq¹⁰</i>
	0.52	-	1.10	-				
	0.55	-	1.12	-				
44	0.48	-	0.97	21.85	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.51	-	0.95	22.32				
	0.49	-	0.98	22.68				
46	0.43	-	1.09	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq¹⁰</i>
	0.44	-	1.14	-				
	0.44	-	1.15	-				
48	0.45	-	1.03	-	<i>Stk-10^{KO}</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq¹⁰</i>
	0.46	-	1.06	-				
	0.48	-	1.11	-				
1	1.72	20.46	1.38	21.84	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol+</i>	<i>frq⁺</i>
	1.78	19.54	1.35	21.89				
	1.81	19.10	1.40	21.84				
7	2.47	18.28	2.67	16.95	<i>Stk-10⁺</i>	<i>Ras-1^{bd+}</i>	<i>chol+</i>	<i>frq⁺</i>
	2.47	18.10	2.52	18.79				
	2.55	17.78	2.63	17.40				
16	0.48	-	1.21	21.61	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.49	-	1.20	21.10				
	0.40	-	1.23	21.15				
18	0.44	55.42	1.31	19.48	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq⁺</i>
	0.45	43.03	1.37	19.37				
	0.46	62.92	1.43	19.27				
20	2.12	19.85	1.62	21.01	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol+</i>	<i>frq⁺</i>
	2.19	19.76	1.67	20.49				
	2.09	19.99	1.62	20.40				
28	2.52	17.63	2.53	16.71			<i>chol+</i>	<i>frq⁺</i>

	2.55	17.35	2.59	17.27	<i>Stk-</i>	<i>Ras-</i>		
	2.58	17.48	2.49	17.07	<i>10⁺</i>	<i>1^{bd+}</i>		
37	0.52	-	1.30	20.70	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>chol-</i> <i>1</i>	<i>frq⁺</i>
	0.53	-	1.31	20.73				
	0.54	-	1.33	20.72				
39	2.90	-	2.97	40.28	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>chol+</i>	<i>frq⁺</i>
	2.92	-	3.06	41.86				
	2.90	-	3.27	39.55				
42	0.43	-	1.12	20.90	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>chol-</i> <i>1</i>	<i>frq⁺</i>
	0.44	-	1.11	20.90				
	0.43	-	1.12	20.74				
45	2.59	18.69	2.74	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>chol+</i>	<i>frq⁺</i>
	2.26	18.29	2.73	-				
	2.48	18.65	2.81	-				
4	0.67	-	2.47	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>chol-</i> <i>1</i>	<i>frq¹⁰</i>
	0.59	-	2.61	-				
	0.62	-	2.64	-				
10	0.52	-	1.16	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>chol-</i> <i>1</i>	<i>frq¹⁰</i>
	0.47	-	1.11	-				
	0.47	-	1.21	-				
12	0.45	-	1.11	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>chol-</i> <i>1</i>	<i>frq¹⁰</i>
	0.49	-	1.12	-				
	0.44	-	1.12	-				
19	0.42	-	1.36	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>chol-</i> <i>1</i>	<i>frq¹⁰</i>
	0.39	-	1.36	-				
	0.47	-	1.42	-				
26	0.52	-	2.93	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>chol-</i> <i>1</i>	<i>frq¹⁰</i>
	0.51	-	2.82	-				
	0.52	-	2.97	-				
29	2.44	-	2.61	-	<i>Stk-</i> <i>10⁺</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>chol⁺</i>	<i>frq¹⁰</i>
	2.42	-	2.73	-				

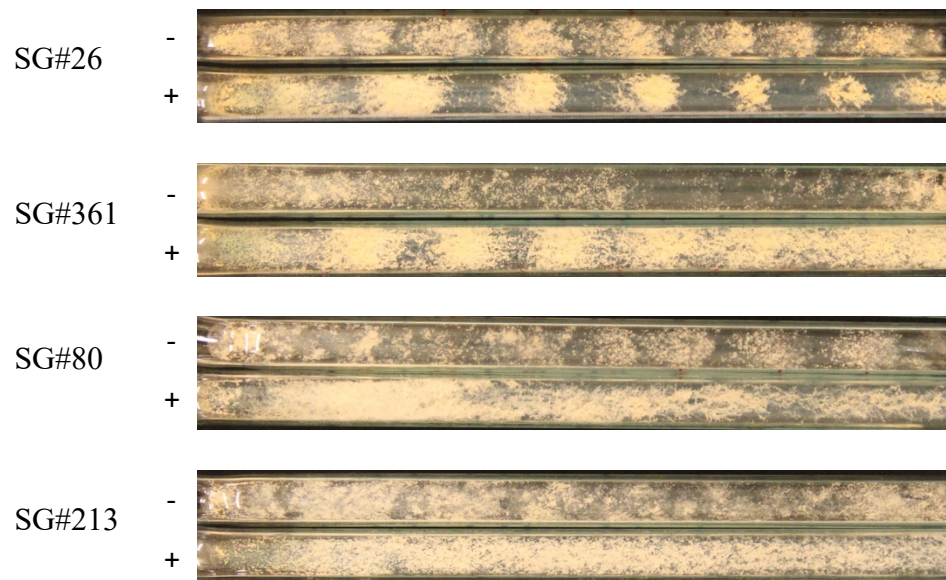
	2.33	-	2.70	-				
33	1.42	-	1.33	-	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol⁺</i>	<i>frq^{l0}</i>
	1.41	-	1.33	-				
	1.40	-	1.33	-				
36	2.55	-	2.56	-	<i>Stk-10⁺</i>	<i>Ras-1^{bd+}</i>	<i>chol⁺</i>	<i>frq^{l0}</i>
	2.58	-	2.57	-				
	2.52	-	2.49	-				
40	0.37	71.02	1.36	-	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.32	100.65	1.37	-				
	0.37	82.85	1.33	-				
47	0.39	75.58	1.29	-	<i>Stk-10⁺</i>	<i>Ras-1^{bd}</i>	<i>chol-1</i>	<i>frq^{l0}</i>
	0.40	58.45	1.31	-				
	0.41	62.28	1.30	-				

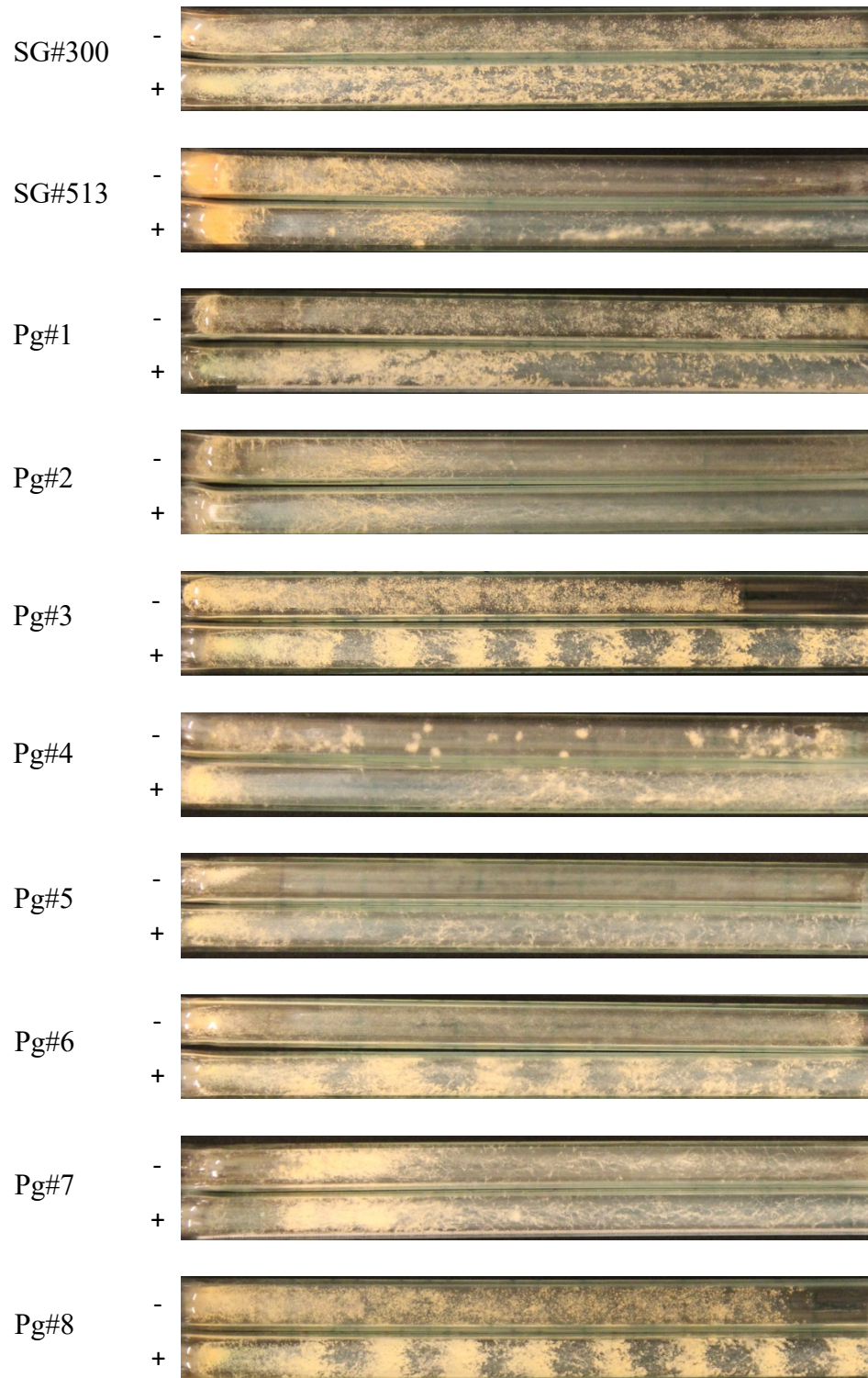
Appendix XXXVIII

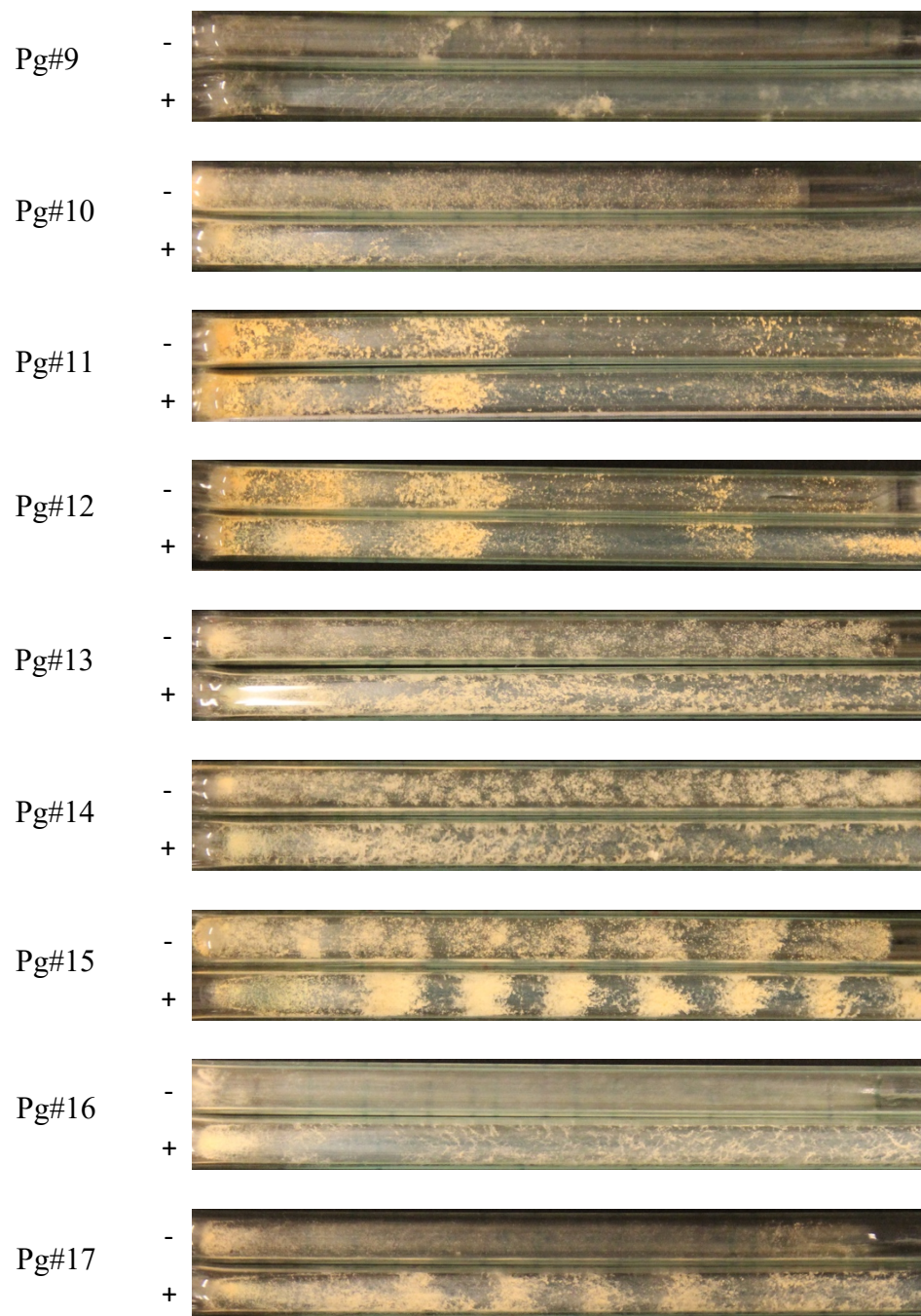
Race tube analysis of *csp-1* progeny strains obtained from genetic cross between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and *Tap42^{KO}* strains.

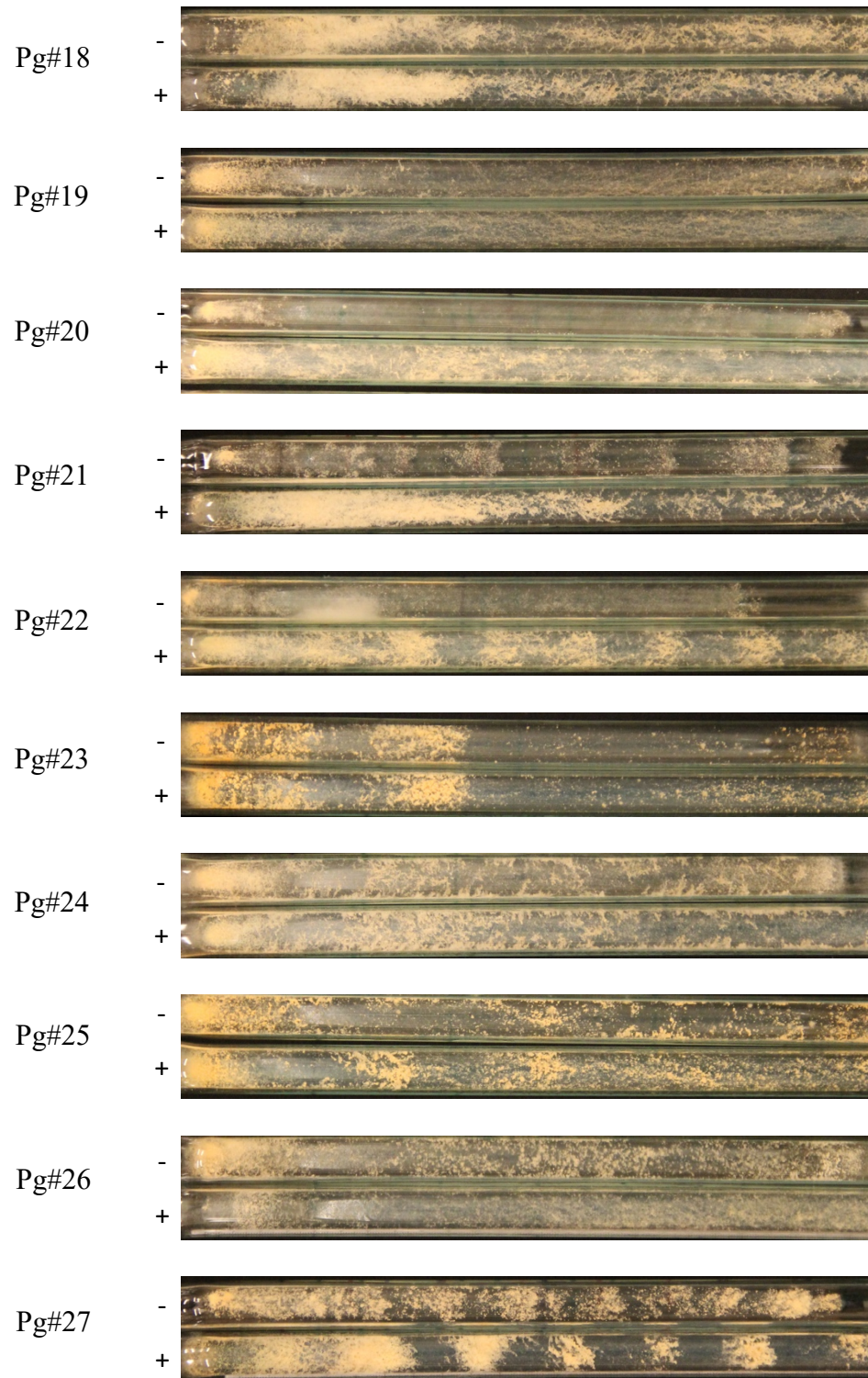
A.

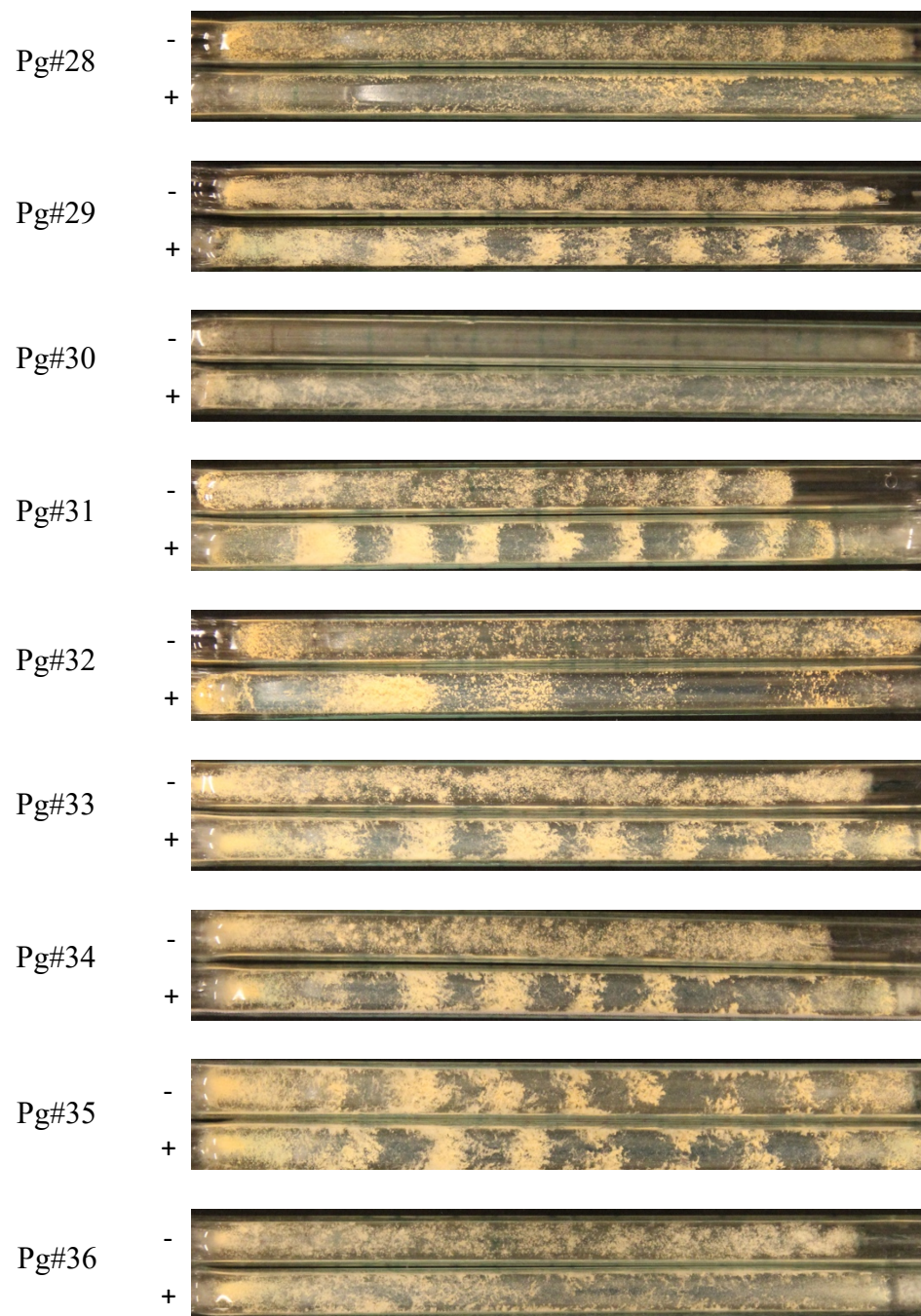
Conidiation phenotypes of progeny strains obtained from the genetic cross performed between SG#300 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat A*) and *Tap42^{KO}* (FGSC#21741) strains. Strains were grown with (+, bottom tube) or without (-, top tube) 100 μ M choline in the medium. One representative replicate tube for each condition and genotype are shown. Growth is from left to right. Periods and growth rates are listed in Appendix XXXVIII – B.

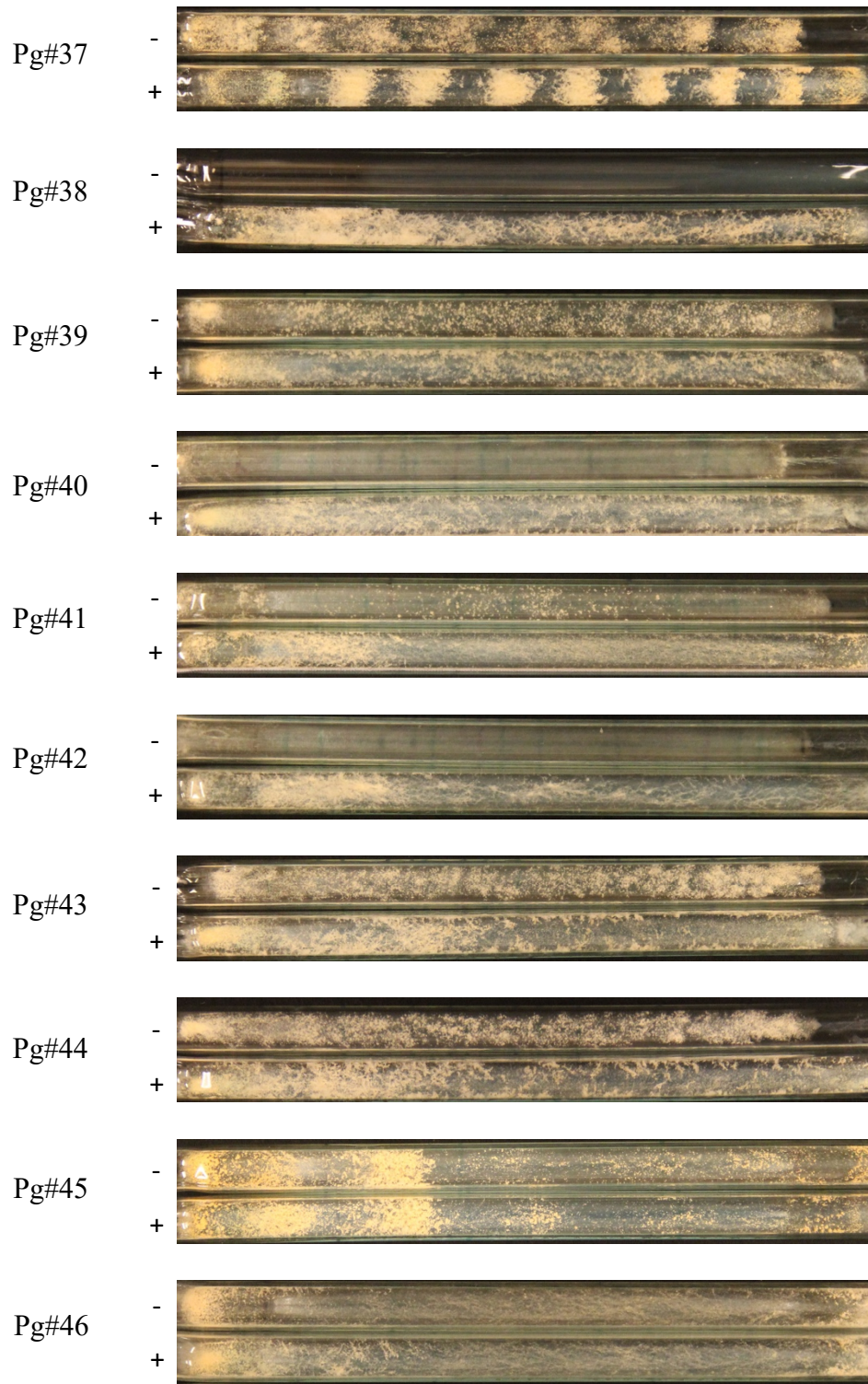


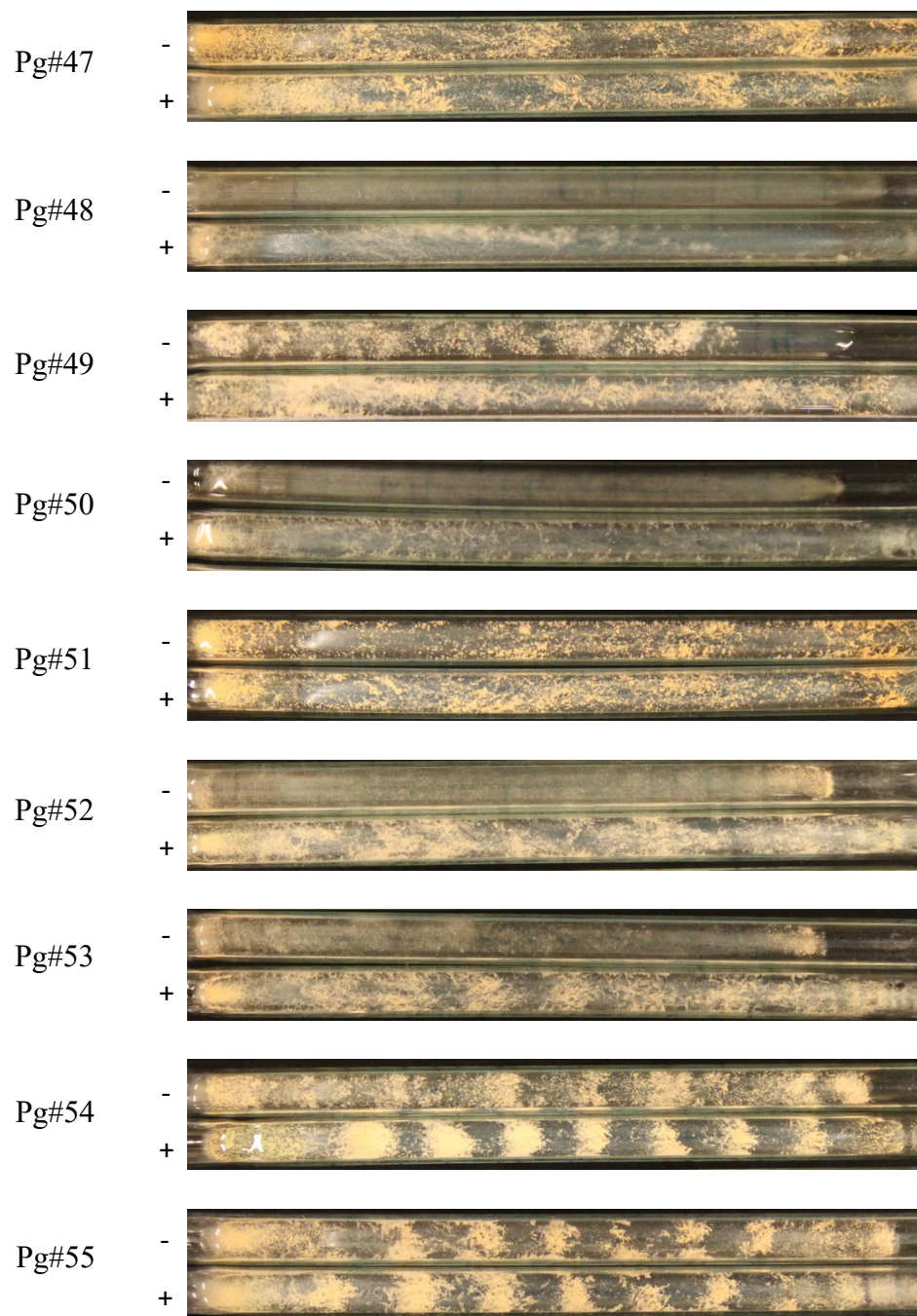


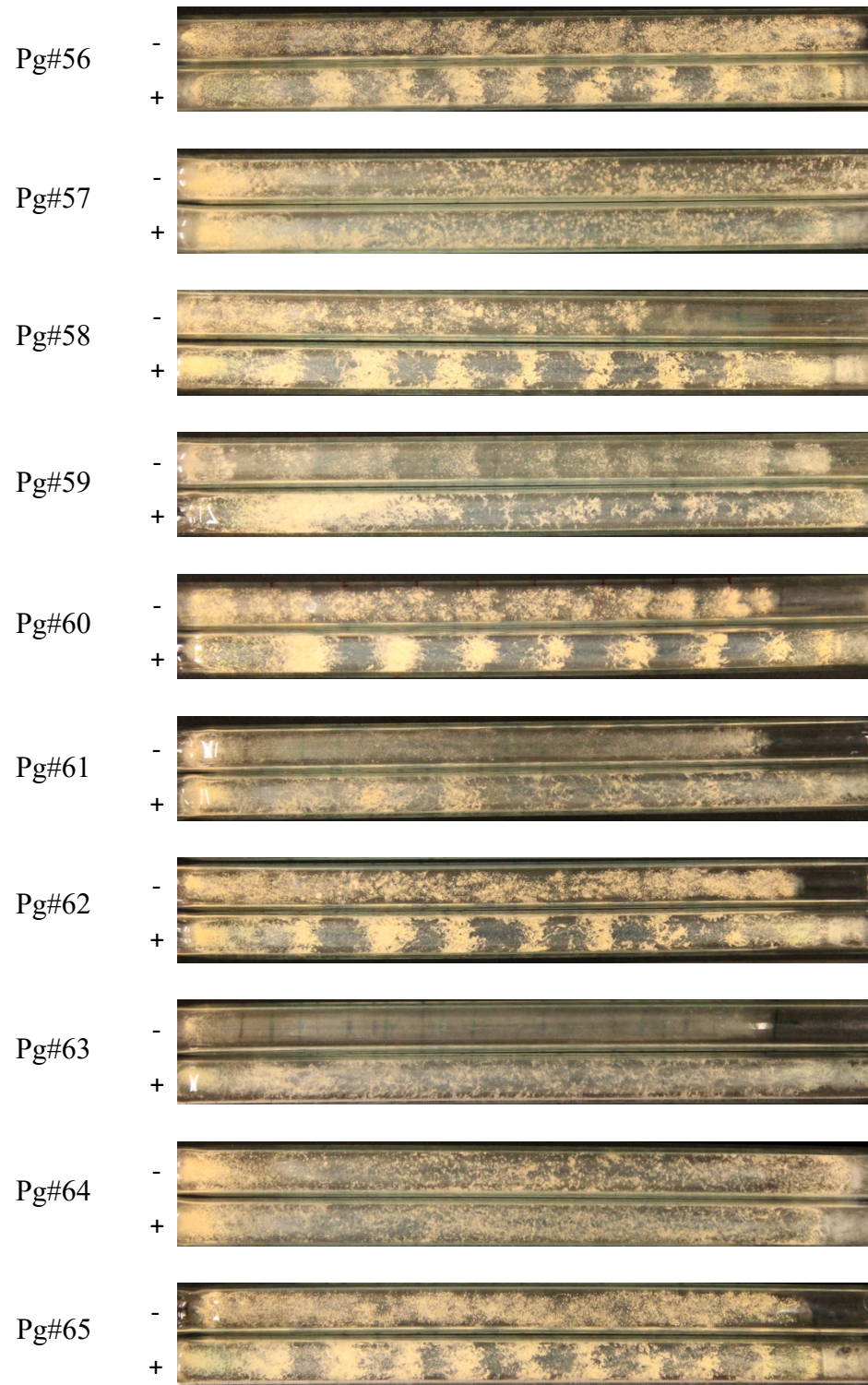


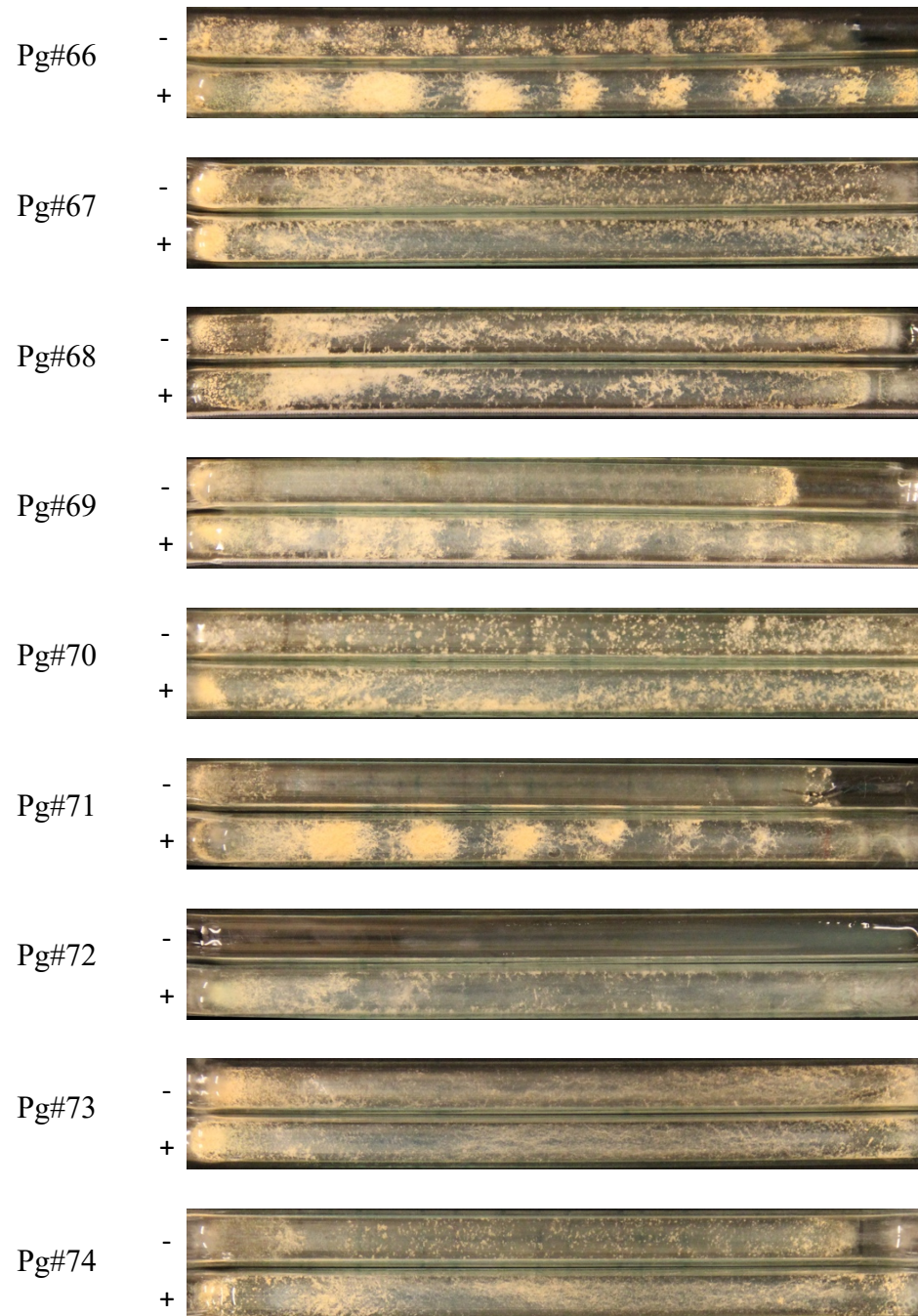


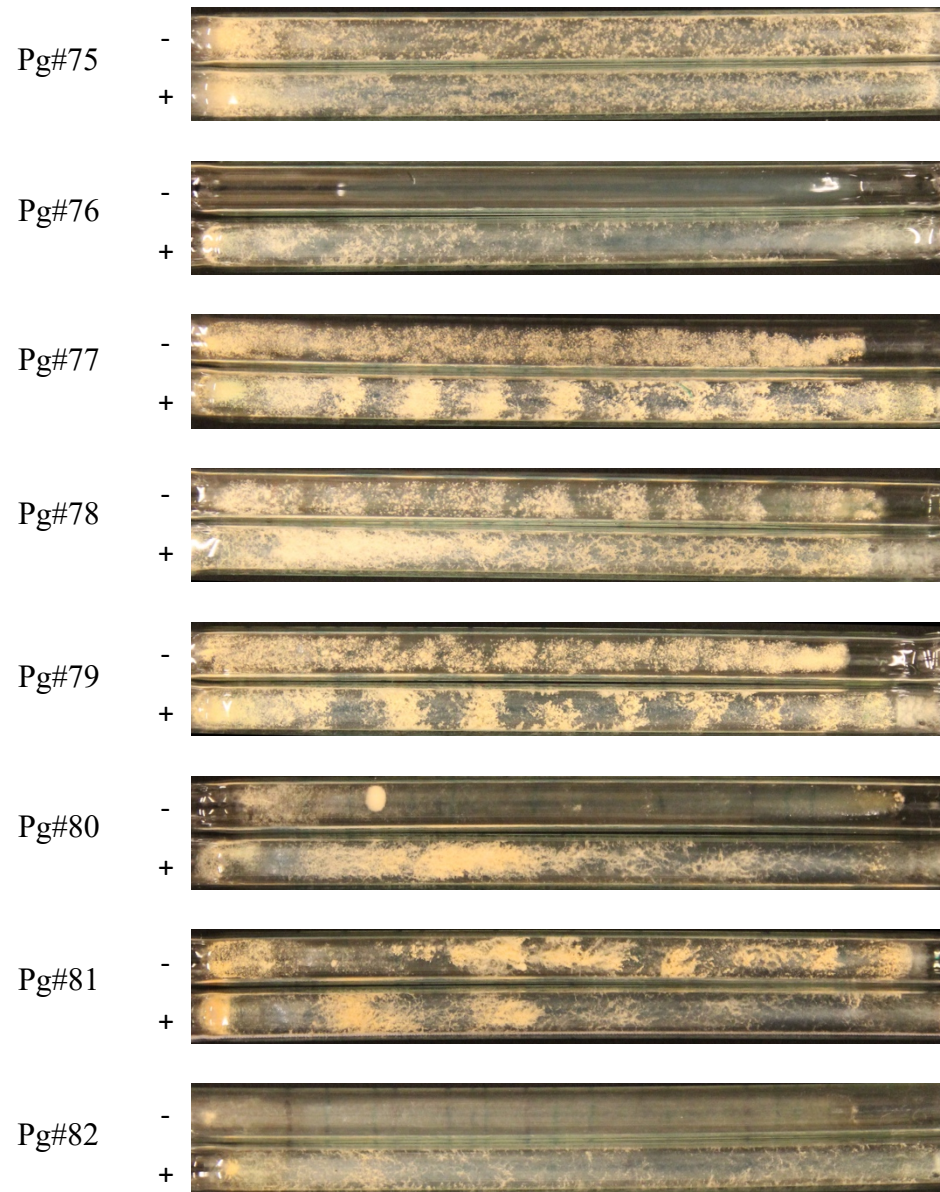












B.

Periods and growth rates of progeny from cross between SG#300 (*csp-1: chol-1 ras-1^{bd}, frq^{l0} mat A*) and Tap42^{KO} strains.

Strain No.	Without Choline		With 100 μ M Choline	
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)
SG#80 (control)	0.44	56.12	1.47	-
SG#213 (control)	0.55	-	1.4	-
SG#26 (control)	0.39	67.87	1.51	20.25
SG#361 (control)	0.41	-	1.37	19.75
SG#300 (control)	0.47	-	1.22	-
SG#513 (control)	2.66	-	3.12	-
1	0.42	-	1.19	-
2	2.41	-	2.9	-
3	0.47	-	1.28	20.5
4	1.08	-	3.3	-
5	0.4	-	1.43	-
6	0.38	-	1.23	21.3
7	1.15	-	1.44	-
8	0.49	-	1.27	20.32
9	0.81	-	2.76	-
10	0.6	-	2.84	-
11	2.56	-	3.19	-
12	1.84	-	3.08	-
13	0.41	-	1.18	-
14	0.46	-	1.35	-
15	0.48	50.33	1.34	19.98
16	0.45	-	1.66	-
17	0.35	-	1.32	20.95
18	1.32	-	1.74	-
19	2.41	-	3.07	-
20	0.42	-	1.37	-
21	0.45	66.07	1.49	-
22	0.44	-	1.43	22.42
23	2.45	-	3.14	-

24	1.16	-	1.35	-
25	2.44	-	2.92	-
26	2.4	-	2.85	-
27	0.51	50.49	1.64	20.11
28	0.57	-	2.47	-
29	0.46	-	1.17	20.42
30	0.42	-	1.34	-
31	0.51	45.58	1.23	20.81
32	0.59	-	2.07	-
33	0.53	-	1.23	21.11
34	0.44	-	1.28	21
35	1.21	21.38	1.24	21.39
36	0.42	-	1.2	-
37	0.48	57.34	1.3	20.2
38	-	-	1.29	-
39	0.65	-	2.72	-
40	0.41	-	1.17	-
41	0.74	-	2.67	-
42	0.42	-	1.36	-
43	0.46	-	1.25	-
44	0.46	-	1.23	-
45	2.55	-	2.64	-
46	2.52	-	2.59	-
47	2.4	-	2.42	-
48	0.8	-	2.67	-
49	0.44	53.98	1.33	-
50	0.44	-	1.29	-
51	2.75	-	2.9	-
52	0.4	-	1.13	-
53	0.42	-	1.27	22.37
54	0.43	74.36	1.21	20.54
55	1.16	21.49	1.22	21.26
56	0.43	-	1.09	21.29
57	2.28	-	2.5	-
58	0.34	-	1.29	20.95
59	0.49	-	1.28	-
60	0.48	46.18	1.34	20.63
61	0.46	-	1.31	24.09
62	0.5	-	1.23	21.29

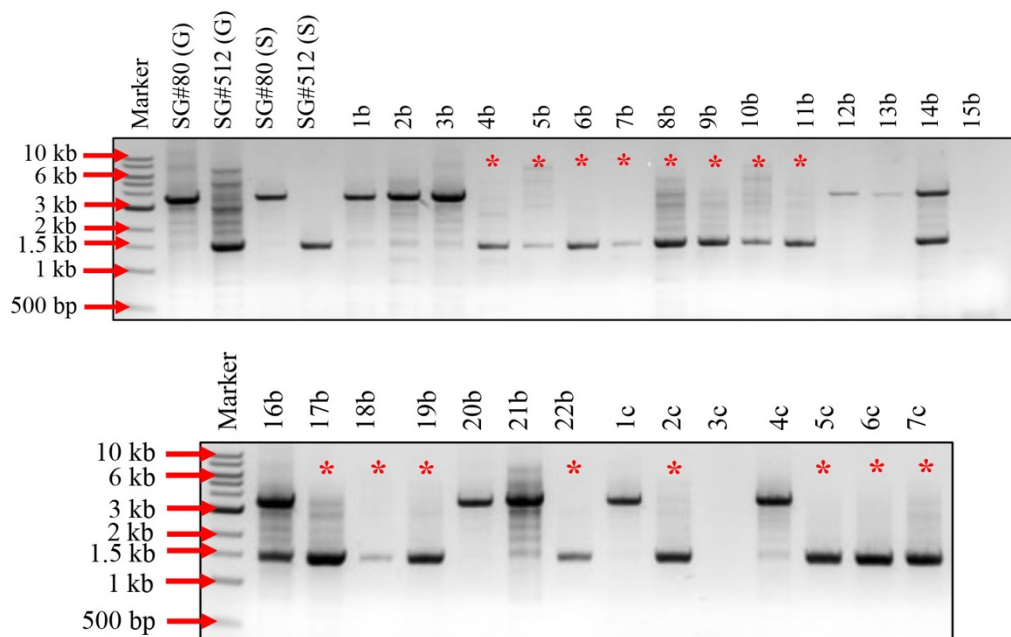
63	0.37	-	1.12	-
64	2.55	-	2.67	-
65	0.43	-	1.05	20.54
66	0.35	-	1.4	20.85
67	2.52	-	2.76	-
68	1.25	-	1.25	-
69	0.41	-	1.12	-
70	0.66	-	2.6	-
71	0.47	-	1.22	22.36
72	-	-	1.17	-
73	2.24	-	2.63	-
74	0.6	-	2.72	-
75	2.48	-	2.64	-
76	-	-	1.16	-
77	0.45	-	1.1	20.57
78	0.43	54.64	1.46	-
79	0.49	-	1.25	21.31
80	0.45	-	1.37	-
81	0.98	-	1.78	-
82	0.41	-	1.21	-

Appendix XXXIX

Analysis of *csp-1* progeny strains obtained from genetic cross between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and *PIK1a^{KO}* strains

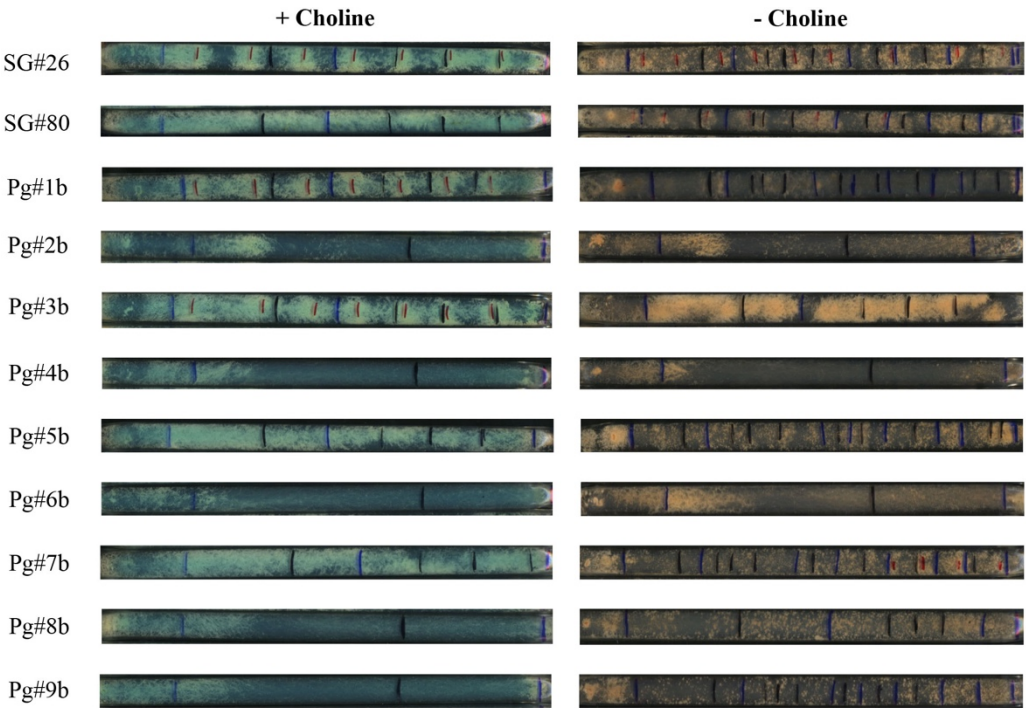
A.

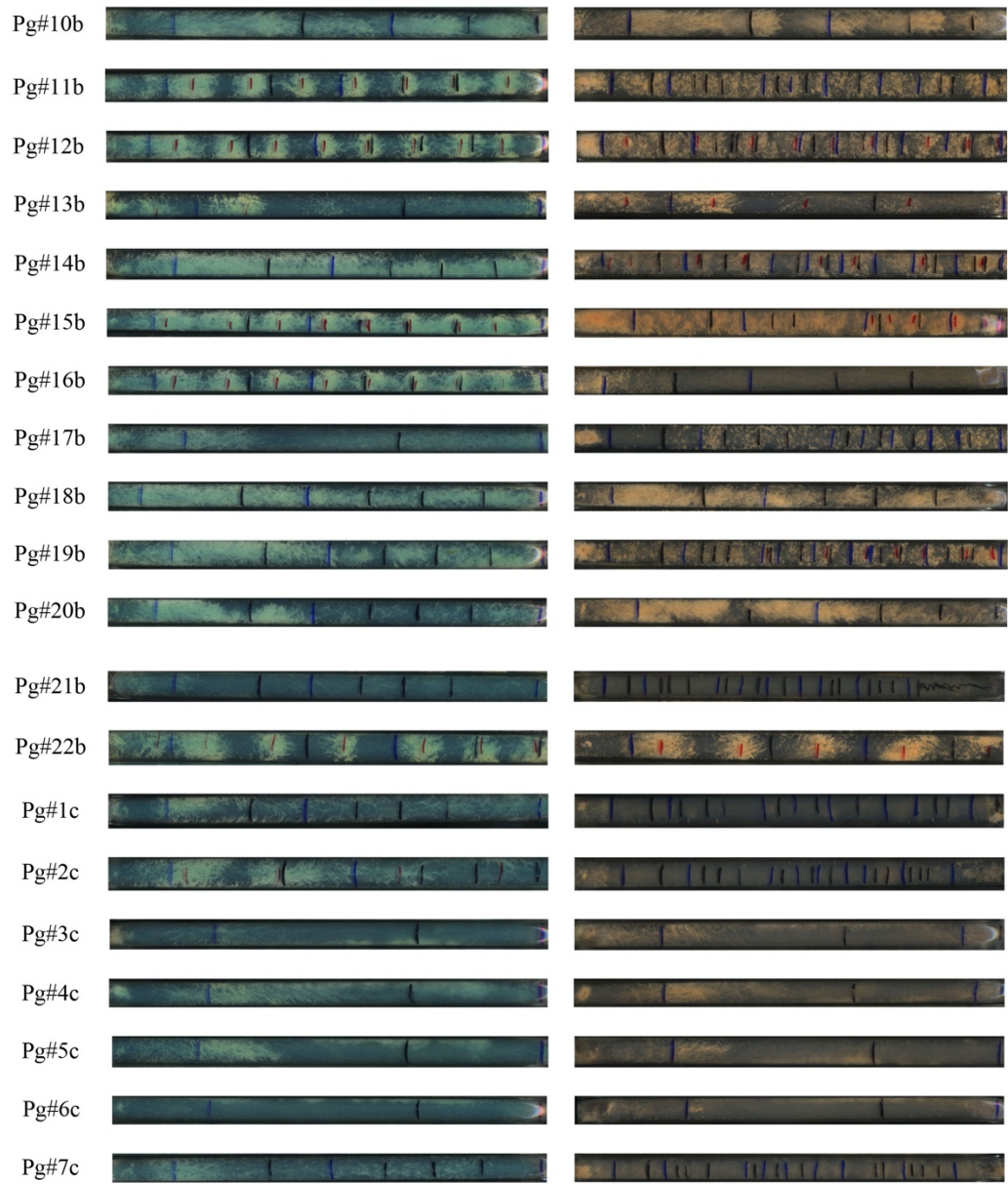
Spore PCR with PIK1aF and PIK1aR primers to identify PIK1a^{KO} mutant strains. Strains carrying the wild type PIK1a gene were recognized by a PCR product of 3562 bp and PIK1a^{KO} mutant strains were identified by a PCR product of ~1500 bp. PIK1a^{KO} mutant strains used for further testing and experiments were marked with (*).



B.

Conidiation phenotypes of progeny strains obtained from the genetic cross performed between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and FGSC#19786 (PIK1a^{KO}) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. One representative replicate tube for each condition and genotype are shown. Growth is from left to right. Genotypes, periods and growth rates are listed in Table 9.11 and Appendix XXXIX – C.





C.

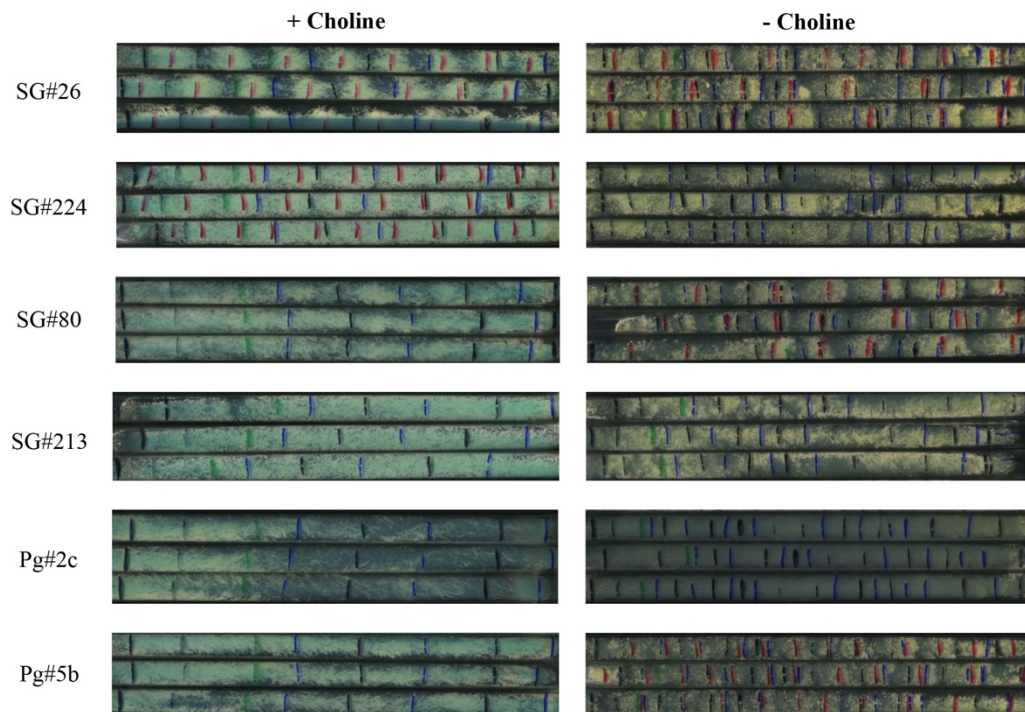
Summary of *csp-1* progeny obtained from cross between SG#80 (*csp-1: chol-1 ras-1^{bd}, frq^{l0} mat a*) and PIK1a^{KO} strains.

Strain Number	Without Choline		With Choline		PIK1 a	Ras-1 ^{bd}	Chol	Frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
SG#26 (control)	0.64	41.75	1.49	21.27	<i>PIK1_a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
SG#80 (control)	0.57	55.55	1.44	-	<i>PIK1_a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq₀^l</i>
1b	0.43	-	1.14	23.99	<i>PIK1_a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
2b	2.72	-	2.88	-	<i>PIK1_a⁺</i>	<i>Ras-1^{bd+}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
3b	1.33	-	1.16	21.45	<i>PIK1_a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
4b	2.98	-	3.01	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
5b	0.48	-	1.21	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq₀^l</i>
6b	2.83	-	3.10	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
7b	0.51	-	1.43	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq₀^l</i>
8b	1.05	-	3.01	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>Chol-1</i>	<i>frq⁺</i>
9b	0.47	-	3.01	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>Chol-1</i>	<i>frq⁺</i>
10b	1.74	-	1.80	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>	<i>frq₀^l</i>
11b	0.46	-	1.52	20.26	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
12b	0.46	57.63	1.25	21.17	<i>PIK1_a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
13b	2.84	20.72	2.95	-	<i>PIK1_a⁺</i>	<i>Ras-1^{bd+}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
14b	0.42	54.17	1.20	-	Heterokaryon			
15b	0.78	20.88	1.22	22.45	Missing spore PCR			
16b	1.63	-	1.21	22.28	Heterokaryon			
17b	0.50	-	2.88	-	<i>PIK1_a^{KO}</i>	<i>Ras-1^{bd+}</i>	<i>Chol-1</i>	<i>frq⁺</i>

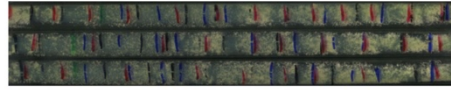
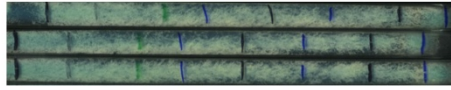
18b	1.41	-	1.22	-	<i>PIK1</i> <i>a^{KO}</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>Chol⁺</i>	<i>frq₀^l</i>
19b	0.36	52.70	1.23	-	<i>PIK1</i> <i>a^{KO}</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>Chol- 1</i>	<i>frq₀^l</i>
20b	1.40	-	1.21	-	<i>PIK1</i> <i>a⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>Chol⁺</i>	<i>frq₀^l</i>
21b	0.33	-	1.11	-	Not classified			
22b	1.93	24.23	1.79	21.10	<i>PIK1</i> <i>a^{KO}</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>Chol⁺</i>	<i>frq⁺</i>
1c	0.46	-	1.12	-	<i>PIK1</i> <i>a⁺</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>Chol- 1</i>	<i>frq₀^l</i>
2c	0.31	-	1.38	40.67	<i>PIK1</i> <i>a^{KO}</i>	<i>Ras-</i> <i>1^{bd}</i>	<i>Chol- 1</i>	<i>frq₀^l</i>
3c	2.81	-	2.77	-	Heterokaryon			
4c	2.64	-	2.77	-	<i>PIK1</i> <i>a⁺</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>Chol⁺</i>	<i>frq₀^l</i>
5c	2.70	-	2.83	-	<i>PIK1</i> <i>a^{KO}</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>Chol⁺</i>	<i>frq₀^l</i>
6c	2.84	-	2.88	-	<i>PIK1</i> <i>a^{KO}</i>	<i>Ras-</i> <i>1^{bd+}</i>	<i>Chol⁺</i>	<i>frq₀^l</i>
7c	0.38	-	1.13	-	Spore PCR indicated as a PIK1a ^{KO} and not a heterokaryon. But formed black coloured sexual spores.			

D.

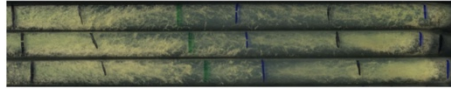
Conidiation phenotypes of selected progeny strains obtained from the genetic cross performed between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and FGSC#19786 (PIK1a^{KO}) strains. Strains were grown with (+, left panel) or without (-, right panel) 100 μ M choline in the medium. Three representative replicate tubes for each condition and genotype are shown. Growth is from left to right. Periods and growth rates are listed in Appendix XXXIX - E.



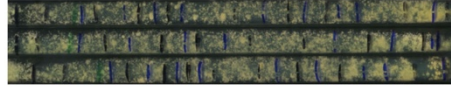
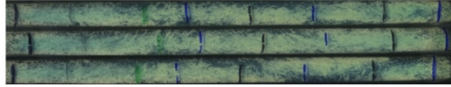
Pg#7b



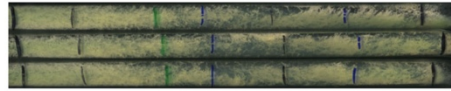
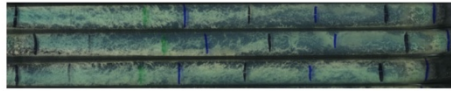
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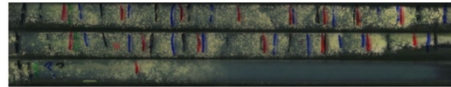
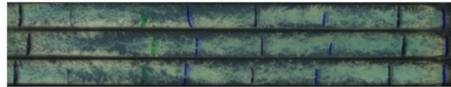
Pg#11b



Pg#18b



Pg#19b



E.

Summary of progeny obtained from cross between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and PIK1a^{KO} strains (2nd round).

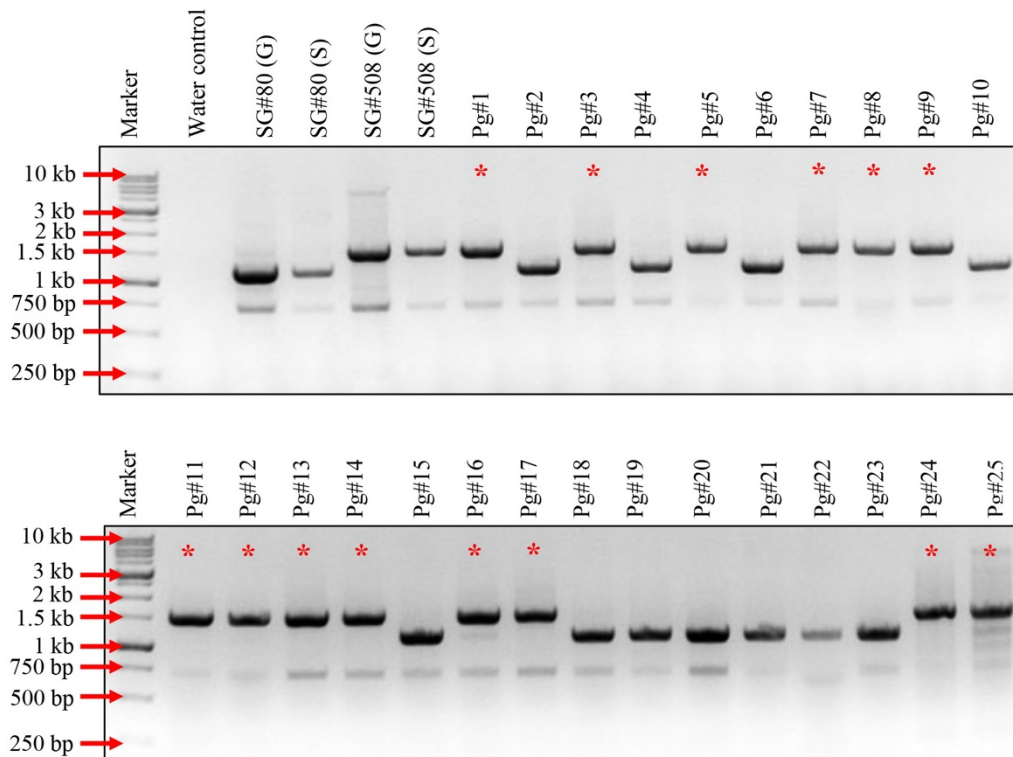
Strain Number	Without Choline		With Choline		PIK 1a	Ras-1 ^{bd}	Chol	Frq
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)				
SG#26 (control)	0.42	84.25	1.37	20.22	<i>PIK 1a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.47	67.23	1.26	20.75				
	0.43	58.59	1.30	20.83				
SG#80 (control)	0.55	56.66	1.34	-	<i>PIK 1a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.42	72.32	1.38	-				
	0.47	70.39	1.33	-				
SG#224 (control)	0.51	-	1.16	18.15	<i>PIK 1a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq⁺</i>
	0.39	-	1.18	21.30				
	0.48	-	1.18	21.50				
SG#213 (control)	0.66	-	1.18	-	<i>PIK 1a⁺</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.60	-	1.21	-				
	0.67	-	1.22	-				
2c	0.38	-	1.37	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.41	-	1.40	-				
	0.36	-	1.40	-				
5b	0.47	42.24	1.42	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.45	42.25	1.41	-				
	0.46	40.56	1.42	-				
7b	0.43	61.80	1.36	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.40	59.10	1.37	-				
	0.47	37.58	1.36	-				
10b	1.93	-	1.80	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
	1.87	-	1.77	-				
	1.93	-	1.84	-				
11b	0.45	-	1.37	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.55	-	1.37	-				
	0.39	-	1.39	-				
18b	1.51	-	1.38	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol⁺</i>	<i>frq¹⁰</i>
	1.56	-	1.44	-				
	1.51	-	1.44	-				
19b	0.54	46.91	1.48	-	<i>PIK 1a^{KO}</i>	<i>Ras-1^{bd}</i>	<i>Chol-1</i>	<i>frq¹⁰</i>
	0.41	67.69	1.46	-				
	-	-	1.45	-				

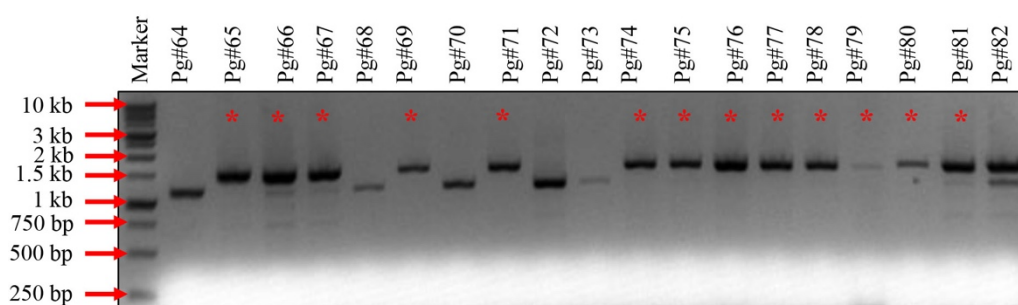
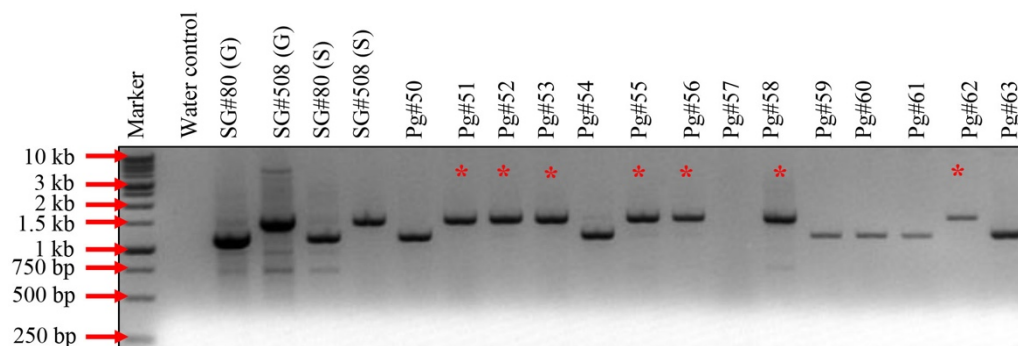
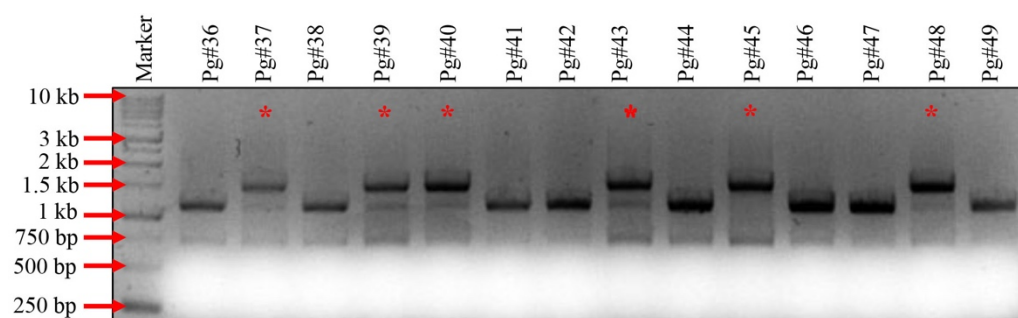
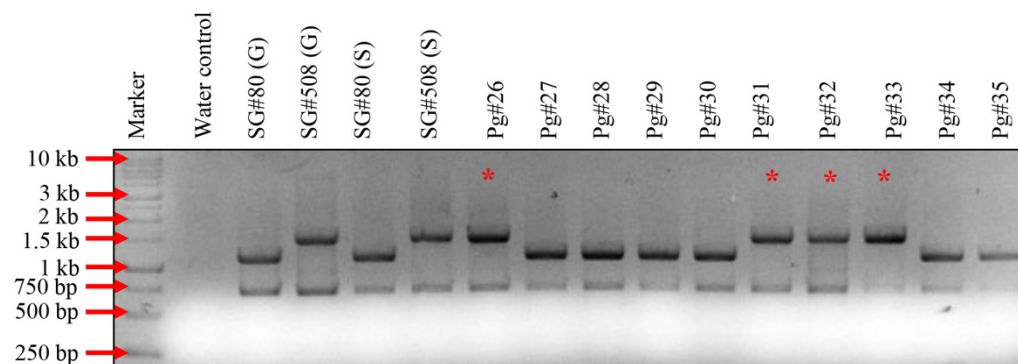
Appendix XL

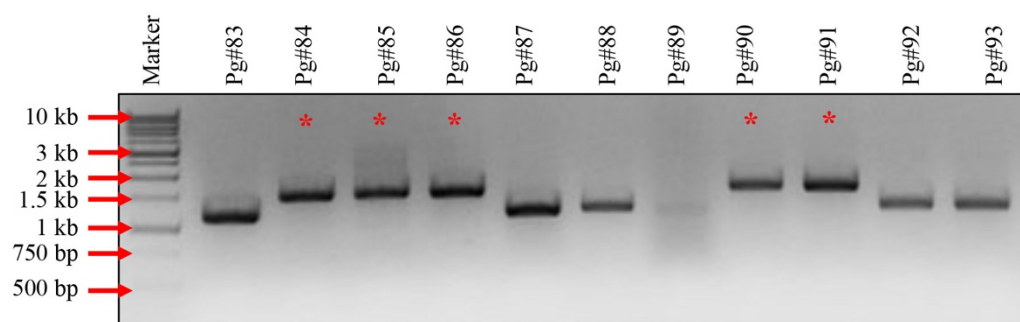
Analysis of *csp-1* progeny strains obtained from genetic cross between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and *FKR5^{KO}* strains

A.

Spore PCR with FKR5F and FKR5R primers to identify FKR5^{KO} mutant strains. Strains carrying the wild type FKR5 gene were recognized by a PCR product of 1161 bp and FKR5^{KO} mutant strains were identified by a PCR product of ~1500 bp. FKR5^{KO} mutant strains used for further testing and experiments were marked with (*).

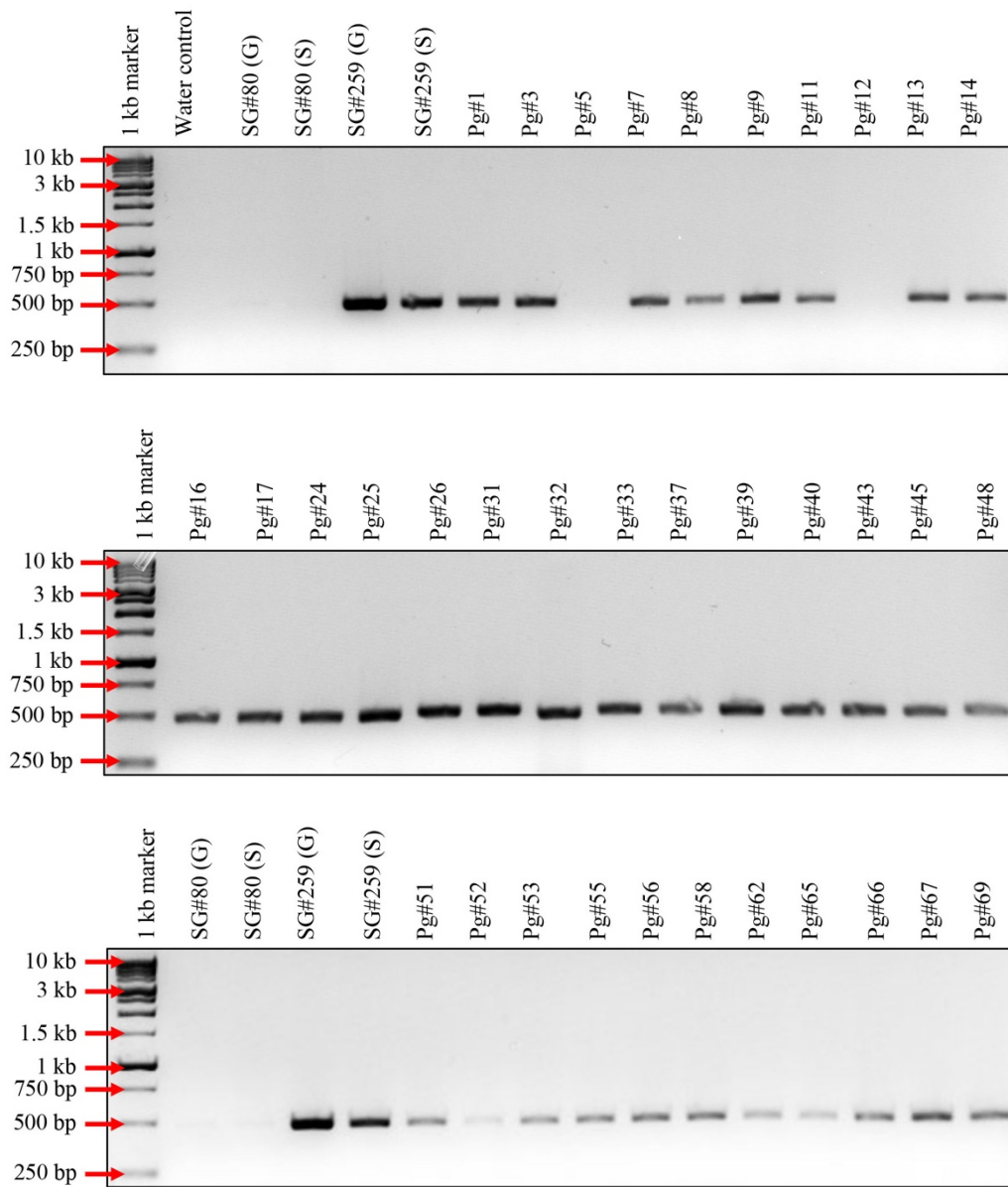


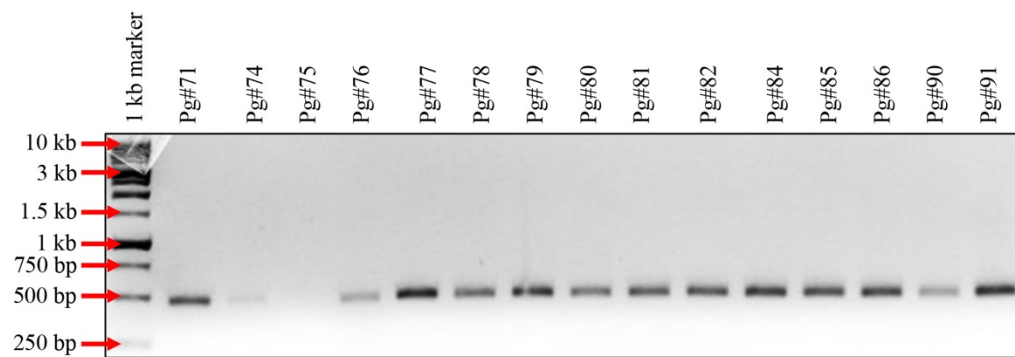




B.

Spore PCR with FrqF and FrqR primers to identify *frq*¹⁰ mutant strains in FKR5^{KO} background. Strains carrying the wild type *frq* gene were recognized by a PCR product of 484 bp.





C.

Periods and growth rates of progeny obtained from cross between SG#80 (*csp-1: chol-1 ras-1^{bd}; frq¹⁰ mat a*) and FKR5^{KO} strains.

Strain Number	Without Choline		With Choline	
	Growth Rate (mm/hr)	Period (hr)	Growth Rate (mm/hr)	Period (hr)
SG#26 (control)	0.51	64.50	1.31	21.31
SG#80 (control)	0.48	70.30	1.26	-
SG#224 (control)	0.49	-	1.15	21.42
SG#213 (control)	0.55	-	1.27	-
1	0.20	-	0.32	-
2	2.62	17.04	2.61	19.09
3	2.66	-	2.65	-
4	0.48	67.66	1.27	-
5	0.48	-	1.23	-
6	0.41	-	1.22	29.05
7	2.75	-	2.48	-
8	0.41	60.33	1.31	-
9	0.44	-	1.22	24.01
10	1.36	20.99	1.31	21.18
11	0.49	-	1.23	-
12	1.33	20.63	1.25	21.00
13	2.66	-	2.69	-
14	0.51	-	1.38	23.95
15	2.64	-	2.67	-
16	2.82	18.63	2.52	18.42
17	0.11	-	0.15	-
18	0.59	-	1.19	-
19	0.14	-	0.15	-

20	2.84	-	2.76	-
21	0.14	-	0.15	-
22	0.09	-	0.09	-
23	0.24	104.33	0.69	-
24	2.68	-	2.63	-
25	2.37	-	2.24	-
26	2.92	-	2.75	-
27	2.63	-	2.43	-
28	0.39	83.42	1.18	20.27
29	0.13	-	0.13	-
30	1.12	-	0.11	-
31	0.49	-	1.26	-
32	0.14	-	0.16	-
33	0.14	-	0.16	-
34	0.10	-	2.06	-
35	0.08	-	0.11	-
36	0.08	-	2.77	-
37	0.13	-	0.16	-
38	2.86	-	2.54	-
39	2.20	-	2.59	-
40	0.08	-	0.07	-