Expression of *Penicillium marneffei* Chitin Synthase Genes in Response to Cell-Wall Stressors

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in Response to Cell-Wall Stressors

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Abstract

Penicillium marneffei is a dimorphic fungus and an opportunistic pathogen responsible for the disease penicilliosis. Efforts to genetically characterize this organism have led to the development of a plasmid-dependent, mutant generating system known as *Agrobacterium tumefaciens* mediated transformation. This system produced one mutant, originally identified as strain I231, which was found to harbor a defective *yakA* gene. As determined by the work of Suwunnakorn et al. (2014), this defective gene resulted in an abnormal phenotype and unique stress responses when compared to a wild-type strain. The *ΔyakA* mutant possessed increased resistance to the anionic dye Congo Red (CR), known to inhibit glucan synthase activity, yet also yielded increased sensitivity to the similarly acting antifungal drug, caspofungin. These seemingly contradictory results, along with a increase in overall chitin content within the mutant, led to this study which seeks to determine overall chitin synthase gene expression in the *ΔyakA* mutant, the wild-type strain, and the complement strain, CY21, in terms of seven known chitin synthase genes. These strains were exposed to CR, the detergent sodium dodecyl sulfate (SDS), the chitin binding agent Calcofluor White (CW), as well as incubated under non-stressed control conditions. Subsequently, RNA from these cultures were isolated and chitin synthase gene expression levels were quantitated through qRT-PCR using the reference gene *benA* (encoding β-tubulin) for normalization. The results of this study indicated that the interrupted *yakA* gene produced no significant effect in terms of chitin synthase gene expression under stressed conditions, but the mutation produced significantly higher expression levels of the chitin synthases in the *ΔyakA* control conditions at both 25°C and

37°C. This response most likely represents a compensatory response to the weakened cell wall caused by an interrupted *yakA* gene.

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CHAPTER 1: INTRODUCTION

1.1 Background

The genus *Penicillium*, as a whole, is recognized as a relatively benign group of often beneficial fungi that rarely cause disease in humans. *Penicillium marneffei* is the exception to this outlook and remains the third most common AIDS-defining illness in the world (Cooper and Haycocks, 2000). Despite this notoriety, *P. marneffei* was unheard of less than sixty years ago, making its first documented appearance in 1956 in Dalat, South Vietnam (Capponi et al., 1956). It was first cultured from a lab-born Chinese bamboo rat, *Rhizomys sinensis*, that had spontaneously and mysteriously died at the Pasteur Institute of Indochina. The examiners noted the unidentified fungus exhibited *Penicillium*-like characteristics, but were subsequently puzzled once they witnessed its yeast-like growth within the deceased rat and its mold-like growth in cultures outside of the rat. One of the first researchers to extensively study *P. marneffei*, Professor Gabriel Segretain, named the fungus based on its *Penicillium*-like nature and the director of the Pasteur Institute at the time, Dr. Hubert Marneffe (Segretain, 1959).

Segretain would not only give *Penicillium marneffei* its name, but he would also become the fungus's first documented case in a human host. While performing his research in 1959, Segretain accidentally inoculated himself with a *P. marneffei*-infected needle. Despite becoming ill, Segretain, who presumably possessed a properly functioning immune system, had little trouble in curing himself with a treatment of oral nystatin (Segretain, 1959). While this first infection was from an obvious source, the first naturally occurring case of *P. marneffei* infection would be documented only 14 years

later. In 1973, a 61-year-old American clergyman undergoing surgery was found to harbor culturable *P. marneffei* inside of his body. Suffering from Hodgkin's disease, the clergyman had resided in Southeast Asia for some time prior to the operation (DiSalvo et al. 1973). In 1984, a similar case occurred where a 59-year-old man underwent lung surgery and *P. marneffei* yeast cells were observed within his lung tissue (Paulter et al., 1984). The spacing between *P. marneffei*-related cases would diminish with the onset of the HIV/AIDS epidemic in 1988. Documented reports of *P. marneffei* infection would span the entire globe, appearing in Europe (Sobottka et al., 1996; Rimek et al., 1999; Julander and Petrini 1997; Kronauer et al., 1993; Borradori et al., 1994; Garbino et al., 2001; Hilmarsodottir et al., 1993; Hilmarsdottir et al., 1994; Depraetere et al., 1998; Hulshof et al., 1990; Kok et al., 1994; Peto et al., 1988; McShane et al., 1998; Vilar et al., 2000) , Asia (Liao et al., 2002; Ranjana et al. 2002; Chang et al., 1995; Chiang et al., 1998; Hien et al., 2001; Huynh et al., 2003; Mohri et al., 2000; Tsunemi et al., 2003), Australia (Heath et al., 1995), Cambodia (Bailloud et al., 2002) and the United States (Nord et al., 1998).

Regardless of well-documented cases and plentiful isolates, the natural reservoir of *P. marneffei* remains unknown (Capponi et al., 1956). Efforts to identify the reservoir began primarily in Thailand with the native rodent species from which it was originally isolated along with several of its relatives. These species included *Rhyzomis sinensis* (Chinese bamboo rat), *Cannomys badius* (lesser bamboo rat), and *Rhyzomis pruinosus* (hoary bamboo rat). *Penicillium marneffei* could be isolated from all of these species and from within the soil that made up their burrows (Ajello et. al., 1995). Despite this, no additional soil or plant-based reservoir could be uncovered though it is believed that non-

isolated, soil-based conidia may become aggregated during Thailand's rainy season and enter human hosts through inhalation (Chariyalerstak et al., 1996; Liu et al., 2009). Additionally, most of the human cases that have been documented occurred in the cities of Thailand where these rodents rarely live. This indicates the possibility of additional carriers such as domestic animals (e.g., dogs) or pest animals that would reside within a proximity that permits human interaction and infection (Cao et. al., 2011; Chaiwun et.al., 2011).

1.2 Biology

Previous studies have indicated the importance of the hyphal and yeast phases of *P. marneffei* in virulence of this pathogenic fungus. Proper functioning and regulation of these phases is vital to the organism's persistence in its hosts (Cooper and Vanittanakorn, 2008). At 25°C, approximately room temperature, *P. marneffei* cells exist as hyphae which are composed of filamentous bodies that grow through lateral branching and apical extension. These multicellular structures reproduce asexually through the production of conidia which are commonly referred to as spores. Once hyphae are exposed to air, specialized cells form aerial stalks. These stalks will swell at their tips, forming globose structures that divide their nuclei. Upon becoming multinucleate, these structures are termed metulae and will next undergo cytokinesis to form uninucleate cells. Budding simultaneously, these metulae will form a second tier of cells that are known as phialides. Interstitial budding of these phialides results in the production of conidia. Growing chains of conidia will be produced through this process (Zuber et al., 2002). When these conidia

are cultured at 37°C, approximately inner-body temperature, they will begin the transition into uninucleate yeast cells (Zuber et al., 2003). At first, the yeast germination process is the same as the hyphal formation with both lateral growth and hyphal branching. Eventually, the hyphae will begin the process of arthroconidiation where septation and nuclear division become coupled together. Through the formation of double septa, the hyphae will separate to form uninucleate yeast cells. These cells will then divide by elongation and binary fission (Boyce and Andrianopoulos, 2013; Adrianopoulos, 2002). The transition from a hyphal to yeast form is believed to be responsible for the ability of *P. marneffei* to survive in host environments and evade immune system responses. After inhalation by a host, the conidia are thought to make their way into the lungs, transition into yeast cells, and eventually take up residence in alveolar macrophages, effectively evading the host immune system. Studies have shown that an inability to make this transition to the yeast phase results in a loss of *P. marneffei* virulence (Canovas and Adrianopoulos, 2007; Boyce et al., 2009; Adrianopoulos, 2002; Todd et al., 2003).

1.3 *Agrobacterium tumefaciens*-Mediated Transformation

Researchers have primarily relied on two methods to both determine the genetic mechanisms that regulate dimorphism in *P. marneffei* and to genetically characterize this fungus in general. These methods are mutant characterization and protein profiling (Zhang et al., 2008; Kumasook et al., 2010; Chandler et al., 2008). The first of these methods relies upon *Agrobacterium tumefaciens*-mediated transformation (ATMT) to induce random mutagenesis in *P. marneffei*. *Agrobacterium tumefaciens* is a Gram-

negative bacterium known to produce crown gall tumors in plants. These tumors are produced due to the tumor-inducing plasmid possessed by strain *A. tumefaciens*. The plasmid directs the random insertion of DNA into a plant's genome and induces uncontrolled growth (Brencic et al., 2005). Researchers have utilized the *A. tumefaciens* plasmid to transform genetically tagged, random inserts into the *P. marneffei* genome. These transformations, while random, will often result in the disruption of a single gene's function. The transformants will be cultured and can be easily examined for phenotypic differences when compared to the *P. marneffei* wild-type strain. Strains of interest will then have their DNA isolated, undergo digestion that targets the tagged sites around the insertions, and be subjected to inverse PCR for amplification. Through these methods, the sites around the ATMT insertions can be electrophoretically isolated, sequenced, and analyzed through the online Basic Local Alignment Search Tool (BLAST) program. Because the genome of *P. marneffei* has been sequenced, BLAST results for a particular sample can usually result in the identification of the gene that was disrupted (Kummasook et al., 2010). The use of this relatively efficient process was first reported by Zhang et al. (2008) and subsequently improved upon by the work of Kummasook et al. (2010).

1.4 *Penicillium marneffei* Mutant I231 – *yakA*

Suwunnakorn et al. (2014) determined that the *P. marneffei* mutant strain I231, produced by the ATMT system of Kummasook et al (2010), contained a defective *yakA* gene. The *yakA* gene codes for a dual-specificity tyrosine phosphorylation regulated

protein kinase. Homologues of the *yakA* gene play roles in growth regulation, carbon source response, and stress response, often being responsible for the regulation of the stress response transcription factors *hsf1* and *msn2/4* (Aranda et al., 2011; Hartley et al., 1994; Lee et al., 2011). *hsf1* plays a role in thermotolerance while *msn2/4* plays a role in various cell-wall-based stress responses (Elfving et al., 2014; Lee et al., 2008; Sadeh et al., 2012; Verna et al., 1997; Garrett et al., 1991; Goyard et al., 2008; Malcher et al., 2010; Moriya et al., 2001).

Suwunnaokorn et al. (2014) determined that the *yakA* mutant strain, designated *ΔyakA*, produces fewer phialides and conidia, a condition that could be partially restored through supplementing the growth medium with glucose (Bulik et al., 2003). Growth medium supplementation was found to increase mRNA levels of the conidial production gene, *abaA*, allowing for a bypass mechanism to promote conidiogenesis (Borneman et al., 2000). Despite these lower overall conidia levels in the *ΔyakA* strain, the defective gene actually resulted in more rapid conidial germination. Based on previous studies, this normally indicates a weakened and more pliable fungal cell wall. To assess the integrity of the mutant's cell wall, it was cultured on media containing a number of different cell-wall stressors including the anionic glucan-binding dye Congo Red (CR), the ionic detergent sodium dodecyl sulfate (SDS), and the β-1,3-glucan synthase inhibiting antifungal drug caspofungin (CAS) (Kopecka and Gabriel, 1992; Verwer et al., 2011; Roncero et al., 1985). The *ΔyakA* strain was, surprisingly more resistant to CR while displaying hypersensitivity to both SDS and CAS. The contradictory nature of these results encouraged further study. Additional assessment of the *ΔyakA* cell-wall relied on the chitin-binding agent Calcofluor White (CW; Hill et al., 2006). This revealed atypical

distribution of chitin throughout the cell-wall, indicating a role for *yakA* role in determining cell-wall ultrastructure. Despite this atypical distribution of chitin, the mutant was actually found to possess overall higher levels of cell-wall chitin when compared to either wild-type or a genetically complemented strain (CY21).

Further cell-wall analysis of the *ΔyakA* cell wall included chitin synthase gene expression studies. The higher chitin content in the mutant strain prompted this study, and determined that two chitin synthases, *chsB* and *chsG*, were found to be expressed at significantly higher levels at both 25°C and 37°C. These results indicate that the *ΔyakA* strain of *P. marneffei* may be activating a compensatory chitin biosynthetic pathway in order to deal with its weakened cell wall (Levin et al., 2011; Liu et al., 2009). Despite additional testing indicating that the *yakA* mutant did not appear to play a role in virulence, these results reveal the importance of this gene, and its corresponding kinase, in terms of proper cell-wall maintenance and stress responses. Because the cell wall is the primary target of antifungal treatments, these investigations may provide significant insight into the complex mechanisms involved in the cell-wall integrity of this pathogenic fungus.

1.5 Chitin

Chitin is a *β*-1,4-linked homopolymer of *N*-acetylglucosamine. It is considered a relatively simple polysaccharide and is present in the cell-wall of every fungus that has been studied to date. It tends to be present in lower concentrations than the other major polymer of the cell-wall, glucan, but is essential to cell-wall viability because it acts as

the structure's skeleton, providing rigidity and binding points for glucan polymers (Borgia and Dodge, 1992; Latge and Beauvais, 2014; Munro, 2013). Despite its relative simplicity, chitin is synthesized and distributed throughout the cell wall by a large number of chitin synthases (Adams, 2004). These synthases can be somewhat specific or possess seemingly ambiguous and overlapping roles (Rogg et al., 2011; Rogg et al., 2012). This hints at the importance of chitin, given that many fungi have evolved with multiple synthases that can compensate for each other if one is either inhibited or defective due to genetic mutation. This large assortment of chitin synthases also appears to be a primary cell-wall integrity compensatory mechanism for most fungi (Lee et al., 2004). A number of studies indicate that cell-wall-based gene mutations, even if they do not involve chitin synthesis or regulation, often result in the overproduction of chitin throughout the cell-wall (Beauvais et al., 2013; Latge, 2007; Lee et al., 2011; Ram et al., 2004). This most likely occurs as a defensive mechanism that is attempting to compensate for a lack of proper cell-wall functionality (Fuchs and Mylonakis, 2009).

CHAPTER 2: SPECIFIC AIMS AND HYPOTHESIS

2.1 Specific Aims

The specific aim of this study is to genetically analyze and quantitate expression of the seven chitin synthase genes that have been identified in the dimorphic pathogenic fungus *P. marneffei* when grown on a medium supplemented with cell-wall perturbing agents. Specifically, two anionic dyes, CR and CW, and the detergent SDS will be employed to assess their affect upon the expression of these genes within the *ΔyakA* mutant strain, the wild-type F4 strain and the genetically complemented *ΔyakA* strain, CY21. Expression levels will be determined through a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) protocol that determines mRNA transcription within a sample at a given time point. This method will be used in conjunction with the mutant, wild-type, and complement strains of *P. marneffei* cultured on media supplemented with one of the three agents that will then subsequently have their RNA subjected to a quantitation protocol to determine levels of relative gene expression compared to the housekeeping β-tubulin gene, *benA*.

2.2 Hypothesis

The mutant *ΔyakA* strain of *P. marneffei* will possess a different chitin synthase gene expression profile when compared to the wild-type F4 strain. Specifically, chitin synthase gene expression, overall, will be higher in the mutant strain under control

conditions and will differ significantly from the wild type in terms of cell-wall stress response.

CHAPTER 3: MATERIALS AND METHODS

3.1 *Penicillium marneffei* Strains and Fungal Culturing

This study utilized three strains of *P. marneffei* including the F4 strain originally isolated from a Chiang Mai AIDS patient (wild-type; CBS 119456) (Pongpom et al., 2005), the *ΔyakA* strain (Suwunnakorn et al., 2014), and the *ΔyakA* complement strain, CY21 (Suwunnakorn et al., 2014). In order to ensure strain continuity, each of these strains was continually cultured in 100 x 15 mm raised ridge Petri dishes (FisherScientific; Pittsburgh, Pensylania) containing potato dextrose agar (PDA) (BD Difco; Franklin Lakes, New Jersey) on a weekly basis and allowed to grow at room temperature. These cultures were used for the initial inoculation phase of all experiments seven days.

3.2 Culture Preparation for Stress Tests and Stress Induction

Seven-day-old stock cultures were used to inoculate 75 cm (25 mL working volume) Nunc EasyFlask filter-capped culture flasks (Fisher Scientific) containing PDA supplemented with 8% glucose (Suwunnakorn et al., 2014). Glucose supplementation was necessary to promote sufficient conidial growth of *ΔyakA* cultures, but was used for all culture strains to ensure consistency across experiments. These cultures were incubated for seven days at 25°C, then subjected to conidial harvesting as previously described (Gifford and Cooper, 2009). The resulting suspension was diluted and a small volume loaded into hemocytometer to determine the conidial concentration. The

microscopic counts were performed using a differential interference contrast (DIC) microscope (Olympus; Center Valley, Pennsylvania). From this count, the proper volume of conidial suspension was calculated such that experimental cultures (described below) would contain a total concentration of 1 x 10^6 conidia/ml.

The conidial suspension was used to properly inoculate a Sabouraud dextrose broth (SDB; BD Difco) to a final concentration of 1 x 10^6 conidia/ml. This culture would normally consist of 250 ml of SDB. After inoculation, the cultures were placed in water baths and incubated at the appropriate temperature and an agitation of 120 rpm for 48 hours.

After the 48 hour incubation period, the cultures were removed from their water baths, aliquoted in 50 ml increments into sterile 250 ml Erlenmeyer flasks, and subjected to the appropriate stressor by first removing the proper volume of culture then replacing that volume with the stressor (refer to Appendix B and Appendix C for stressor volumes and total concentrations). After adding the stressors, the cultures were placed back into their same water bath and incubated for an additional 4 hours at the previous temperature and agitation. After 4 hours, the cultures were taken out of the water baths, quickly poured into 50 ml conical tubes, and centrifuged at 6500 rpm for 15 minutes at 4°C. After being centrifuged, the broth was poured off of each sample so that only the cell pellet remained. These cell pellets were aliquotted into 700-µl portions which were pipetted into 1.5 ml microcentrifuge tubes and stored at – 20°C for next day use or at – 80°C for extended storage.

3.3 RNA Isolation and Spectrophotometry

The 700 µl cell pellets (described above) were thawed on ice and used to carry out RNA isolations employing the RNeasy Plant Mini Kit (Qiagen; Valencia, California) according to the manufacturer's protocol. For the cell lysis portion of the protocol, ZR bashing bead lysis tubes containing 0.5 mm beads (Fisher Scientific) were used in conjunction with a Mini-Beadbeater (BioSpec Products; Bartlesville, Oklahoma) with a protocol of speed 42 and 40 seconds for two cycles. Final RNA samples were placed in 1.5 ml microcentrifuge tubes and stored at –80°C when not in use.

The concentration and purity of each RNA samples was determined using a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific; Waltham, Massachusetts). All blanks and sample measurements were assessed using 2 µl samples and were recorded as 260/280 nm wavelength values and as ng/µl concentrations.

3.4 Primer Assessment through RT-PCR, Gel Electrophoresis, and Standard Curve Generation

All primers chosen for this study were previously described by Suwunnakorn et al. (2014). A list of all primers used, their sequences, and the genes they code for is provided in Appendix A. The quality of all primers was assessed through an initial RT-PCR reaction using a Qiagen OneStep RT-PCR kit (Qiagen) according to manufacturer protocol (refer to appendices D and E) and a MJ Mini Personal Thermocycler (Bio-Rad; Hercules, California) with the manufacturer's suggested program. The RT-PCR product

sizes were determined through gel electrophoresis. A 1% agarose gel was used in conjunction with a Horizon 11·14 Life Technologies gel electrophoresis box (Grand Island, New York). For all samples, 2 µl of EZ Vision dye (Amresco; Solon, Ohio) and 5 µl PCR product were used. Ladders (1kb; Amresco) were used to assess sample size. The gel was subjected to a current of 60 volts for 60 minutes and banding patterns visualized using an UltraCam Digital Imager (Vexcel; Boulder, Colorado) equipped with Foto/PrepI UV Transilluminator (Fotodyne; Hartland, Wisconsin). Images (Appendix H) were captured with the PowerShot A620 (Canon; Lake Success, New York)

Primer quality was additionally assessed through RNA dilution series and their resulting standard curves produced through qRT-PCR. Wild-type control RNA was used for the standard curve production. The dilution series were begun by creating 8 µl samples that contained a total RNA concentration of 50 ng/µl (which would yield a total of 100 ng of RNA in the first sample after using 2 µl). The 50 ng/µl samples were then diluted by half by pipetting 4 µl into the next series of tubes which contained 4 µl of nuclease-free water. This process was repeated two more times to create four levels of dilution series. Once the RNA dilution series were complete, qRT-PCR master mixes were prepared for each of the three genes being studied. In each well of the 96-well plate, 18 µl of Bio-Rad iTaq Universal SYBR Green One-step kit master mix (to manufacturer specifications) (Bio-Rad; Hercules, California) was added. 2 µl of sample were added to each master mix for a total reaction volume of 20 µl. All dilution series were loaded in triplicate, the plate was centrifuged briefly in the Beckman GPR centrifuge (Beckman Coulter; Brea, California) at 600 rpm and 4°C. The plate was then loaded into the iQ5 Real-Time PCR System Thermocycler (Bio-Rad), the plate setup was created, and the

qRT-PCR reactions were ran to manufacturer specifications in triplicate (Appendix G) and the resulting standard curves were generated using the iQ5 software (refer to Appendix I for standard curves).

3.5 qRT-PCR

Relative gene expression of the *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* genes in each sample was assessed using a Bio-Rad iTaq Universal SYBR Green One-step kit to manufacturer specifications (requiring 18 µl of master mix and 2 µl of sample) (Bio-Rad), the Bio-Rad iQ5 Real-Time PCR System Thermocycler (Bio-Rad; Hercules, California), and the manufacturer's recommended one-step qRT-PCR program (Appendix F and G). Samples were processed in triplicate for all unknowns and for all non-template controls (which received 2 µl nuclease-free-water instead of a 2 µl RNA sample). Normalized gene expression was determined using the iQ5 software in conjunction with the gene expression results for the housekeeping gene, *benA*. The resulting data from this normalization was exported to both Microsoft Excel (Microsoft; Redmond, Washington) (for graph generation) and to SPSS (for statistical analyses).

3.6 Statistical Data Analysis

All data analyses was performed using SPSS software (Version 17.0; IBM; Armonk, New York). For each round of normalized gene expression data that was produced, a general multivariate MANOVA was first run to determine any statistically

significant relationships between and among the gene expression values and the different conditions (mold, yeast, stressor, etc.). Based on these results, univariate ANOVA models were ran with the Post-Hoc Bonferroni and Tukey tests to look for more specific relationships between the different conditions. All statistical results were retyped into custom charts produced through Microsoft Excel (Microsoft; Redmond, Washington).

CHAPTER 4: RESULTS

4.1 Overview

All of the qRT-PCR gene expression study results were organized and visualized into bar graphs using Microsoft Excel software. These graphs allowed for easy comparison of results, measured in normalized fold expression, under the different stressor conditions and between the different *P. marneffei* strains. Briefly, the relative expression of seven chitin synthase genes (*chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB*) was determined in two separately conducted experiments - qRT-PCR Rounds 1 and 2, respectively. Every experimental condition within each round was performed in triplicate and the results averaged. Hence, for each round, the resulting expression data was normalized to the expression of β-tubulin (*benA*). The data from each of the two rounds are presented herein. Overall, the results from each round were generally similar. Therefore, a general summary of the combined results is provided below.

The data generated in this study is best interpreted by looking at chitin synthase gene expression as a whole rather that as individual gene expression levels. This was determined through the statistical tests used to analyze the Round 1 and Round 2 data (Appendices J and K). The wild-type strain under control conditions, at both 25°C and 37°C tended to have lower overall chitin synthase fold expression levels when compared to the treatment conditions. The *ΔyakA* strain, at 25°C and 37°C, had proportionally higher chitin synthase fold expression levels under the control conditions when compared to the wild-type strain. Under the treatment conditions, however, the fold expression

levels for the *ΔyakA* strain were similar to those observed in the wild type. The genetically complemented CY21 strain, at both 25°C and 37 °C also expressed similar fold expression levels, under both control and treatment conditions, when compared to the wild type.

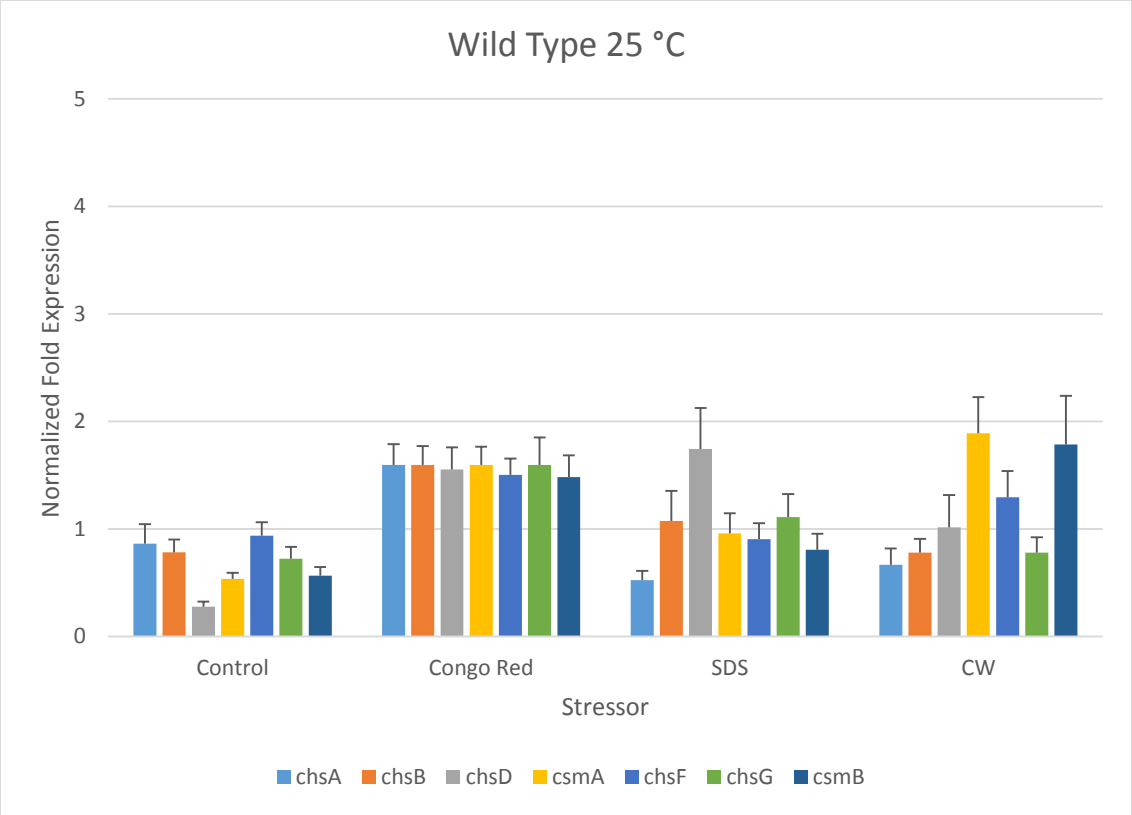


Figure 1: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the F4 (wild type) strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. No statistical analyses were performed for the combined data.

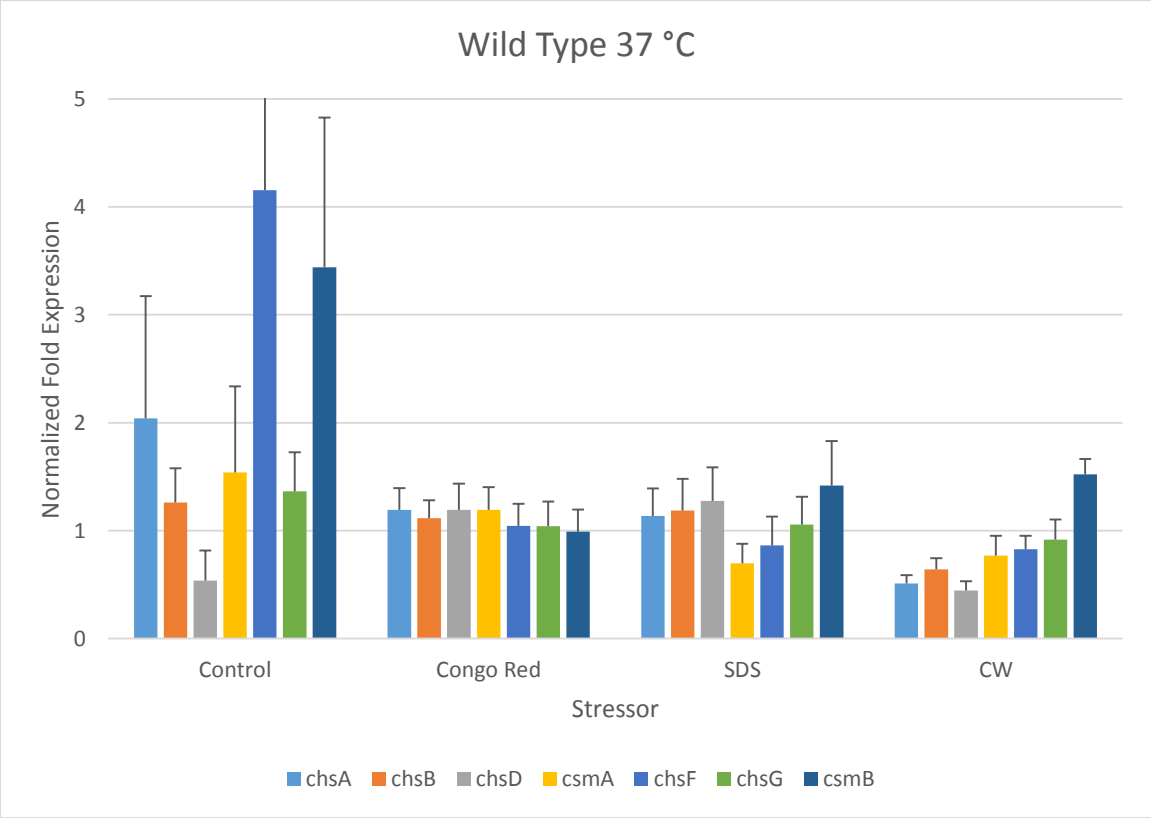


Figure 2: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the F4 (wild type) strain of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. No statistical analyses were performed for the combined data.

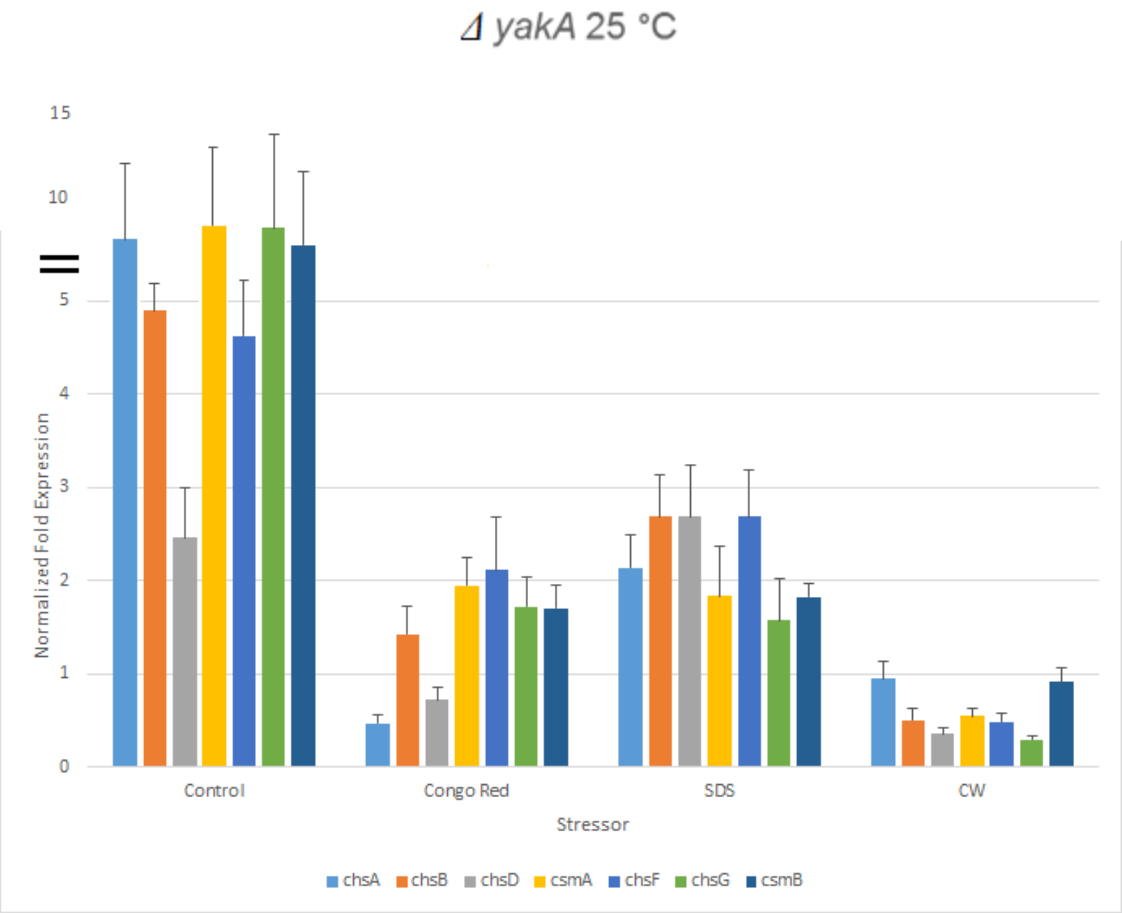


Figure 3: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the *ΔyakA* of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. No statistical analyses were performed for the combined data.

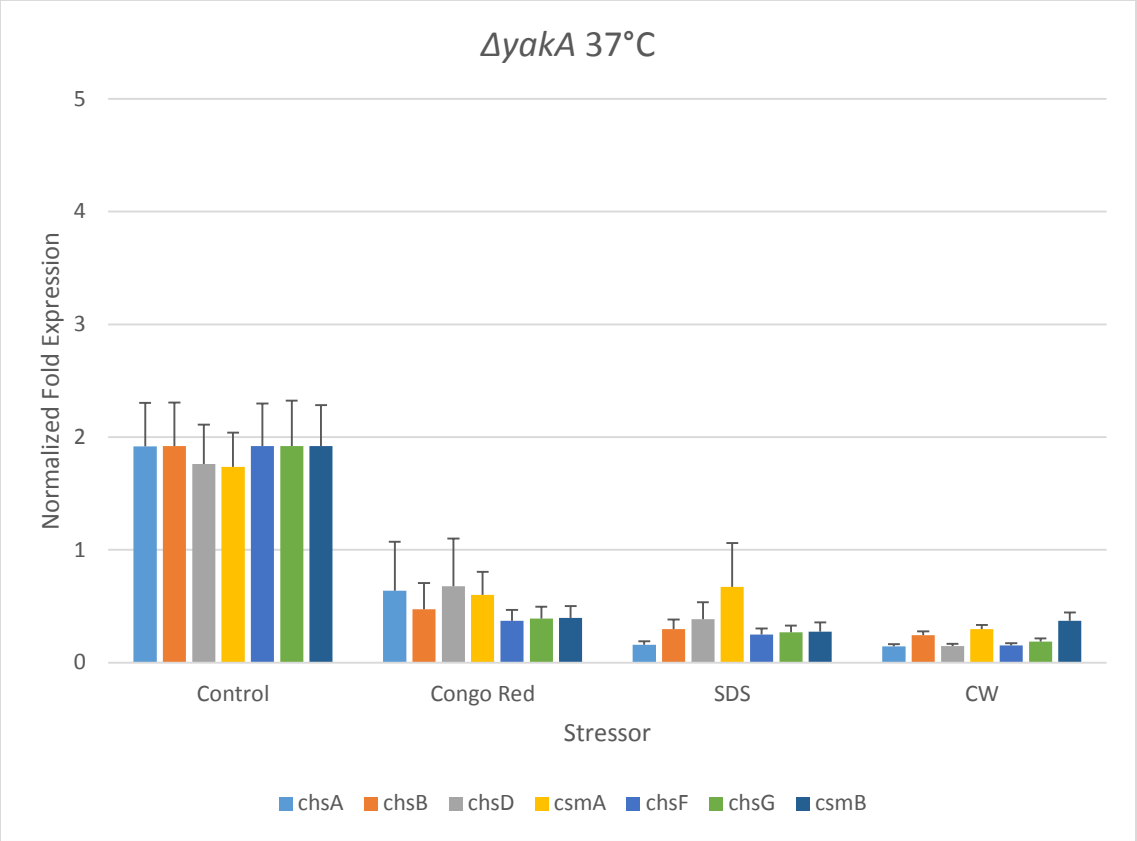


Figure 4: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the *ΔyakA* of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. No statistical analyses were performed for the combined data.

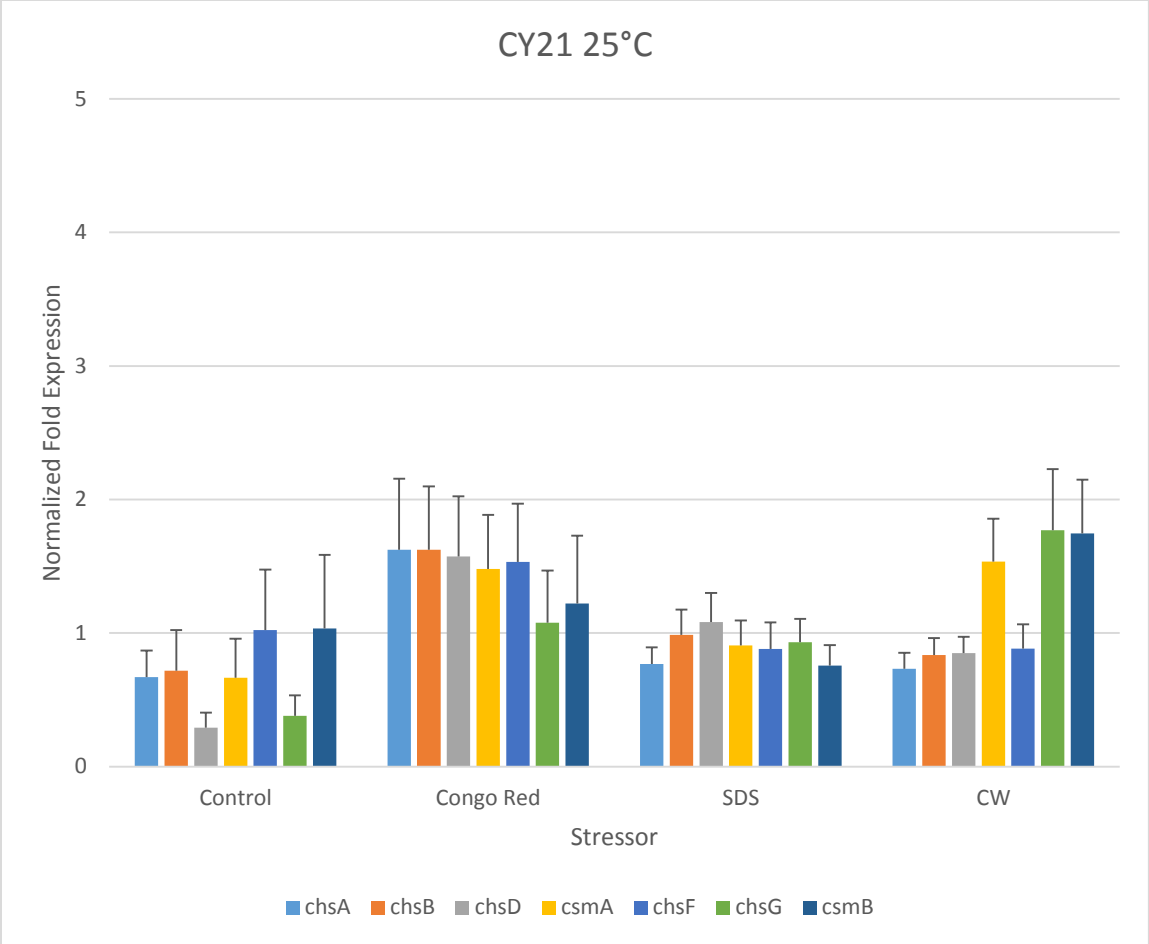


Figure 5: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the CY21 of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. No statistical analyses were performed for the combined data.

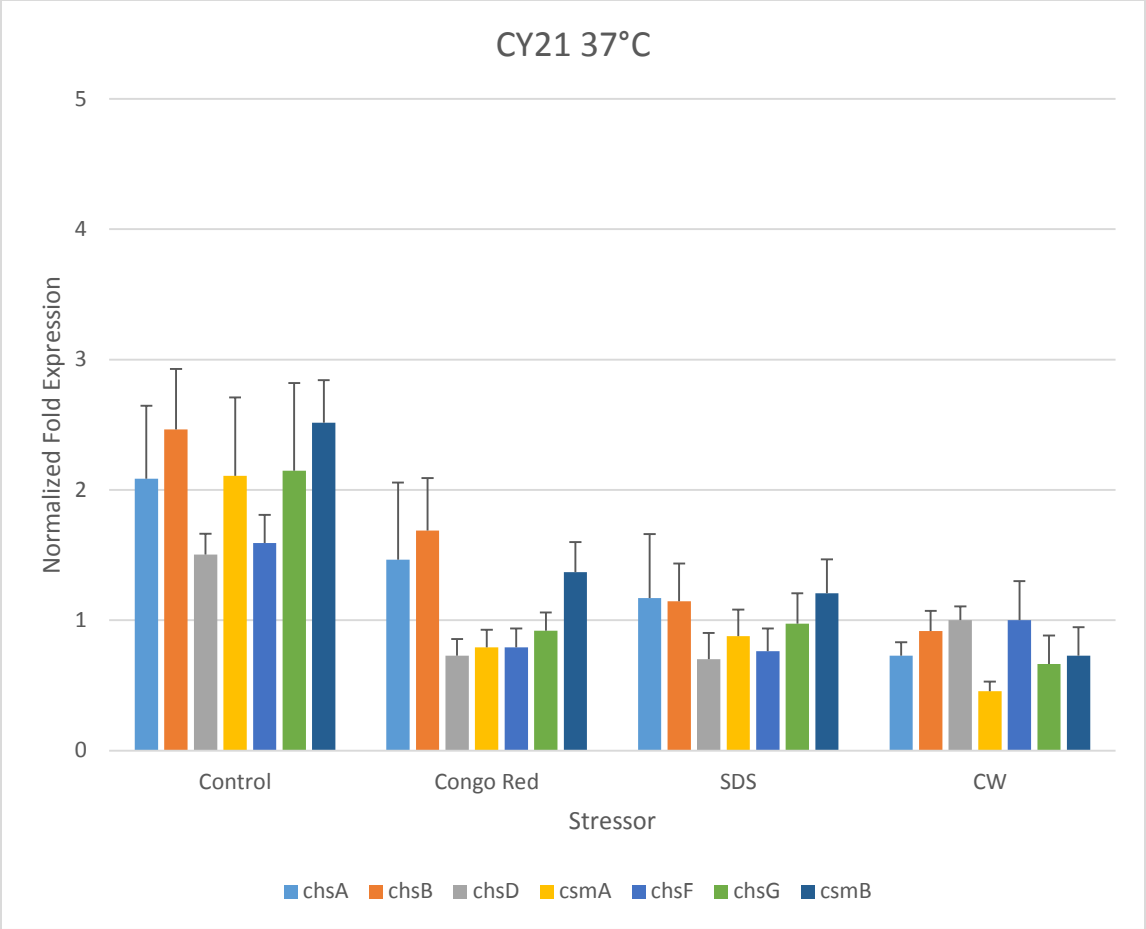


Figure 6: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the CY21 of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. No statistical analyses were performed for the combined data.

Combined Results

25°C	Wild Type	*ΔyakA*	CY21
Control	0.2 - 1.0	↑	≈
CR	1.4 - 1.6	≈	≈
SDS	0.5 - 1.8	≈	≈
CW	0.6 - 1.9	≈	≈

37°C	Wild Type	*ΔyakA*	CY21
Control	0.5 - 4.2	≈	≈
CR	1 - 1.2	↓	≈
SDS	0.6 - 1.5	↓	≈
CW	0.4 - 1.5	↓	≈

Figure 7: Summary chart illustrating the general patterns of gene expression for each group of chitin synthase genes under each set of possible conditions for the combined data. The normalized relative gene expression range for each wild type condition is listed. An up arrow (↑) indicates a higher fold gene expression range for that strain under that particular condition whereas a down arrow (↓) indicates a lower fold gene expression range for that strain under that particular condition. An approximate equal sign indicates that the indicated gene expression range is approximately equivalent to that of the wild-type.

4.2 Chitin Synthase Gene Expression Results from qRT-PCR Round 1

For the first round of data, the F4 strain at 25°C under control conditions (non-stressed conditions) possessed average chitin synthase gene expression levels between 0.2 fold expression (*chsD*) and 0.9 fold expression (*chsB*) with the remaining five chitin synthase genes falling somewhere between this range (Figure 1). The F4 strain subjected to CR possessed overall higher levels of fold expression with all seven chitin synthase gene falling between 1.1 and 1.4 fold expression. Subjected to SDS, the expression of all chitin synthases was still higher when compared to the control conditions, but somewhat lower when compared to the CR condition falling mostly between 0.4 and 1.0, with the exception of *chsD* which exhibited a fold expression of 1.7. When subjected to CW, strain F4 had four chitin synthases (*chsD*, *csmA*, *chsF*, and *csmB*) with higher fold expression when compared to the control condition ranging between 0.8 and 2.0 and the remaining three synthases with lower fold expressions ranging between 0.4 and 0.5 (Figure 1).

At 37°C, strain F4 possessed very similar, though somewhat lower, overall patterns of chitin synthase gene expression for the three stress conditions which ranged from 0.4 to 1.4 fold expression. The control condition at 37°C, however, possessed noticeably higher levels of hold expression with the lowest (*chsD*) being 0.8 and the highest (*chsF*) being 7.6 along with 6.0 for *csmB* and 3.1 for *chsA* (Figure 2).

When the *ΔyakA* strain was incubated at 25°C, the control condition revealed higher levels of the chitin synthase gene expression overall when compared to the F4

strain incubated under the same conditions. These fold expressions ranged from 0.6 (*csmB*) up to 3.8 (*chsB*) with *chsD*, *csmA*, *chsF*, and *chsG* all possessing fold expression higher than 1.5. Under the stress conditions, chitin synthase gene expression of the *ΔyakA* strain at 25°C was overall, similar to that seen in the F4 strain with fold expressions ranging between 0.4 and 1 for CR, 2.1 and 0.4 for SDS, and 0.3 and 1.6 for CW (Figure 3).

At 37°C, under control conditions, the *ΔyakA* strain exhibited *chs* fold expressions all between 0.6 and 1.0, whereas the stress conditions generated rather low fold expressions overall, with most ranging between 0.006 and 0.3. Two of the four exceptions belonged to *chsA* (1.0) and *chsD* (1.0) under CR stress conditions and the remaining two belonged to *chsD* (0.6) and *csmA* (1.0) under SDS stress conditions (Figure 4).

The genetically complemented *ΔyakA* strain, designated as CY21, possessed an overall more balanced set of gene expression levels when compared to either the F4 strain or the *ΔyakA* strain. At 25°C under control conditions, three of the chitin synthase genes (*chsA*, *chsD*, and *chsG*) exhibited fold expressions between 0.2 and 0.5 with the remaining four chitin synthase genes possessing fold expression between 0.7 and 1.6. CY21 displayed fold expressions between 1.1 and 1.4 when subjected to CR, between 0.4 and 1.0 when subjected to SDS, and between 0.6 and 1.0 when subjected to CW (Figure 5).

At 37°C under control conditions, CY21 expressed all but one of its chitin synthase genes between 2.2 and 2.9 fold with the exception, *chsF*, at 1.3 fold expression. The fold expressions were similar across the stress conditions with all of the chitin

synthase genes possessing fold expressions between 0.5 and 1.0, with the exception of three (*chsA*, *chsB*, and *csmB* under CR) which fell between 1.4 and 1.8 (Figure 6).

Overall, the results for round 1 indicated that the F4 strain under control conditions at 25°C had relatively lower chitin synthase gene expression when compared to the stress conditions. No discernible patterns are present for individual genes amongst the different conditions. This was supported by statistical analyses (Appendix J). At 37°C, the control condition had possessed much higher fold expressions for several of the chitin synthase genes, but very similar patterns of fold expression for the stress conditions were displayed. For the *ΔyakA* strain incubated at 25°C under control conditions, the chitin synthase gene fold expressions were noticeably higher when compared to the F4 strain while the fold expressions for the stress conditions were somewhat similar to F4 at 25°C. At 37°C, *ΔyakA* once again exhibited relatively higher fold expressions when compared to the stress conditions, but not as high as those observed by the F4 strain incubated under similar conditions. In addition, gene expression patterns of strain CY21incubated at 25°C were similar to those produced by strain F4 at 25°C. Moreover, incubation of CY21 at 37°C resulted in a higher fold expression of *chs* genes under control conditions, much like the F4 and *ΔyakA* strain*s* when compared to the stress conditions. Collectively, these general observations are supported by statistical analyses (Appendix J).

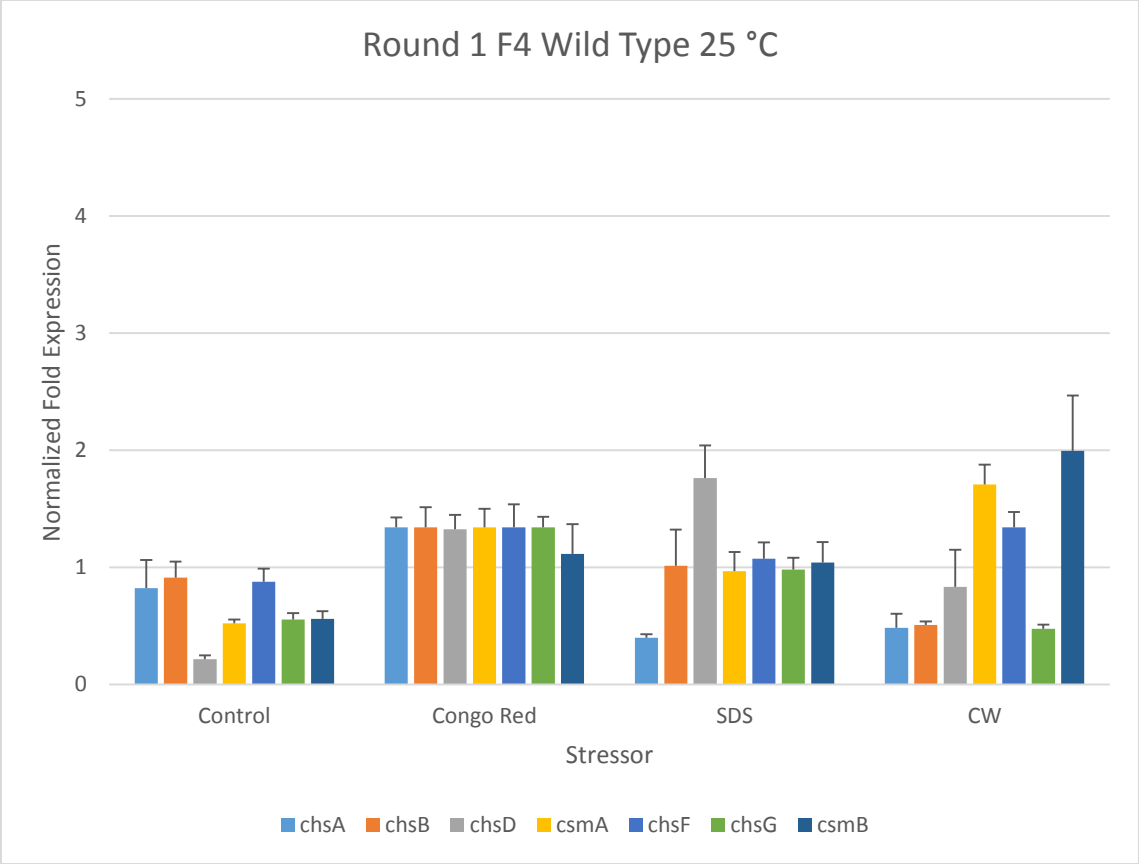


Figure 8: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the F4 (wild type) strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix J.

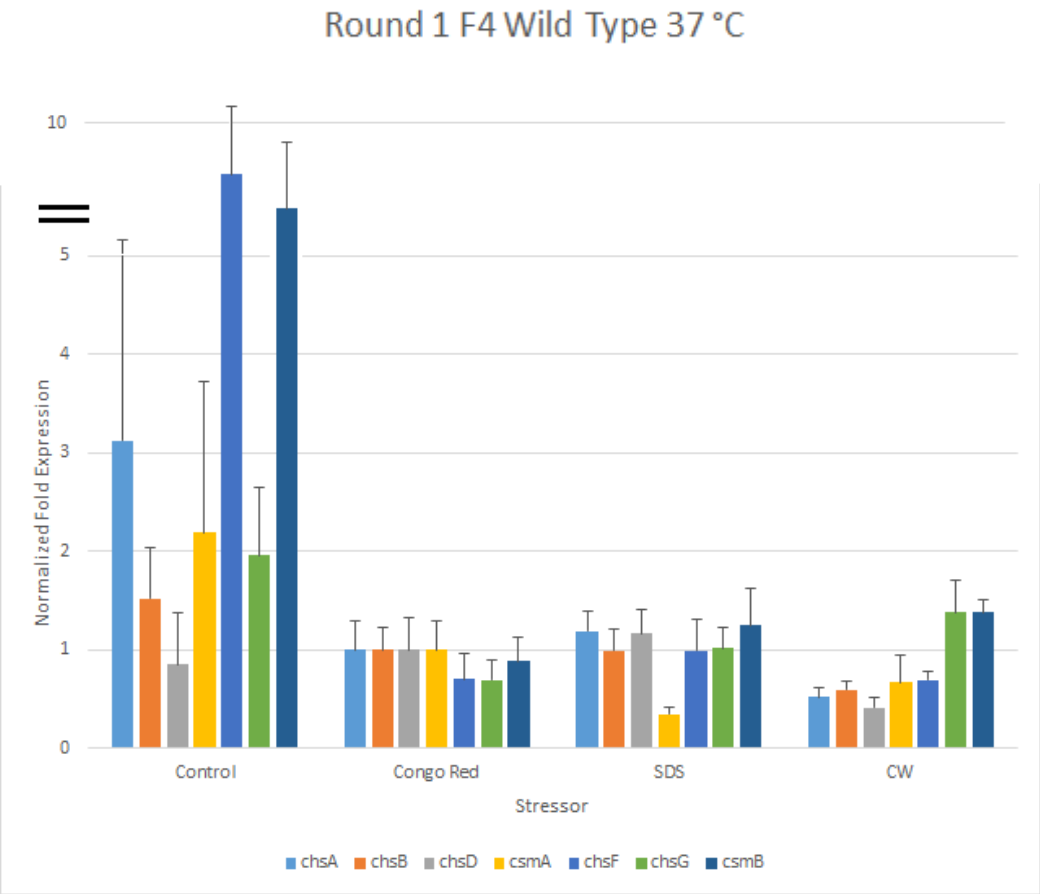


Figure 9: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the F4 (wild type) strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix J.

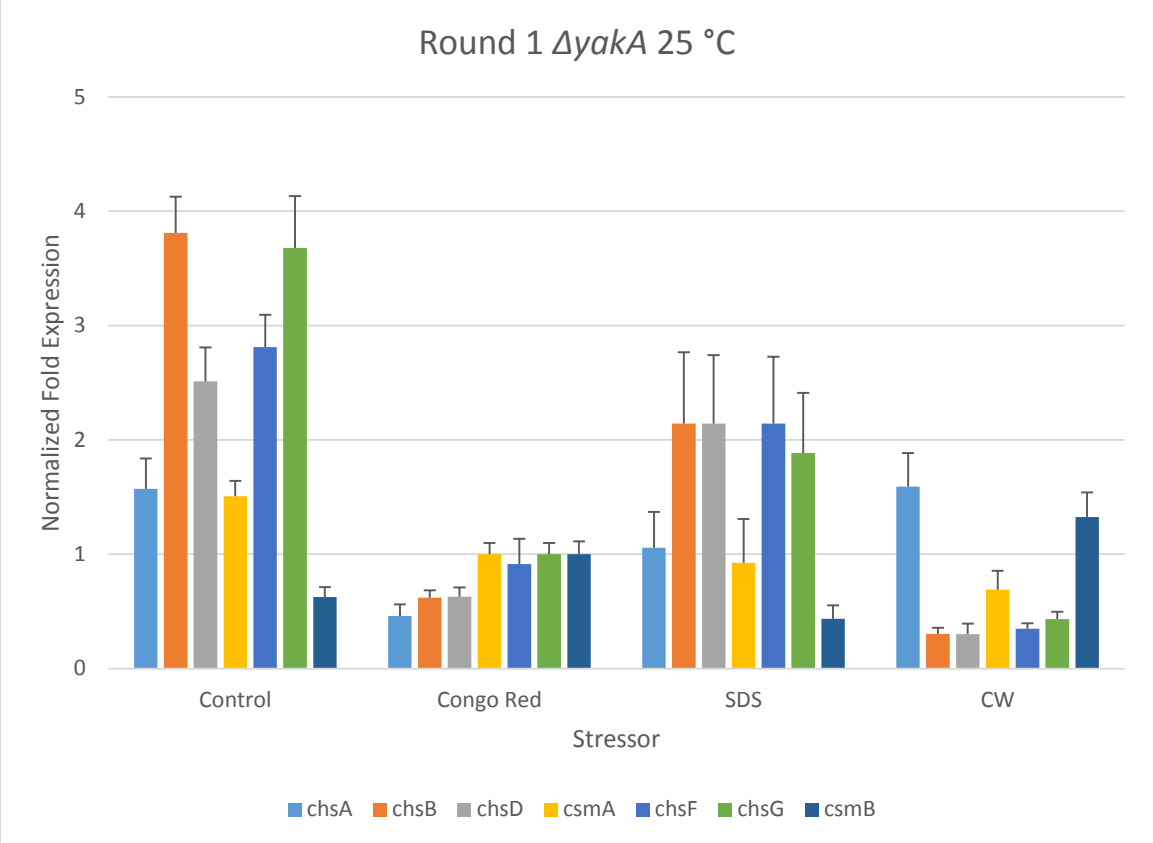


Figure 10: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the *ΔyakA* strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix J.

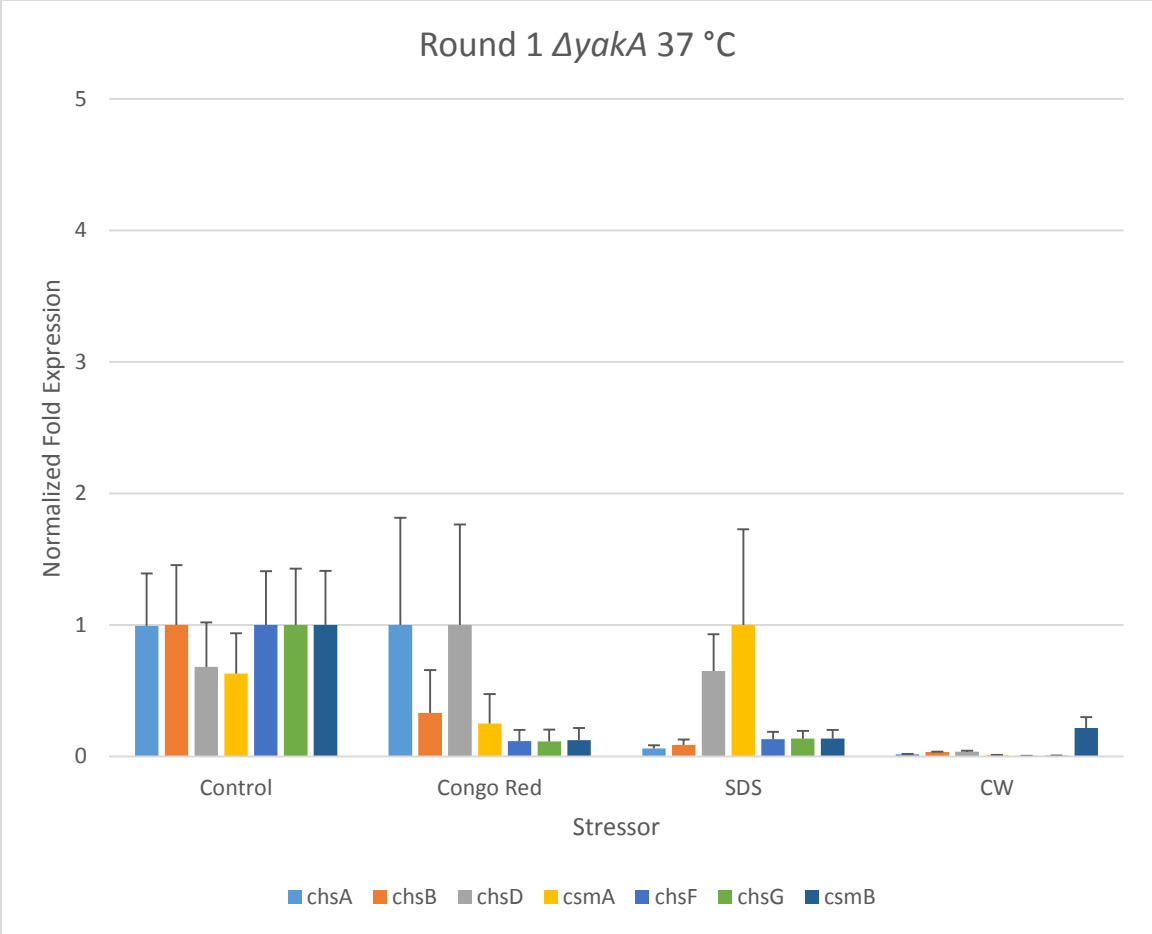


Figure 11: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the *ΔyakA* strain of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix J.

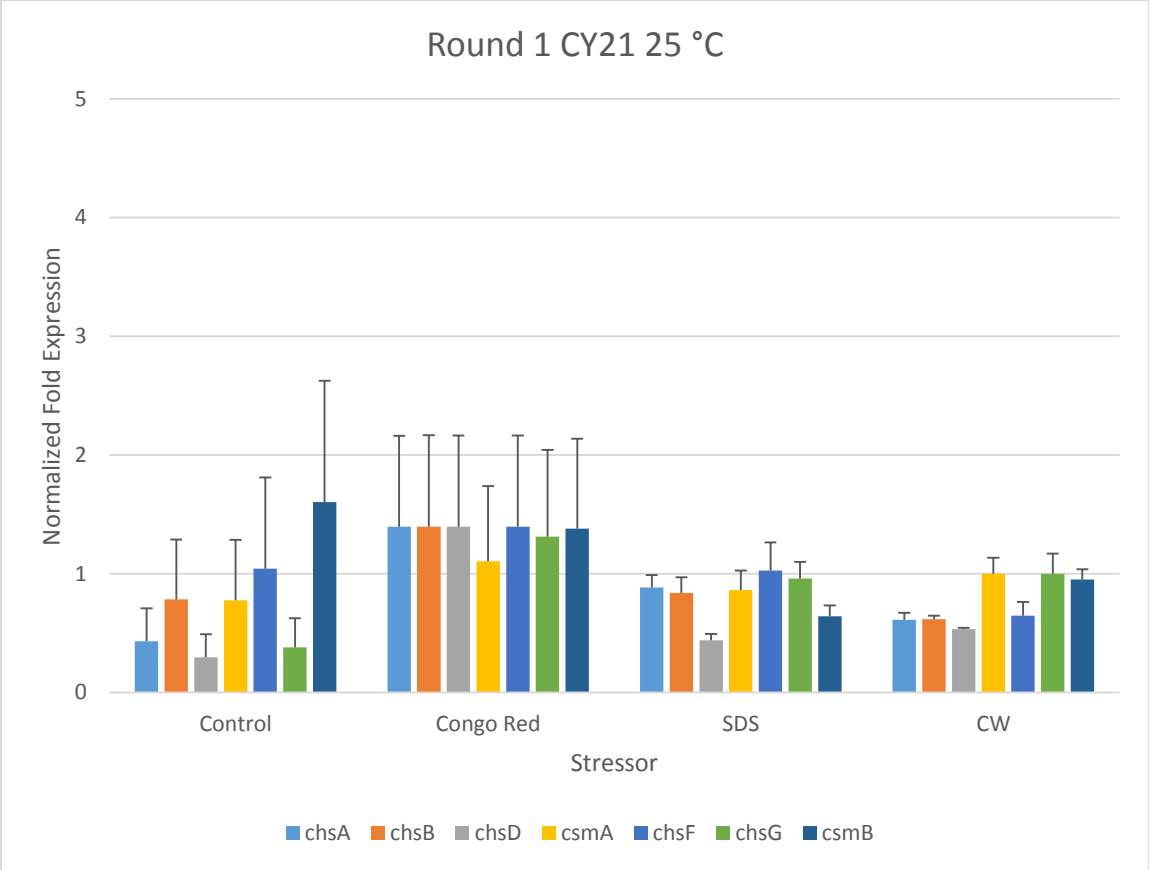


Figure 12: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the CY21 strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix J.

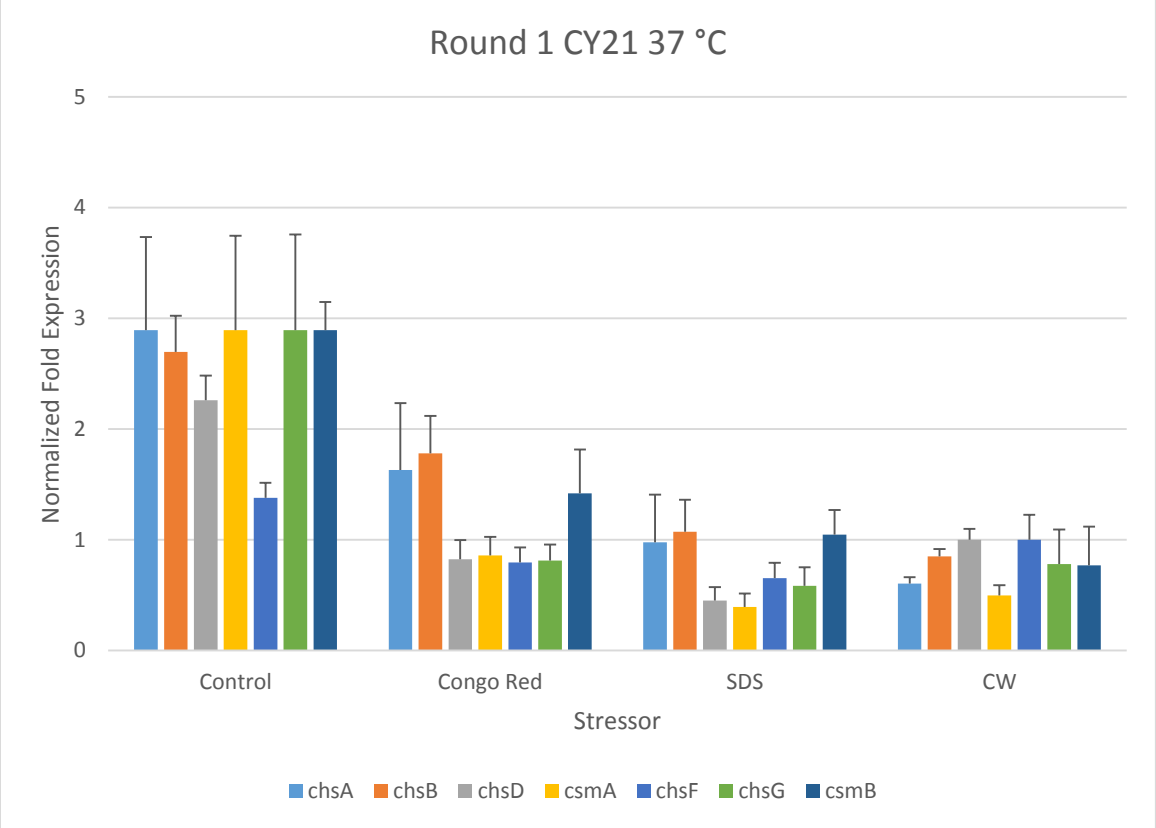


Figure 13: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the CY21 strain of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix J.

Round 1

25°C	Wild Type	*ΔyakA*	CY21
Control	0.2 - 1.0	↑	≈
CR	1.1 - 1.4	≈	≈
SDS	0.4 - 1.8	≈	≈
CW	0.4 - 2.0	≈	≈

37°C	Wild Type	*ΔyakA*	CY21
Control	0.8 - 7.6	↓	↓
CR	0.6 - 1.0	↓	≈
SDS	0.9 - 1.7	↓	≈
CW	0.4 - 1.4	↓	≈

Figure 14: Summary chart illustrating the general patterns of gene expression for each group of chitin synthase genes under each set of possible conditions for the Round 1 data. The normalized relative gene expression range for each wild type condition is listed. An up arrow (↑) indicates a higher fold gene expression range for that strain under that particular condition whereas a down arrow (↓) indicates a lower fold gene expression range for that strain under that particular condition. An approximate equal sign indicates that the indicated gene expression range is approximately equivalent to that of the wild-type.

4.3 Chitin Synthase Gene Expression Results from qRT-PCR Round 2

Overall, the second round of qRT-PCR data revealed a similar pattern of gene expression amongst the different strains and conditions when compared to the first round. For the F4 strain at 25°C under control conditions, the chitin synthase genes possessed very similar patterns of fold expressions ranging from 0.3 (*chsD*) up to 1.0 (*chsF*). Under the CR condition all seven chitin synthases displayed very similar fold expressions ranging only from 1.6 to 1.9. When subjected to SDS, there was a little more variability amongst the chitin synthase genes with the lowest fold expression being 0.5 (*csmB*) and the highest being 1.7 (*chsD*), wherase the CW condition two of the chitin synthase gene fold expressions were 1.5 (*csmA*) and 2.1 (*csmB*). The remainder varied between 0.8 and 1.3 (Figure 7).

At 37°C, the F4 strain under control conditions all chitin synthase gene fold expressions fell between 0.7 and 1.0, except for *chsD* which exhibited a fold expression of 0.2. Again, the *chs* fold expressions under CR exposure showed little variability, ranging from 1.1 to 1.4. The fold expressions in response to SDS exposure ranged from 1.0 to 1.6, except that *chsF* exhibited a fold expression of 0.7. Finally, the CW conditions induced *chs* fold expressions between 0.4 and 1.0, except for *csmB* displayed had a fold expression of 1.6 (Figure 8).

The *ΔyakA* strain at 25°C under control conditions exhibited the highest fold expressions of this study in the second round. Fold expression for *chsA*, *csmA*, *chsG*, and *csmB* all fell between 14.9 and 17.3. Fold expressions for *chsB*, *chsD*, and *chsF* were 6.0,

2.4, and 6.4 respectively. Under the CR stress condition, *chsA* and *chsD* displayed fold expressions at 0.4 and 0.8, whereas the remaining five chitin synthase genes exhibited fold expressions between 2.2 and 3.3. Under the SDS stress condition, all the fold expressions, except for *chsG* (which was1.2), fell between 2.7 and 3.3. For the CW stress condition, all *chs* fold expressions were below 1.0, ranging from 0.2 up to 0.6 (Figure 9).

At 37°C under control conditions, all *chs* genes in the *ΔyakA* strain exhibited, very similar fold expressions, ranging from 2.7 to 2.9. All three of the stress conditions again displayed low *chs* fold expressions between 0.1 and 1.0, with the *chs* fold expressions in response to CR exposure being slightly higher overall (Figure 10).

In the second round of results for the CY21 complement strain, essentially the same expression patterns as in the first round were generated. At 25°C under control conditions, all seven of the chitin synthase gene fold expressions ranged from 0.3 to 1.0. Under the CR stress condition, *chsG* and *csmB* displayed fold expression of 0.8 and 1.0, respectively, while the remaining five genes exhibited fold expressions between 1.6 and 1.9. Under the SDS stress condition, *chsD* revealed a fold expression of 1.7, whereas the remaining six chitin synthase genes showed fold expressions between 0.6 and 1.1. Under the CW stress condition, *csmA*, *chsG*, and *csmB* exhibited fold expressions of 2.0, 2.5, and 2.5, respectively, with the remaining fold expressions between 0.8 and 1.2 (Figure 11).

At 37°C under control conditions for the CY21 strain, the expression levels of *chsA*, *csmA*, and *chsG* were between 1.2 and 1.4 fold, whereas expression of *chsB*, *chsF*, and *csmB* fell between 1.8 and 2.3 fold. The expression of *chsD* was the lowest at 0.7 fold. Under the CR stress condition, *chsA*, *chsB*, and *csmB* expression fell between 1.3

and 1.6 fold. The expression of the remaining four *chs* genes fell between 0.6 and 1.0 fold. Under the SDS conditions, the expression of all seven chitin synthase genes ranged between 0.8 and 1.4 fold. Under the CW condition, the expression folds fell between 0.8 and 1.0 for *chsA*, *chsB*, *chsD* and *chsF*, whereas those of *csmA*, *chsG*, and *csmB* fell between 0.4 and 0.7 for (Figure 12).

Overall, the results for round 2 of this study were similar to the first round with the exception of the fold expressions for the F4 strain at 37°C. At 25°C under control conditions, the F4 strain once again possessed mostly lower expression levels when compared to all of the stress conditions. At 25°C under control conditions, the *ΔyakA* strain displayed much higher fold expressions than the F4 strain or any other strain in the rest of this study. However, the pattern remained the same in that chitin synthase gene fold expression was higher in the control, yet similar in the stress conditions. At 37°C under control conditions, the *ΔyakA* strain again demonstrated had higher levels of chitin synthase gene expression in the control condition and lower fold expressions in the stress conditions. The latter was similar to the F4 strain under the same conditions. The complement strain CY21 at 25°C, like in the first round, demonstrated lower levels of expression in the control conditions and the same stress response expression patterns that have been seen in the other strains and in the first round. At 37°C, CY21 displayed a balanced set of fold expressions in which both the control conditions and the stress conditions ranged from about 0.5 to 2 fold.

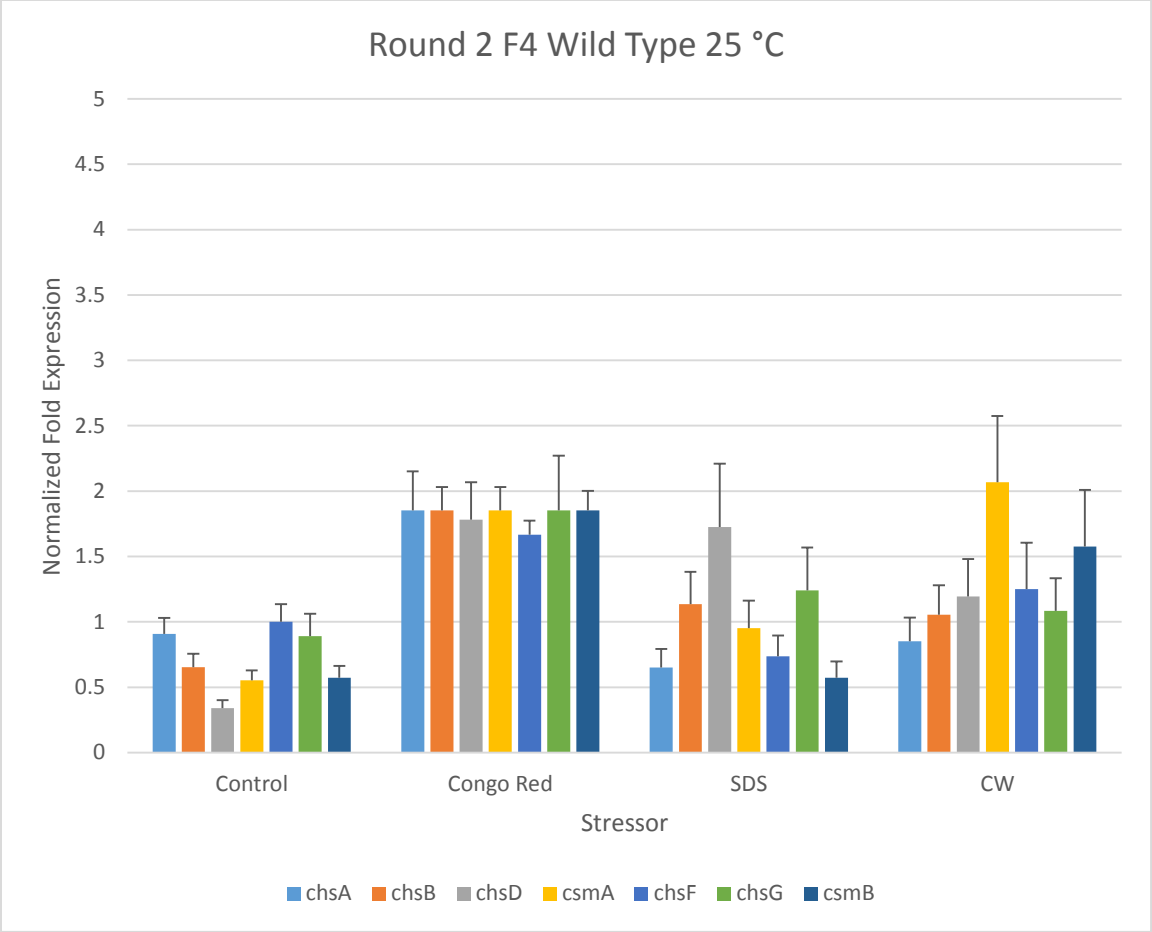


Figure 15: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the F4 (wild type) strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix K.

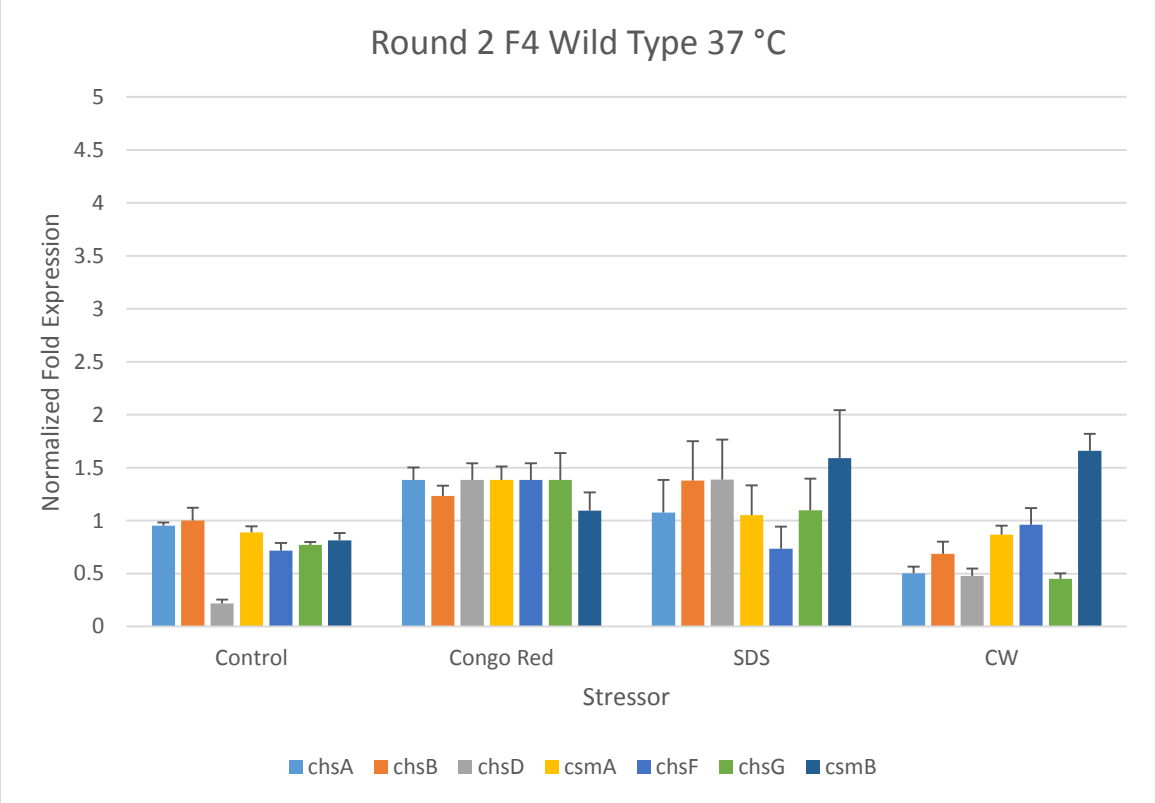


Figure 16: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the F4 (wild type) strain of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix K.

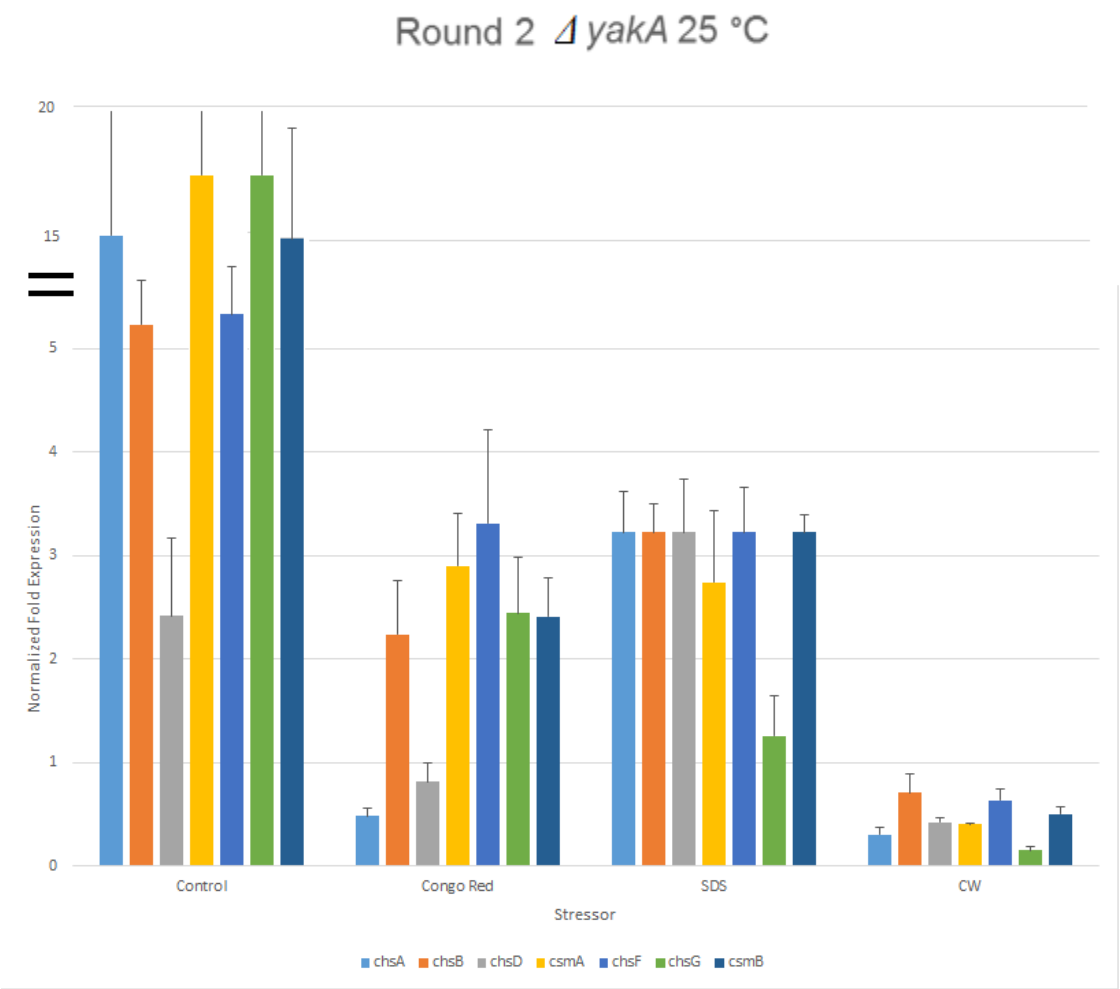


Figure 17: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the *ΔyakA* strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix K.

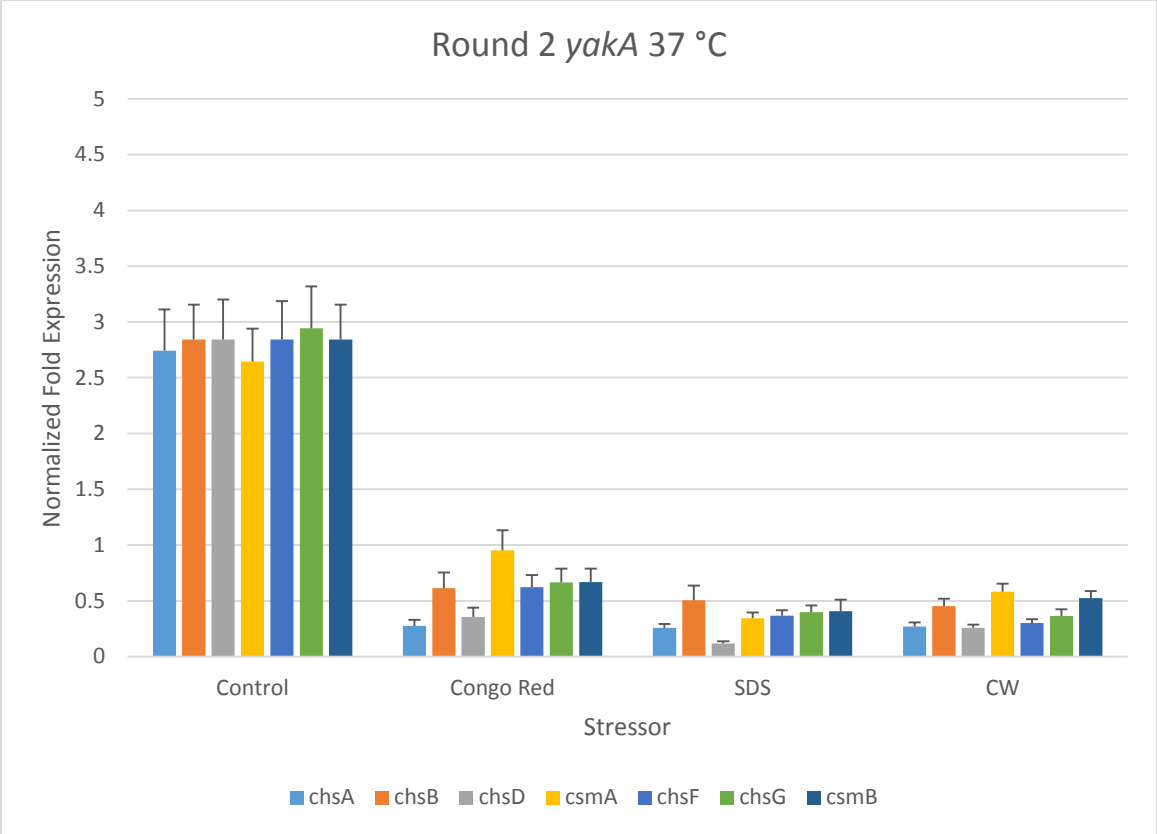


Figure 18: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the *ΔyakA* strain of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix K.

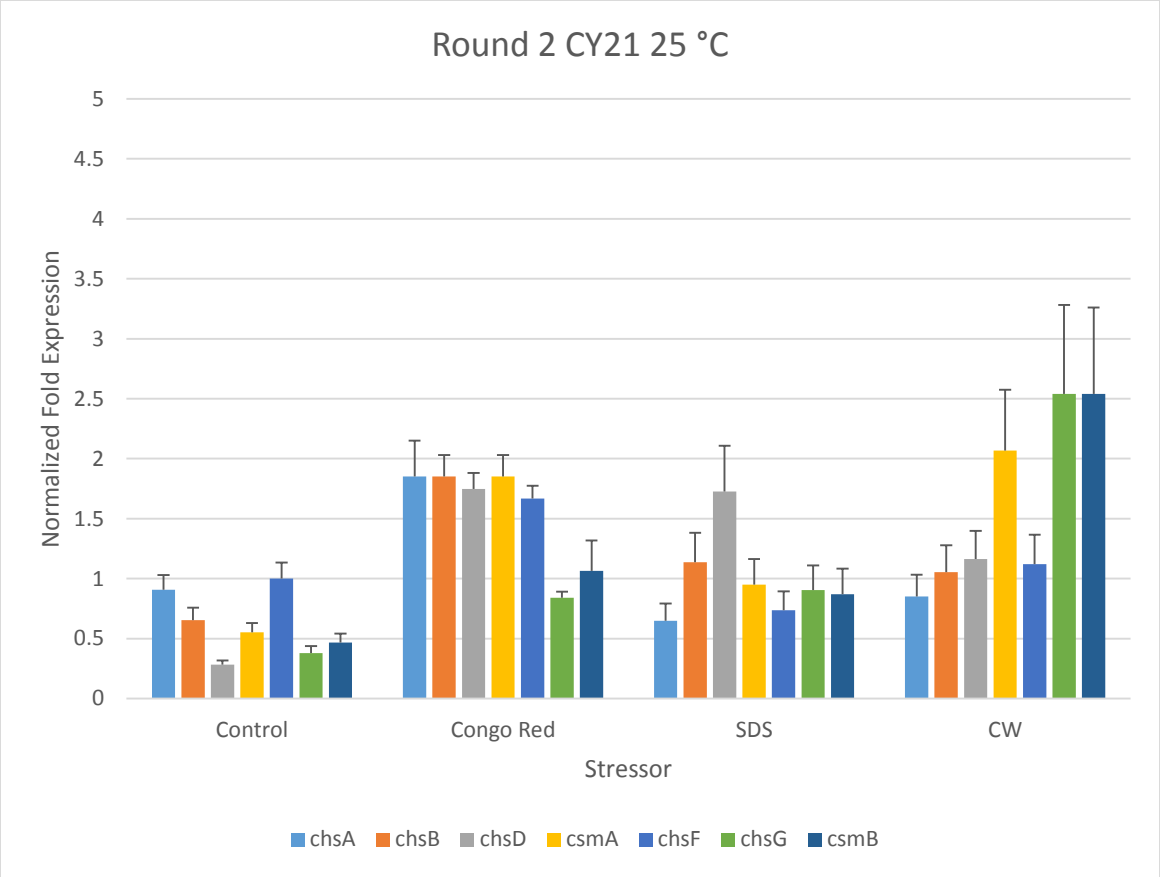


Figure 19: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the CY21 strain of *P. marneffei* at 25°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix K.

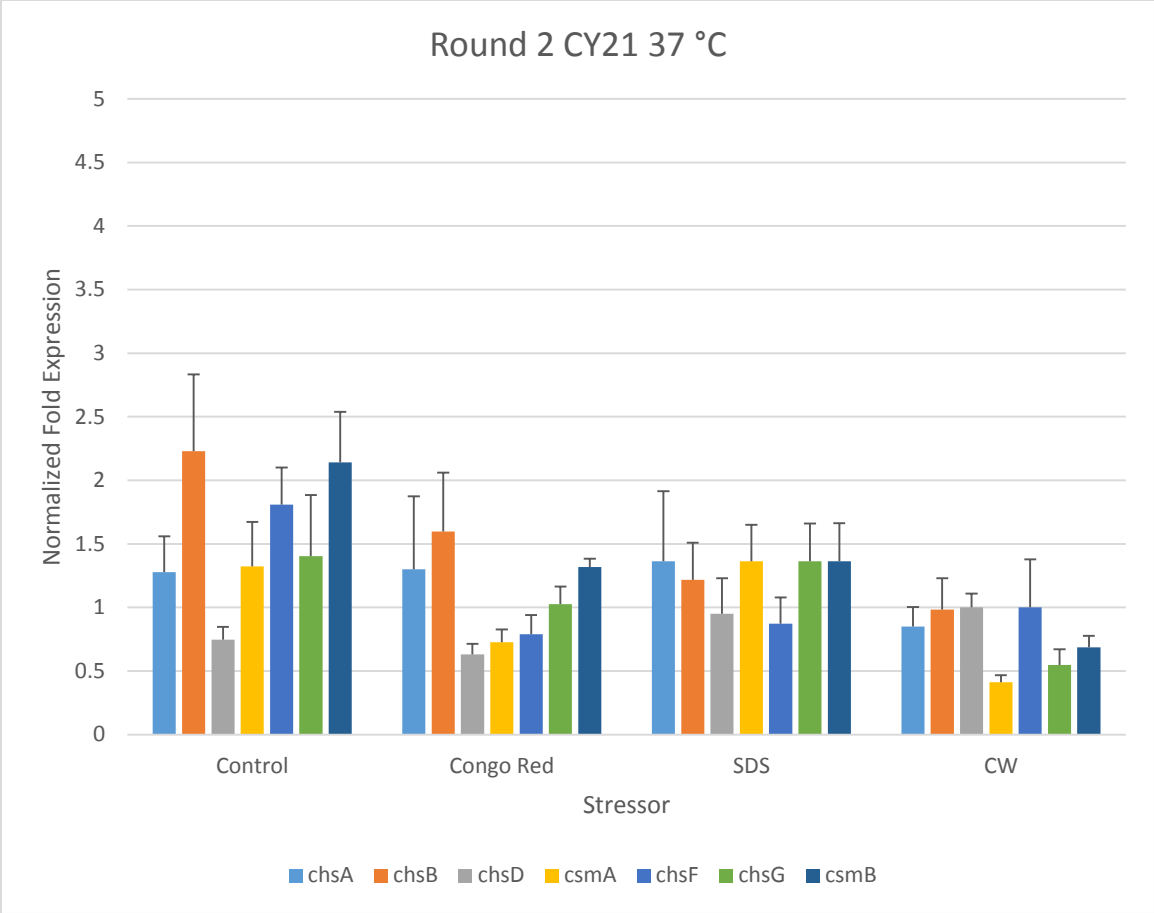


Figure 20: Normalized, relative gene expression of the chitin synthase genes *chsA*, *chsB*, *chsD*, *csmA*, *chsF*, *chsG*, and *csmB* in the CY21 strain of *P. marneffei* at 37°C. Expression levels were quantified while subjected to cell-wall stress due to Congo Red, sodium dodecyl sulfate (SDS), or Calcofluor White (CW), as well as under non-stressed (control) conditions. All results were normalized to the *benA* gene and performed in triplicate. Error bars represent one standard deviation. Statistical analyses and interaction terms for Round 1 data can be found in Appendix K.

Round 2

25°C	Wild Type	*ΔyakA*	CY21
Control	0.3 - 1.0	↑	≈
CR	1.6 - 1.9	≈	≈
SDS	0.5 - 1.8	≈	≈
CW	0.8 - 2.0	≈	≈

37°C	Wild Type	*ΔyakA*	CY21
Control	0.2 - 1.0	↑	≈
CR	1.0 - 1.4	↓	≈
SDS	0.7 - 1.6	↓	≈
CW	0.4 - 1.7	↓	≈

Figure 21: Summary chart illustrating the general patterns of gene expression for each group of chitin synthase genes under each set of possible conditions for the Round 2 data. The normalized relative gene expression range for each wild type condition is listed. An up arrow (↑) indicates a higher fold gene expression range for that strain under that particular condition whereas a down arrow (↓) indicates a lower fold gene expression range for that strain under that particular condition. An approximate equal sign indicates that the indicated gene expression range is approximately equivalent to that of the wild-type.

CHAPTER 5: DISCUSSION

The graphical presentations of the qRT-PCR data from both rounds of this study reveal a simple, yet informative pattern. At both 25°C and 37°C, the *ΔyakA* strain under control conditions possessed significantly higher expression levels of all of the chitin synthase genes that were studied. However, it is important to note that any attempt to analyze these seven chitin synthase genes individually or in terms of their interactions amongst each other yielded no significance (P value = 0.05). Therefore, it was necessary to consider them as a whole, and this model provided a much more insightful picture of the interactions that are occurring in this study. When subjected to multivariate statistical analyses, only the strain versus condition model yielded significance. This significance occurred for samples at both 25°C and 37°C and for both rounds of data. Models that looked at strain versus gene and gene versus condition yielded no significant interactions (P values > 0.05). Based on these outcomes, the next step was to subject the mold (25°C) and yeast (37°C) samples to univariate analysis with Tukey and Bonferroni Post-hoc tests. These analyses would allow for the identification of the interactions that produced the significant multivariate models and provide a more detailed insight into the study as a whole. Examination of the mold sample data from the first round of qRT-PCR analysis, the *ΔyakA* strain control condition had significant interactions with all of the other possible strain-condition combinations except for the stress response due to SDS. This reveals the statistical significance of the observed outcome of this portion of the study and provides additional evidence that the deletion of the *yakA* gene resulting in increased chitin synthase gene expression is not a random occurrence. This significance actually

did not occur in the 37°C samples for the first round. Significant interactions at 37°C for the first round always involved either the wild-type strain under control conditions or CY21 under control conditions. For the second round of data, the *ΔyakA* control conditions at both 25°C and 37°C produced significant interactions with every other possible combination of strain and condition. Furthermore, all three strains at 37°C in the second round of data produced a large number of significant interactions. This most-likely occurred due to the consistency amongst the fold expression values that is present at 37°C for Round 2.

Both the graphical representations and the statistical analyses of this study make it easy to see that an interrupted *yakA* gene results in significantly higher levels of chitin synthase gene expression under non-stressed control conditions. These outcomes also show that this interrupted gene seemingly has no impact on how these chitin synthases react to cell-wall stress conditions. Between all three strains, there is virtually no difference in stress responses to any of the three stressors that were used. Taken together, these two facts indicate that while an interrupted *yakA* gene may not interfere with the regulation of chitin synthase gene expression in terms of stress responses, it appears to invoke a general compensatory response to deal with its weakened cell wall. The cell-wall deformities caused by the interrupted *yakA* gene do not directly involve chitin synthase activity. Rather, the increased chitin synthase activity that is witnessed is a natural response that is carried out by a group of rather unspecialized synthases that can shift their roles when the cell is in danger of dying. It is likely, based on the literature review for this study, that any number of gene deletions, not just *yakA*, could have caused a very similar chitin synthase response to the one that is seen here. The actual effect

caused by the *yakA* gene interruption most likely involves glucan synthase gene expression as determined by the master thesis work of Sarah Eisnaugle (unpublished data). The stressed condition results reveal that there does not seem to be any effect on the stress response of these chitin synthases. What seems to have been observed in this study is an attempt by the fungus to reinforce its cell wall. Although there is not a direct correlation of these results with the virulence attributes of *P. marneffei*, understanding these type of compensatory interactions may enhance the development of antifungal treatments.

CHAPTER 6: APPENDICES

Appendix A: List of primers used for both RT-PCR and qRT-PCR experiments:

Gene	qRT-pcr Primer Sequence	Description
chsA-F	TTATACAGACAATCGCACTCCC	chitin synthase
chsA-R	GTTGATTCCTTCGACCGAGTAC	chitin synthase
chsB-F	GCGTTGCAATTTATCCTCTCG	chitin synthase
chsB-R	TCCACCGCCTTTCAGTTTATC	chitin synthase
chsD-F	GTGTCATCGCGGTTTTCATC	chitin synthase
chsD-R	TTAACGAGAGCCCAGGAAAAG	chitin synthase
csmA-F	CCCCTCCCATACACAATCTTC	chitin synthase
csmA-R	CAGACAAATCAAAGCCTGCG	chitin synthase
chsF-F	AGCCGAGGGTGAATTTGATAG	chitin synthase
chsF-R	GGTAGGCATATGGCGCTG	chitin synthase
chsG-F	CTACCCTGTTCCTAGTGCTATTC	chitin synthase
chsG-R	CGTAAGTTGTAACCGTTGTGC	chitin synthase
csmB-F	GTATCGTTGATCCCCAGTTAGG	chitin synthase
csmB-R	GGAGTAGATAGTGGTAAGATAGAA	chitin synthase
benA-F	GCTCCGGTGTCTACAATGGC	β-tubulin (used for normalization)
benA-R	AGTTGTTACCAGCACCGGAC	β-tubulin (used for normalization)

Appendix B: Stressor concentrations and respective inoculation volumes for cell-wall stress experiments performed at 25°C:

Stressor	Inoculation Volume	Concentration in Culture
CR	350 µl	70 µg ml^{-1}
Sodium Dodecyl Sulfate (SDS)	250 µl	0.02%
CW	300 µl	60 µg ml^{-1}

Appendix C: Stressor concentrations and respective inoculation volumes for cell-wall stress experiments performed at 37°C:

Stressor	Inoculation Volume	Concentration in Culture
CR	200 µl	40 µg ml^{-1}
Sodium Dodecyl Sulfate (SDS)	220 µl	0.0175%
CW	175 µl	35 µg ml^{-1}

Appendix D: Qiagen One-Step RT-PCR Protocol used to assess primer quality.

QIAGEN One-Step RT-PCR Protocol	Volume/reaction	Final Concentration
RNase-free water	Variable	_
5x Qiagen OneStep RT-PCR Buffer	10 µl	1x
dNTP Mix	2 µl	400 µM of each dNTP
Forward Primer	Variable	0.6 µM
Reverse Primer	Variable	0.6 µM
OneStep RT-PCR Enzyme Mix	2 µl	_
Template RNA	Variable	1 pg - 2 µg/reaction
Total Volume	**50 µl**	

Appendix E: Qiagen One-Step RT-PCR thermal cycler program used to assess primer quality.

Thermal Cycler Program for RT-PCR	Time/Cycles	Temperature
Reverse Transcription	30 min	50 °C
Initial PCR activation step	15 min	95 °C
Cycle: 3 steps		
1 Denaturing:	0.5-1 min	94 °C
2 Annealing:	0.5-1 min	50-68 °C
3 Extension:	1 min	72 °C
Number of Cycles:	25-40	
Final Extension:	10 min	72 °C
Hold	∞	4°C

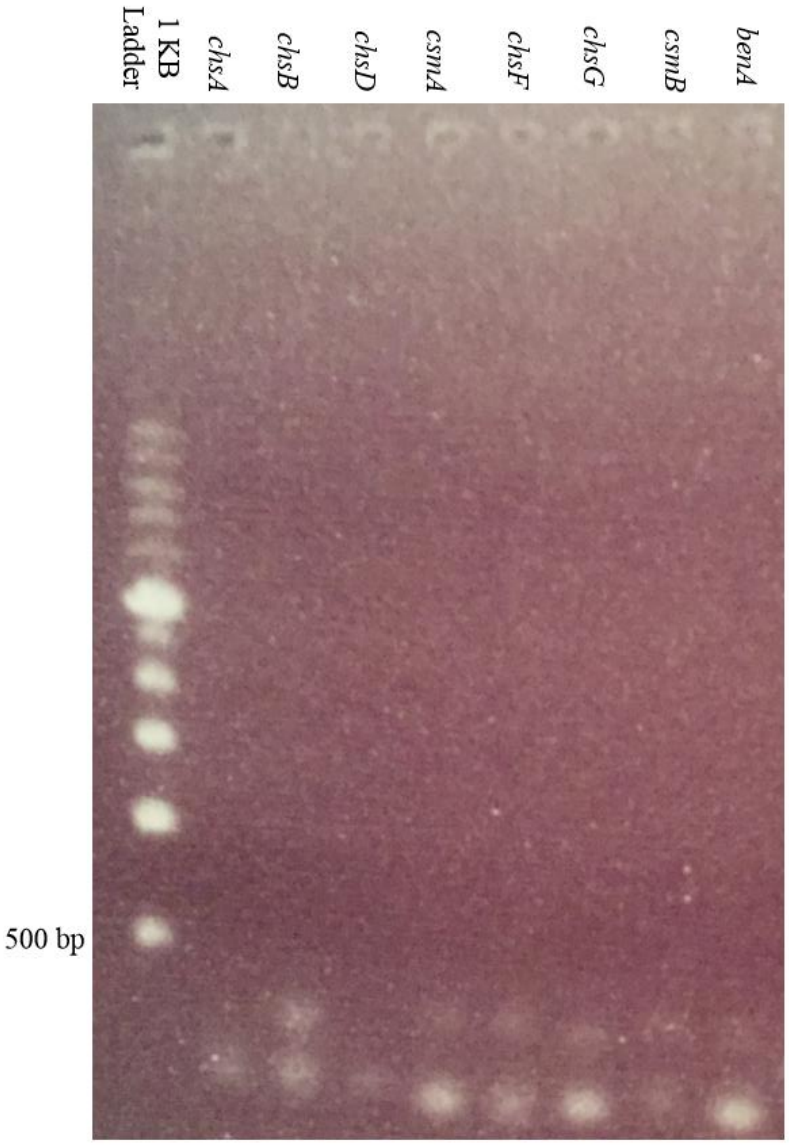
Appendix F: iTaq Universal SYBR Green One-Step kit protocol used used with the Bio-Rad iQ5 Real-Time PCR System Thermocycler for all qRT-PCR experiments.

iTaq Universal SYBR Green One-Step Kit Protocol	Volume per 20 µl Reaction
iTaq universal SYBR Green reaction mix	10 µl
iscript reverse transcriptase	0.25 µl
Forward primer (working stock concentration: 10 µM)	2 µl
Reverse primer (working stock concentration: 10 µM)	2 µl
Nuclease-free water	3.75 µl
RNA (working stock concentration: 50 ng/µl)	2 µl
Total	20 µl

Appendix G: iTaq Universal SYBR Green One-Step kit thermal cycler program used with the Bio-Rad iQ5 Real-Time PCR System Thermocycler for all qRT-PCR experiments.

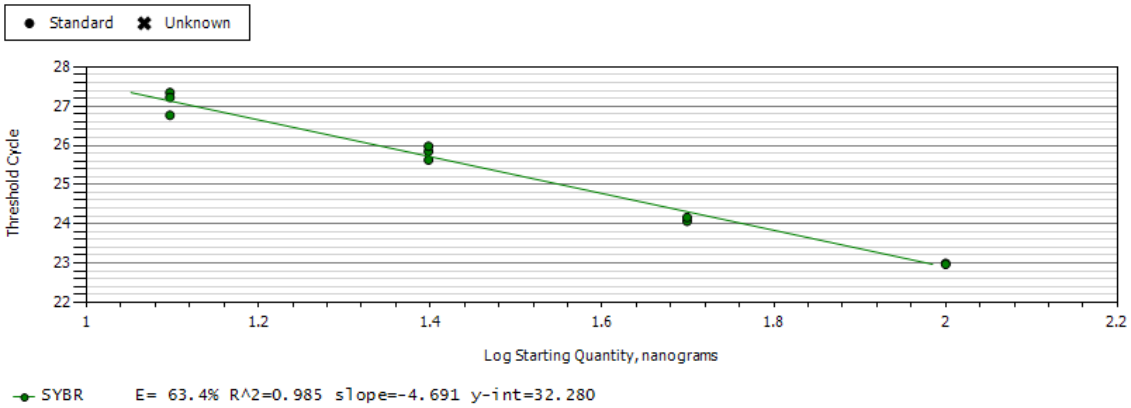
iTaq Universal SYBR Green One-Step Kit Thermal Cycler Program	**Time/Cycles**	**Temperature**
Reverse Transcription Reaction	10 min	50 °C
Polymerase Activation and DNA Denaturation	5 min	95 °C
Cycle: 2 steps		
Denaturation	10 sec	95 °C
Annealing/Extension + Plate Read	30 sec	60 °C
Number of Cycles	45	
Melt Curve	1 min	95 °C
	1 min	55 °C
Repeat this step x 81 cycles	10 sec	55 °C

Appendix H: RT-PCR products of wild-type, non-stressed control RNA samples. Lane 1 contains a 1 kilobase molecular weight ladder. All resulting bands were between 200 and 400 kilobases as would be expected for these type of PCR products.

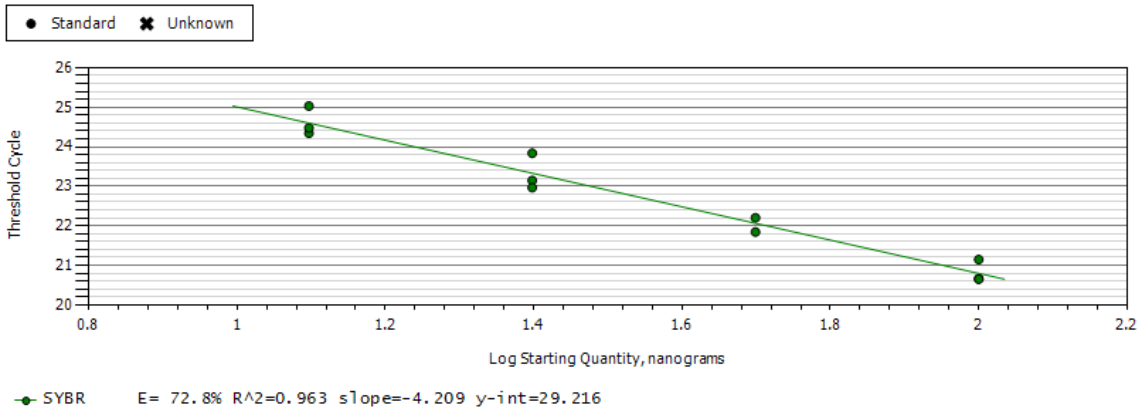


Appendix I: Standard curves produced for assessing primer quality using the Bio-Rad iQ5 Real-Time PCR System software for each of the genes analyzed in this study.

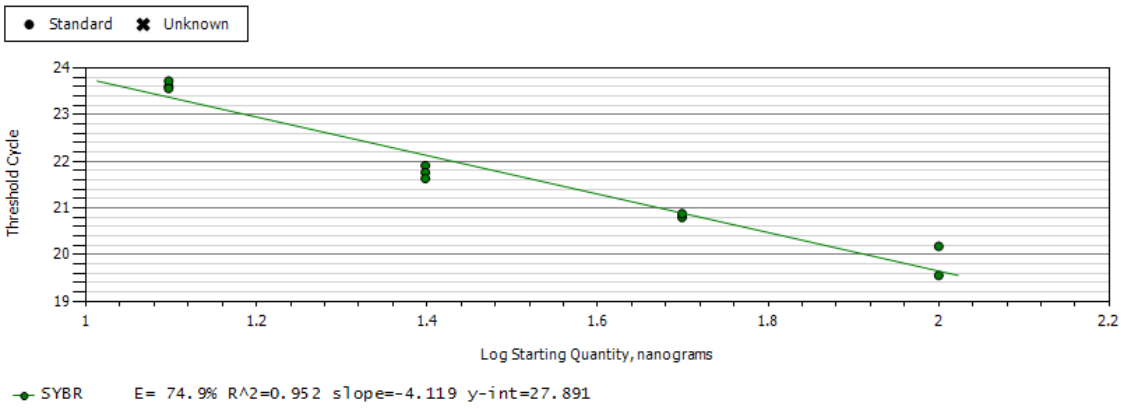
chsA



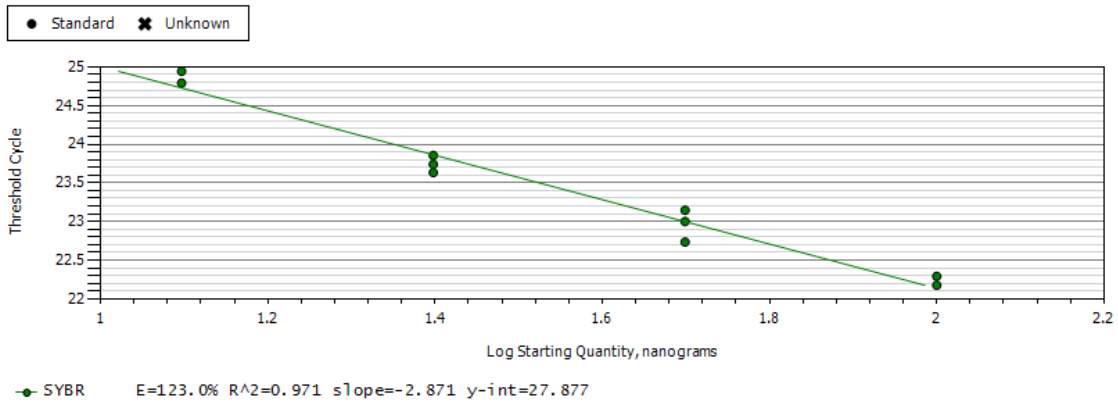
chsB



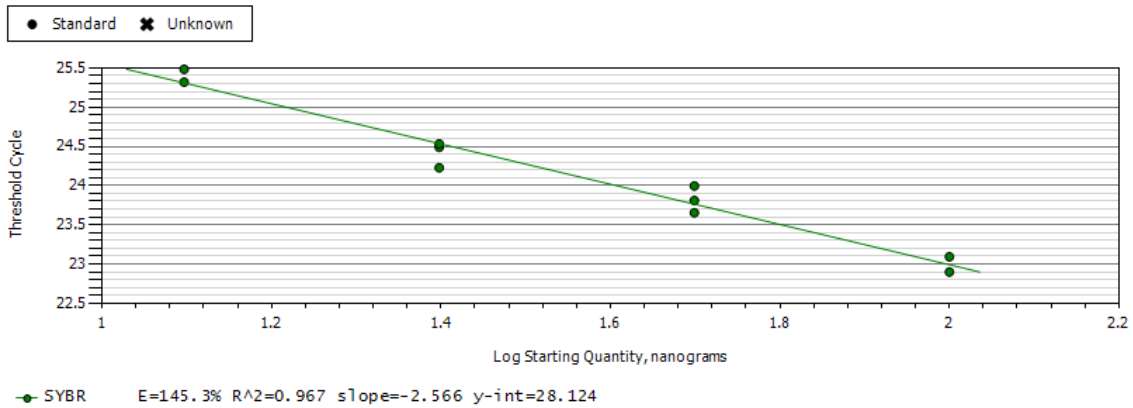
chsD



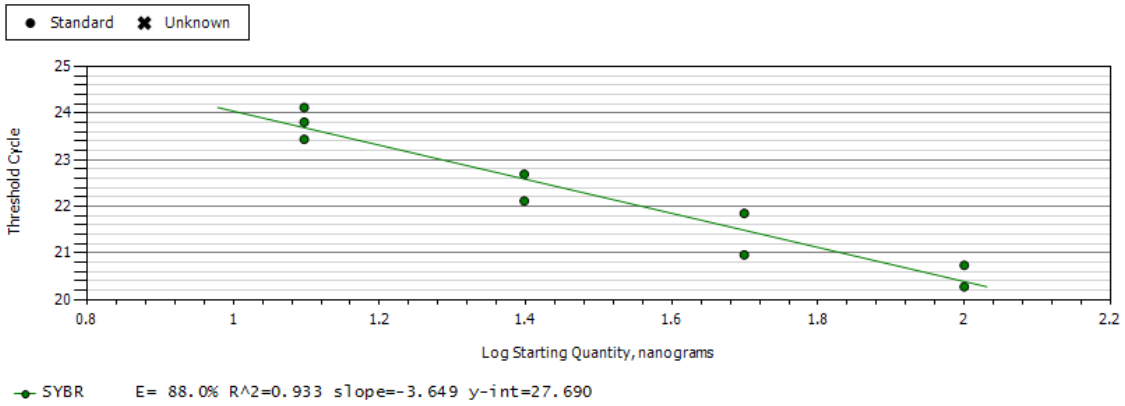
csmA



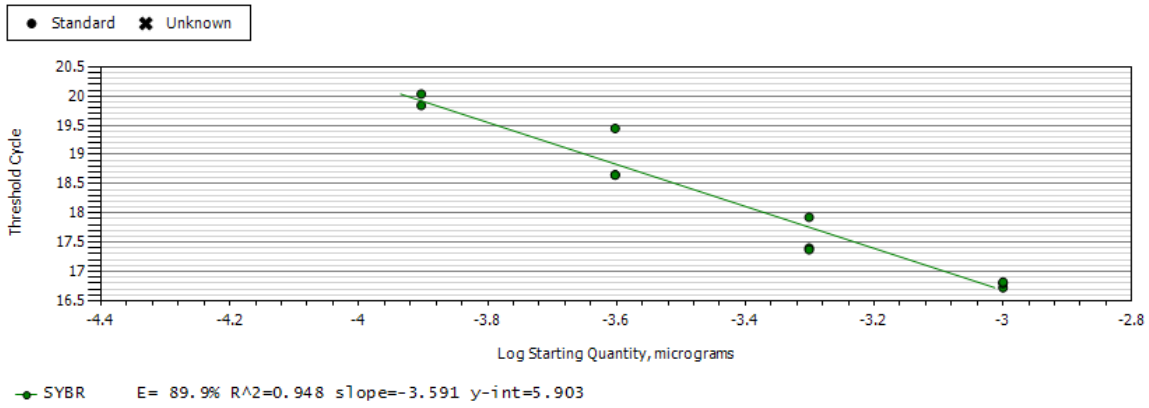
chsF



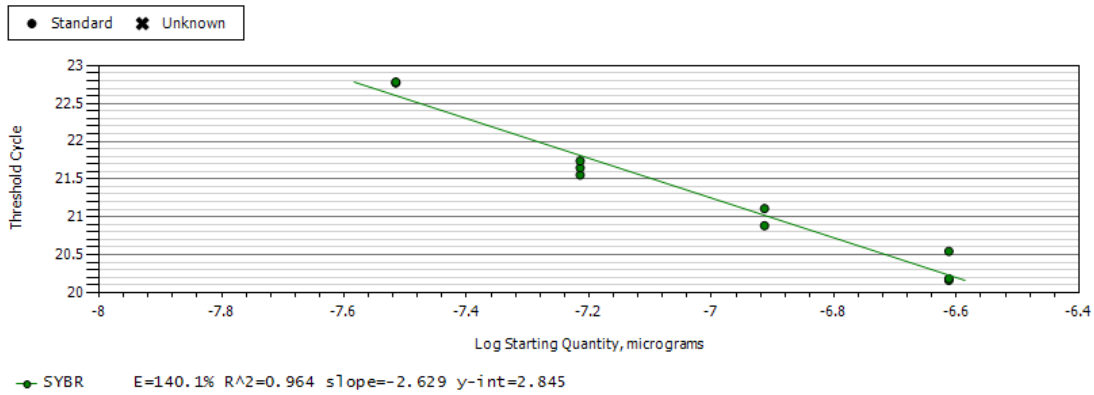
chsG



csmB



benA



Appendix J: Statistical analyses for round 1 qRT-PCR data. Only significant post-Hoc test results ($p < .05$) have been listed. Refer to the key below for post-hoc chart categories:

Term	Meaning
Mold	Gene expression for all samples at 25°C
Yeast	Gene expression for all samples at 37°C
StrainCond 1	F4 WT Control
StrainCond 2	F4 WT CR
StrainCond 3	F4 WT SDS
StrainCond 4	F4 WT CW
StrainCond 5	*yakA* Control
StrainCond 6	*yakA* CR
StrainCond 7	*yakA* SDS
StrainCond 8	*yakA* CW
StrainCond 9	CY21 Control
StrainCond 10	CY21 CR
StrainCond 11	CY21 SDS
StrainCond 12	CY21 CW

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.876	250.711	2.000	71.000	0.000
	Wilks' Lambda	0.124	250.711	2.000	71.000	0.000
	Hotelling's Trace	7.062	250.711	2.000	71.000	0.000
	Roy's Largest Root	7.062	250.711	2.000	71.000	0.000
Strain	Pillai's Trace	0.380	8.457	4.000	144.000	0.000
	Wilks' Lambda	0.625	9.419	4.000	142.000	0.000
	Hotelling's Trace	0.593	10.376	4.000	140.000	0.000
	Roy's Largest Root	0.579	20.840	2.000	72.000	0.000
Condition	Pillai's Trace	0.523	8.502	6.000	144.000	0.000
	Wilks' Lambda	0.499	9.824	6.000	142.000	0.000
	Hotelling's Trace	0.957	11.169	6.000	140.000	0.000
	Roy's Largest Root	0.908	21.781	3.000	72.000	0.000
Strain * Condition	Pillai's Trace	0.533	4.355	12.000	144.000	0.000
	Wilks' Lambda	0.498	4.939	12.000	142.000	0.000
	Hotelling's Trace	0.948	5.530	12.000	140.000	0.000
	Roy's Largest Root	0.878	10.542	6.000	72.000	0.000

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mold	18.508	11.000	1.683	6.358	0.000
	Yeast	67.831	11.000	6.166	9.720	0.000
Intercept	Mold	100.343	1.000	100.343	379.194	0.000
	Yeast	97.989	1.000	97.989	154.454	0.000
Strain	Mold	2.932	2.000	1.466	5.540	0.006
	Yeast	18.786	2.000	9.393	14.806	0.000
Condition	Mold	1.937	3.000	0.646	2.440	0.071
	Yeast	40.009	3.000	13.336	21.021	0.000
Strain * Condition	Mold	13.638	6.000	2.273	8.590	0.000
	Yeast	9.037	6.000	1.506	2.374	0.038
Error	Mold	19.053	72.000	0.265		
	Yeast	45.678	72.000	0.634		
Total	Mold	137.903	84.000			
	Yeast	211.498	84.000			
Corrected Total	Mold	37.561	83.000			
	Yeast	113.510	83.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.822	142.836	2.000	62.000	0.000
	Wilks' Lambda	0.178	142.836	2.000	62.000	0.000
	Hotelling's Trace	4.608	142.836	2.000	62.000	0.000
	Roy's Largest Root	4.608	142.836	2.000	62.000	0.000
Strain	Pillai's Trace	0.244	4.374	4.000	126.000	0.002
	Wilks' Lambda	0.758	4.600	4.000	124.000	0.002
	Hotelling's Trace	0.316	4.819	4.000	122.000	0.001
	Roy's Largest Root	0.307	9.667	2.000	63.000	0.000
Gene	Pillai's Trace	0.067	0.365	12.000	126.000	0.973
	Wilks' Lambda	0.934	0.360	12.000	124.000	0.975
	Hotelling's Trace	0.070	0.354	12.000	122.000	0.976
	Roy's Largest Root	0.040	0.420	6.000	63.000	0.863
Strain * Gene	Pillai's Trace	0.196	0.569	24.000	126.000	0.945
	Wilks' Lambda	0.814	0.561	24.000	124.000	0.949
	Hotelling's Trace	0.218	0.554	24.000	122.000	0.953
	Roy's Largest Root	0.132	0.692	12.000	63.000	0.753

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mold	7.026	20.000	0.351	0.725	0.786
	Yeast	31.270	20.000	1.564	0.198	0.286
Intercept	Mold	100.343	1.000	100.343	207.029	0.000
	Yeast	97.989	1.000	97.989	75.065	0.000
Strain	Mold	2.932	2.000	1.466	3.025	0.056
	Yeast	18.786	2.000	9.393	7.196	0.002
Gene	Mold	0.922	6.000	0.154	0.317	0.926
	Yeast	3.230	6.000	0.538	0.412	0.868
Strain * Gene	Mold	3.172	12.000	0.264	0.545	0.876
	Yeast	9.254	12.000	0.771	0.591	0.842
Error	Mold	30.535	63.000	0.485		
	Yeast	82.239	63.000	1.305		
Total	Mold	137.903	84.000			
	Yeast	211.498	84.000			
Corrected Total	Mold	37.561	83.000			
	Yeast	113.510	83.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.880	201.218	2.000	55.000	0.000
	Wilks' Lambda	0.120	201.218	2.000	55.000	0.000
	Hotelling's Trace	7.317	201.218	2.000	55.000	0.000
	Roy's Largest Root	7.317	201.218	2.000	55.000	0.000
Gene	Pillai's Trace	0.091	0.443	12.000	112.000	0.942
	Wilks' Lambda	0.911	0.437	12.000	110.000	0.945
	Hotelling's Trace	0.096	0.431	12.000	108.000	0.948
	Roy's Largest Root	0.068	0.637	6.000	56.000	0.700
Condition	Pillai's Trace	0.497	6.174	6.000	112.000	0.000
	Wilks' Lambda	0.516	7.197	6.000	110.000	0.000
	Hotelling's Trace	0.914	8.230	6.000	108.000	0.000
	Roy's Largest Root	0.886	16.547	3.000	56.000	0.000
Gene * Condition	Pillai's Trace	0.322	0.596	36.000	112.000	0.962
	Wilks' Lambda	0.703	0.589	36.000	110.000	0.964
	Hotelling's Trace	0.388	0.582	36.000	108.000	0.967
	Roy's Largest Root	0.249	0.774	18.000	56.000	0.720

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mold	8.489	27.000	0.314	0.606	0.922
	Yeast	53.000	27.000	1.963	1.817	0.030
Intercept	Mold	100.343	1.000	100.343	193.291	0.000
	Yeast	97.989	1.000	97.989	90.687	0.000
Gene	Mold	0.922	6.000	0.154	0.296	0.936
	Yeast	3.230	6.000	0.538	0.498	0.807
Condition	Mold	1.937	3.000	0.646	1.244	0.302
	Yeast	40.009	3.000	13.336	12.342	0.000
Gene * Condition	Mold	5.631	18.000	0.323	0.603	0.882
	Yeast	9.254	18.000	0.542	0.502	0.946
Error	Mold	30.535	56.000	0.519		
	Yeast	82.239	56.000	1.081		
Total	Mold	137.903	84.000			
	Yeast	211.498	84.000			
Corrected Total	Mold	37.561	83.000			
	Yeast	113.510	83.000			

Dependent Variable: Mold					
Source	Type III Sum of Squares	df	Mean Suare	F	Sig.
Corrected Model	18.508	11.000	1.683	6.358	0.000
Intercept	100.343	1.000	100.343	379.194	0.000
Strain Condition	18.508	11.000	1.683	6.358	0.000
Error	19.053	72.000	0.265		
Total	137.903	84.000			
Corrected Total	37.561	83.000			

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Mold	Tukey HSD	1	5	0
		2	5	0.013
		3	5	0
		4	5	0.001
		5	1	0
			2	0.013
			3	0
			4	0.001
			6	0
			8	0
			9	0
			10	0.02
			11	0
			12	0
		6	5	0
		8	5	0
		9	5	0
		10	5	0.02
		11	5	0
		12	5	0
	Bonferroni	1	5	0
		2	5	0.018
		3	5	0.001
		4	5	0.001

		5	1	0
			2	0.018
			3	0.001
			4	0.001
			6	0
			8	0
			9	0
			10	0.027
			11	0
			12	0
		6	5	0
		8	5	0
		9	5	0
		10	5	0.027
		11	5	0
		12	5	0

Dependent Variable: Yeast					
Source	Type III Sum of Squares	df	Mean Suare	F	Sig.
Corrected Model	67.831	11.000	6.166	9.720	0.000
Intercept	97.989	1.000	97.989	154.454	0.000
Strain Condition	67.831	11.000	6.166	9.720	0.000
Error	45.678	72.000	0.634		
Total	211.498	84.000			
Corrected Total	113.510	83.000			

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Yeast	Tukey HSD	1	2	0
			3	0
			4	0
			5	0
			6	0
			7	0
			8	0
			10	0
			11	0
			12	0
		9	2	0.011
			3	0.022
			4	0.005
			5	0.011
			6	0
			7	0
			8	0
			11	0.003
			12	0.005
	Bonferroni	1	2	0
			3	0
			4	0
			5	0
			6	0

			7	0
			8	0
			10	0
			11	0
			12	0
		9	2	0.014
			3	0.03
			4	0.007
			5	0.014
			6	0
			7	0
			8	0
			11	0.004
			12	0.006

Appendix K: Statistical analyses for round 1 qRT-PCR data. Only significant post-Hoc test results ($p < .05$) have been listed. Refer to the key below for post-hoc chart categories:

Term	Meaning
Mold	Gene expression for all samples at 25 °C
Yeast	Gene expression for all samples at 37 °C
StrainCond 1	F4 WT Control
StrainCond 2	F4 WT CR
StrainCond 3	F4 WT SDS
StrainCond 4	F4 WT CW
StrainCond 5	*yakA* Control
StrainCond 6	*yakA* CR
StrainCond 7	*yakA* SDS
StrainCond 8	*yakA* CW
StrainCond 9	CY21 Control
StrainCond 10	CY21 CR
StrainCond 11	CY21 SDS
StrainCond 12	CY21 CW

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.949	656.923	2.000	71.000	0.000
	Wilks' Lambda	0.051	656.923	2.000	71.000	0.000
	Hotelling's Trace	18.505	656.923	2.000	71.000	0.000
	Roy's Largest Root	18.505	656.923	2.000	71.000	0.000
Strain	Pillai's Trace	0.450	10.453	4.000	144.000	0.000
	Wilks' Lambda	0.567	11.641	4.000	142.000	0.000
	Hotelling's Trace	0.733	12.828	4.000	140.000	0.000
	Roy's Largest Root	0.689	24.808	2.000	72.000	0.000
Condition	Pillai's Trace	0.738	14.038	6.000	144.000	0.000
	Wilks' Lambda	0.265	22.314	6.000	142.000	0.000
	Hotelling's Trace	2.763	32.238	6.000	140.000	0.000
	Roy's Largest Root	2.759	66.218	3.000	72.000	0.000
Strain * Condition	Pillai's Trace	1.052	13.321	12.000	144.000	0.000
	Wilks' Lambda	0.131	20.825	12.000	142.000	0.000
	Hotelling's Trace	5.219	30.447	12.000	140.000	0.000
	Roy's Largest Root	4.936	59.237	6.000	72.000	0.000

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mold	672.509	11.000	61.145	17.941	0.000
	Yeast	34.623	11.000	3.148	40.302	0.000
Intercept	Mold	404.294	1.000	404.294	118.625	0.000
	Yeast	96.517	1.000	96.517	1235.812	0.000
Strain	Mold	166.232	2.000	83.116	24.387	0.006
	Yeast	0.287	2.000	0.143	1.835	0.167
Condition	Mold	119.131	3.000	39.710	11.651	0.000
	Yeast	13.157	3.000	4.386	56.155	0.000
Strain * Condition	Mold	387.227	6.000	64.538	18.936	0.000
	Yeast	21.180	6.000	3.530	45.198	0.000
Error	Mold	245.389	72.000	3.408		
	Yeast	5.623	72.000	0.078		
Total	Mold	1322.272	84.000			
	Yeast	136.763	84.000			
Corrected Total	Mold	917.979	83.000			
	Yeast	40.246	83.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.742	88.959	2.000	62.000	0.000
	Wilks' Lambda	0.258	88.959	2.000	62.000	0.000
	Hotelling's Trace	2.870	88.959	2.000	62.000	0.000
	Roy's Largest Root	2.870	88.959	2.000	62.000	0.000
Strain	Pillai's Trace	0.363	6.985	4.000	126.000	0.002
	Wilks' Lambda	0.639	7.776	4.000	124.000	0.002
	Hotelling's Trace	0.561	8.560	4.000	122.000	0.001
	Roy's Largest Root	0.555	17.494	2.000	63.000	0.000
Gene	Pillai's Trace	0.089	0.489	12.000	126.000	0.918
	Wilks' Lambda	0.912	0.484	12.000	124.000	0.921
	Hotelling's Trace	0.094	0.479	12.000	122.000	0.924
	Roy's Largest Root	0.071	0.741	6.000	63.000	0.619
Strain * Gene	Pillai's Trace	0.128	0.360	24.000	126.000	0.997
	Wilks' Lambda	0.873	0.362	24.000	124.000	0.997
	Hotelling's Trace	0.143	0.363	24.000	122.000	0.997
	Roy's Largest Root	0.127	0.666	12.000	63.000	0.777

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mold	219.979	20.000	10.999	0.993	0.483
	Yeast	2.317	20.000	0.116	0.192	1.000
Intercept	Mold	404.294	1.000	404.294	36.491	0.000
	Yeast	96.517	1.000	96.517	160.312	0.000
Strain	Mold	166.232	2.000	83.116	7.502	0.001
	Yeast	0.287	2.000	0.143	0.238	0.789
Gene	Mold	18.814	6.000	3.136	0.238	0.943
	Yeast	1.299	6.000	0.216	0.360	0.902
Strain * Gene	Mold	34.933	12.000	2.911	0.263	0.993
	Yeast	0.731	12.000	0.061	0.101	1.000
Error	Mold	698.000	63.000	11.079		
	Yeast	37.929	63.000	0.602		
Total	Mold	1322.272	84.000			
	Yeast	136.763	84.000			
Corrected Total	Mold	917.979	83.000			
	Yeast	40.246	83.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.802	111.406	2.000	55.000	0.000
	Wilks' Lambda	0.198	111.406	2.000	55.000	0.000
	Hotelling's Trace	4.051	111.406	2.000	55.000	0.000
	Roy's Largest Root	4.501	111.406	2.000	55.000	0.000
Gene	Pillai's Trace	0.087	0.425	12.000	112.000	0.951
	Wilks' Lambda	0.914	0.419	12.000	110.000	0.953
	Hotelling's Trace	0.092	0.414	12.000	108.000	0.955
	Roy's Largest Root	0.066	0.612	6.000	56.000	0.719
Condition	Pillai's Trace	0.348	3.930	6.000	112.000	0.001
	Wilks' Lambda	0.653	4.354	6.000	110.000	0.001
	Hotelling's Trace	0.530	4.771	6.000	108.000	0.000
	Roy's Largest Root	0.528	9.850	3.000	56.000	0.000
Gene * Condition	Pillai's Trace	0.151	0.253	36.000	112.000	1.000
	Wilks' Lambda	0.853	0.252	36.000	110.000	1.000
	Hotelling's Trace	0.168	0.251	36.000	108.000	1.000
	Roy's Largest Root	0.135	0.419	18.000	56.000	0.978

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Mold	202.733	27.000	7.509	0.588	0.933
	Yeast	15.313	27.000	0.567	1.274	0.220
Intercept	Mold	404.294	1.000	404.294	31.654	0.000
	Yeast	96.517	1.000	96.517	216.771	0.000
Gene	Mold	18.814	6.000	3.136	0.246	0.959
	Yeast	1.299	6.000	0.216	0.486	0.816
Condition	Mold	119.131	3.000	39.710	3.109	0.034
	Yeast	13.157	3.000	4.386	9.850	0.000
Gene * Condition	Mold	64.788	18.000	3.599	0.282	0.998
	Yeast	0.857	18.000	0.048	0.107	1.000
Error	Mold	715.246	56.000	12.772		
	Yeast	24.934	56.000	0.445		
Total	Mold	1322.272	84.000			
	Yeast	136.763	84.000			
Corrected Total	Mold	917.979	83.000			
	Yeast	40.246	83.000			

Dependent Variable: Mold					
Source	Type III Sum of Squares	df	Mean Suare	F	Sig.
Corrected Model	672.590	11.000	61.145	17.941	0.000
Intercept	404.294	1.000	404.294	118.625	0.000
Strain Condition	672.590	11.000	61.145	17.941	0.000
Error	245.389	72.000	3.408		
Total	1322.272	84.000			
Corrected Total	917.979	83.000			

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Mold	Tukey HSD	1	5	0
		2	5	0
		3	5	0
		4	5	0
		5	1	0
			2	0
			3	0
			4	0
			6	0
			7	0
			8	0
			9	0
			10	0
			11	0
			12	0
		6	5	0
		7	5	0
		8	5	0
		9	5	0
		10	5	0
		11	5	0
		12	5	0
	Bonferroni	1	5	0
		2	5	0

		3	5	0
		4	5	0
		5	1	0
			2	0
			3	0
			4	0
			6	0
			7	0
			8	0
			9	0
			10	0
			11	0
			12	0
		6	5	0
		7	5	0
		8	5	0
		9	5	0
		10	5	0
		11	5	0
		12	5	0

Dependent Variable: Yeast					
Source	Type III Sum of Squares	df	Mean Suare	F	Sig.
Corrected Model	34.623	11.000	3.148	40.302	0.000
Intercept	96.517	1.000	96.517	1235.812	0.000
Strain Condition	34.623	11.000	3.148	40.302	0.000
Error	5.623	72.000	0.078		
Total	136.763	84.000			
Corrected Total	40.246	83.000			

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Yeast	Tukey HSD	1	2	0.019
			5	0
			9	0
		2	1	0.019
			4	0.038
			5	0
			6	0
			7	0
			8	0
			12	0.027
		3	5	0
			6	0.008
			7	0
			8	0
		4	2	0.038
			5	0
			9	0
		5	1	0
			2	0
			3	0
			4	0
			6	0
			7	0
			8	0

			9	0
			10	0
			11	0
			12	0
		6	2	0
			3	0.008
			5	0
			9	0
			11	0.005
		7	2	0
			3	0
			5	0
			9	0
			10	0.001
			11	0
		8	2	0
			3	0
			5	0
			9	0
			10	0.001
			11	0
		9	1	0
			4	0
			5	0
			6	0
			7	0
			8	0
			10	0.049
			12	0
		10	5	0
			7	0.001
			8	0.002
			9	0.049
		11	5	0
			6	0.005
			7	0
			8	0
		12	2	0.027
			5	0
			9	0
	Bonferroni	1	2	0.027

			5	0
			9	0
		2	1	0.027
			5	0
			6	0
			7	0
			8	0
			12	0.038
		3	5	0
			7	0.011
			8	0
		4	5	0
			9	0
		5	1	0
			2	0
			3	0
			4	0
			6	0
			7	0
			8	0
			9	0
			10	0
			11	0
			12	0
		6	2	0
			3	0.011
			5	0
			9	0
			11	0.006
		7	2	0
			3	0
			5	0
			9	0
			10	0.001
			11	0
		8	2	0
			3	0
			5	0
			9	0
			10	0.002
			11	0

		9	1	0
			4	0
			5	0
			6	0
			7	0
			8	0
			12	0
		10	5	0
			7	0.001
			8	0.002
		11	5	0
			6	0.006
			7	0
			8	0
		12	2	0.038
			5	0
			9	0

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