Expression of *Penicillium marneffei* Glucan 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 an opportunistic, dimorphic fungal pathogen that causes penicilliosis in AIDS patients. In an effort to genetically characterize this organism, Kummasook et al. (2010) developed an *Agrobacterium tumefaciens*-mediated transformation system. This system allowed for the production of thousands of *P. marneffei* mutant strains that could be further assessed in order to determine gene-to-function relationships. Subsequently, Suwunnakorn et al. (2014) characterized one of these mutants, I231, and determined the presence of an interrupted *yakA* gene which codes for a dual-specificity tyrosine phosphorylation-regulated protein kinase. These investigators demonstrated that the *yakA* mutant produced fewer conidia, possessed increased chitin content, and exhibited unique cell-wall stress responses when compared to the wild-type strain of *P. marneffei.* The present study seeks to investigate these stress responses in the *yakA* mutant (*ΔyakA*), the wild-type (F4) strain, and a *yakA* genetically complemented strain (CY21) at the transcriptional level. Using qRT-PCR, expression of three glucan synthase genes (*fksP*, *ags2*, and *kre6*) was quantified using the *benA* (β-tubulin) gene as a reference gene for normalization. The three strains were subjected to cell-wall stressors, including the anionic dye Congo Red, the detergent sodium dodecyl sulfate, and the glucan synthase-specific antifungal drug caspofungun, prior to determining glucan synthase gene expression. The results of this study indicated that the *yakA* mutant possessed relatively larger stress responses than the wild type, indicating that the *yakA* gene and its resulting protein may be responsible for the regulation of glucan synthase gene expression. This reasoning is reinforced by the presence of

increased chitin in the *yakA* mutant, a characteristic common to fungal mutants lacking proper glucan regulation in their cell walls.

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CHAPTER 1: INTRODUCTION

1.1 General Background

The genue *Penicillium* represents a grouping of fungi that is mostly considered benign in regards to causing human disease. However, the species *Penicillium marneffei* diverges from this pattern. This pathogenic fungus is the causative agent of a systemic infection known as penicilliosis that occurs most often in immunocompromised individuals of Southeast Asia (Cooper and Haycocks, 2000). In addition to its characteristic pathogenicity, *P. marneffei* is known for its temperature-dependent, dimorphic nature. This dimorphism is defined by the this species ability to grow as filamentous, multinucleate hyphae at 25°C and its transition to a uninucleate yeast phase at 37°C. Dimorphism in *P. marneffeii* is a key virulence factor and represents one of the most intensely studied characteristics of both its genetic and morphological composition (Chandler et al., 2002; Vanittanakorn et al., 2006).

1.2 History, Epidemiology, and Medical Significance

Despite its prominence as a human pathogen, *P. marneffei* was first isolated in the Chinese bamboo rat, *Rhizomys sinensis*, in 1956. This bamboo rat was a lab animal held at the Pasteur Institute of Indochina in Dalat, South Vietnam (Capponi et al., 1956). After spontaneous death of several rats, tissue biopsies revealed *Penicillium*-like growth. Interestingly, when the biopsies were grown at room temperature, mold growth was present wherase those samples observed directly from the tissue biopsies indicated that

the fungus grew as fission yeast. The genus of this fungus was designated as *Penicillium* and the species name *marneffei* was appointed after the Pasteur Institute's director Dr. Hubert Marneffe (Segretain, 1959).

Initial isolation of *P. marneffei* from *Rhizomys sinensis* did not aid in determining the natural reservoir of the fungus(Capponi et al., 1956). To this day, all laboratory isolates of *P. marneffei* can be traced back to samples taken from either infected humans or animals. In addition to *Rhizomys sinensis*, two additional rodent species, native to Thailand and Southeast Asia, have been shown to carry the fungus. These species are the hoary bamboo rat (*Rhizomys pruinosus*) and the lesser bamboo rat (*Cannomys badius*). Additionally, *P. marneffei* was isolated from the soil in a rat burrow (Ajello et al., 1995; Vanittanakorn et al., 1996). Despite these findings, most *P. marneffei* human infections occur in cities where bamboo rats do not normally live. This hints at the potential involvement of additional, more domestic hosts such as dogs, although the link of transmission, in regards to dogs or bamboo rats, still remains unknown (Cao et al., 2011; Chaiwun et al., 2011).

Several years after the first *P. marneffei* isolation, the first human case occurred. In 1959, Professor Gabriel Segretain accidentally inoculated himself with a contaminated needle containing *P. marneffei*. He developed axillary lymphadenopathy shortly thereafter, but a combination of nystatin treatment and immunocompetency permitted a full recovery. Like with the bamboo rat isolates, Segretain was able to isolate *P. marneffei* from a nodule that was present at his site of infection (Segretain, 1959). It would not be till 1973 that the first naturally occurring case of *P. marneffei* infection was documented. This case involved a 61 year old American minister who had resided in

Southeast Asia for some time. Suffering from Hodgkin's disease, the man had to undergo a splenectomy. This surgery revealed the presence of *P. marneffei* (DiSalvo et al. 1973).

Penicillium marneffei cases would continue to surface over the coming years in Southeast Asia. With the onset of the HIV/AIDS epidemic in 1988, however, the occurrence of human penicilliosis would dramatically increase. The increased occurrence of air travel throughout Southeast Asia would see this disease occurring in individuals from around the planet. No longer were individuals from or who had traveled to Thailand being affected, but rather individuals from Cambodia (Bailloud et al., 2002), China (Chandler et al., 2002), India (Ranjana et al. 2002) , Taiwan (Chang et al., 1995; Chiang et al., 1998), Germany (Sobottka et al., 1996; Rimek et al., 1999), Sweden (Julander and Petrini 1997), Switzerland (Kronauer et al., 1993; Borradori et al., 1994; Garbino et al., 2001), Vietnam (Hien et al., 2001; Huynh et al., 2003), France (Hilmarsodottir et al., 1993; Hilmarsdottir et al., 1994), the United States (Nord et al., 1998), Australia (Heath et al., 1995), Belgium (Depraetere et al., 1998), Japan (Mohri et al., 2000; Tsunemi et al., 2003), the Netherlands (Hulshof et al., 1990; Kok et al., 1994), and the United Kingdom (Peto et al., 1988; McShane et al., 1998; Vilar et al., 2000). Despite the variation in infected individuals, *P. marneffei* was still most devastating within the country of Thailand and has become the third most common AIDS-defining illness (Cooper and Haycocks, 2000).

1.3 Biology, Dimorphism, and Pathogenicity

Penicillium marneffei is an ascomyceteous fungus that displays a characteristic dimorphism. It is capable of growing as a filamentous mold at 25°C (room temperature) and as a fission yeast at 37°C (human body temperature).

At 25°C *P. marneffei* conidia, or spores, will undergo an expansion to create germ tubes. These germ tubes will generate hyphae, which grow apically and through lateral branching, while septation produces individual cells and results in multicellular hyphal growth. Formation of aerial stalks allows production of the conidiophores which are the sites for development of additional conidia. The aerial stalks will swell at their tips and promote their nuclei to divide. These globose structures and their multinucleate structure are known as metulae. The metulae then make way for the formation of phialides which are the product of the metulae undergoing cytokinesis to form uninucleate cells. These cells bud to form a second tier known as the phialides. Through interstitial budding, the phialides generate conidia chains (Zuber et al., 2002).

When *P. marneffei* conidia are cultured at 37°C, the germination process resembles that which occurs at 25°C. Initially, the spores will undergo hyphal growth and, like before, grow both laterally and through hyphal branching. After some time, however, these hyphae will undergo arthroconidiation where nuclear and cellular division cycles will become coupled. This results in the formation of double septa surrounding each of the cells. Full arthroconidia are produced once these cells separate. These arthroconidia then reproduce by binary fission to produce uninucleate yeast cells (Adrianopoulos, 2002).

The dimorphic nature of *P. marneffei* is a key aspect of its virulence. A well-established notion as to how the organisms makes its way into a host's body relies heavily upon this transition from mold to yeast (Canovas and Adrianopoulos, 2007; Boyce et al., 2009; Cooper and Vanittanakorn, 2008). It is believed that *P. marneffei* grows in the soil in the mold phase. The heavy rains that Thailand experiences each year may be responsible for aerosolizing the conidia of mold phase *P. marneffei* (Chariyalertsak et. al., 1997). These conidia are most likely inhaled by an individual and make their way into the lungs. Once the conidia enter the body they will eventually transition into the yeast phase. These yeast cells have been shown to be capable of residing within host macrophages to evade the immune system (Liu et. al., 2009). A number of studies have indicated that a lack of the mold to yeast transition will inhibit the virulent nature of *P. marneffei*. This is the reason that researchers hope to better understand the genetic machinery that drive this dimorphic switch (Adrianopoulos, 2002; Todd et al., 2003).

1.4 *Agrobacterium tumefaciens*-Mediated Transformation

The generation of mutant fungal strains is a common model for genetic characterization. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is an effective technique for mutagenesis of *P. marneffei*. This transformation technique relies on the Gram-negative bacterium *Agrobacterium tumefaciens* which is responsible for crown gall tumors in plants. These tumors are produced due to the bacteria's tumor inducing (Ti) plasmid. *Agrobacterium tumefaciens* produces these tumors in plants in response to the production of amino acid-sugar complexes known as opines. These opines serve as a carbon source for *A. tumefaciens* once it is in the plant (Brencic et al.,

2005). The ATMT mutagenesis technique developed by Kummasook et al. (2010) made use of this T-DNA (transfer DNA) plasmid by randomly inserting it into *P. marneffei* and potentially causing the disruption of one or more genes, leading to mutation.

Zhang et al. (2008) was the first group to attempt ATMT in *P. marneffei*. Different *A. tumefaciens* strains and binary vectors were tested to determine which combination produced the highest levels of mutagenesis. More recently, Kummasook et al. (2010) sought to improve upon the *P. marneffei* ATMT method established by Zhang et al. The *A. tumefaciens* strain AGL1-pUPRS0 was co-cultivated with pre-germinated *P. marneffei* conidia. Co-cultivation with this strain produced bleomycin resistant tranformants with an efficiency of approximately 123 ± 3.27 transformants per plate for conidia and 239 ± 13.12 for pre-germinated conidia when 5×10^4 conidia was used for each. Southern blot analysis could determine that 95% of these transformants possessed a single copy of T-DNA indicating a single insertion. These transformants could then have their sequences amplified through inverse PCR and analyzed through the GenBank database to determine the gene that was interrupted by the T-DNA insertion. This ATMT procedure represented an efficient and relatively cost effective means of generating and screening *P. marneffei* deletion mutants on a large scale.

1.5 Strain Δ*yakA* of *Penicillium marneffei*

In 2014, Suwunnakorn et al. (2014) characterized a previously isolated ATMT mutant strain of *P. marneffei* originally designated as I231. This strain possessed an interrupted gene (*yakA*) encoding a protein kinase. The *yakA* gene codes for a dual-specificity tyrosine phosphorylation-regulated protein kinase responsible for growth control in

response to the variability of carbon sources (Aranda et al., 2011; Moriya et al., 2001; Garrett et al., 1991; Goyard et al., 2008; Lee et al., 2011; Malcher et al., 2010). Suwunnakorn et al. (2014) designated strain I231 as Δ*yakA* and subsequently determined that the interruption was responsible for morphogenic abnormalities, stress response variations, and disruptions in chitin distribution and chitin gene expression when compared to the *P. marneffei* wild-type strain (strain F4). At 25°C Δ*yakA* exhibited a reduced colony diameter, abnormal grey-white colonies, and reduced conidia production when compared to either wild-type or previously engineered complement strains (*CY21*). At 37°C, Δ*yakA* exhibited yeast growth that appeared the same as both the wild-type and the complement strains, indicating that morphological defects were restricted to the mycelial phase. In addition to morphological abnormalities, Δ*yakA* exhibited variation in its sensitivity to cell-wall perturbing agents including the cytoplasmic stain Congo Red (CR), the anionic membrane disrupting agent sodium dodecyl sulfate (SDS), and the lipopeptide antifungal drug caspofungin (CAS). Because *yakA* homologs have been shown to activate the stress-responsive transcription factors including the thermotolerance related Hsf1 and the less specific stress gene Msn2, this aspect of the mutant's nature was carefully assessed (Sadeh et al., 2012; Sadeh et al., 2011; Elfving et al., 2014; Hartley et al., 1994; Lee et al., 2008; Liu et al., 2013). Specifically, Δ*yakA* exhibited hypersensitivity to SDS at 25°C and 37°C, a slight increase in sensitivity to CAS at 25 °C and 37 °C, but an increase in resistance to CR at 25°C and 37°C. Suwunnakorn et al. (2014) remarked that these results were somewhat contradictory because both CAS and CR affect glucan synthesis, but Δ*yakA* exhibited increased sensitivity to the one agent (CAS) but increased resistance to the other (CR). Moreover,

Suwunnakorn et al. (2014) investigated chitin deposition throughout the Δ*yakA* cell-wall and the expression level of chitin synthesis genes (the *chs* family of genes). Using the chitin-binding fluorescent dye Calcofluor White and measuring cellular glucosamine content, they determined that Δ*yakA* exhibits abnormal chitin distribution at 25°C but increased chitin content possibly as a compensatory response to a defective, weakened cell-wall (Hill et al., 2006; Verwer et al., 2011). Wanting to analyze this increased chitin content further, Suwunnakorn et al. (2014) measured levels of genetic expression of the *chs* genes. Using quantitative reverse-transcription PCR (qRT-PCR), they found that genetic expression of two of the *chs* genes, *chsB* and *chsG*, was significantly increased in the Δ*yakA* strain when compared to the wild-type strain, whereas the remaining five *chs* genes remained at wild-type levels of expression. This finding may reflect the previously mentioned concept of a compensatory mechanism within the fungus that is attempting to compensate for a weakened or abnormally functioning cell-wall (Bulik et al., 2003). Additionally, the Δ*yakA* mutant possessed significantly lower expression levels of the *abaA* gene which plays a significant role in phialide development and the production of conidia (Borneman et al., 2000). Overall, the research performed by Suwunnakorn et al. (2014) determined that the Δ*yakA* strain of *P. marneffei* was a conidiation mutant that possessed additional defects in its germination abilities and its cell-wall integrity. Additionally, the *yakA* gene appears to play uncertain roles in the regulation and deposition of the cell-wall constituent chitin. Despite no apparent effects on virulence, this mutation may provide insight into the cell-wall growth dynamics of the *P. marneffei* mold phase.

1.6 The Fungal Cell-wall

All fungal organisms rely upon their cell-wall for protection from the external environment. The two structural carbohydrate polymers that are the basis of fungal cell-wall integrity are chitin and glucan (Borgia and Dodge, 1992). Specifically, chitin and β-(1,3)-glucan create an interwoven matrix that provides the foundation of most fungal cell wall not only in terms of outright physical protection, but also complex adaptability to ever-changing environmental and genetic conditions (Munro, 2013; Verna et al., 1997; Damveld et al., 2004; Latge, 2007). This adaptability is a necessity for fungi such as *P. marneffei* by their very nature. In order for *P. marneffei* to carry out its phase transitions, it has to be capable of manipulating virtually all of its organelles, perhaps most importantly the cell-wall which not only provides an outer layer of physical cover, but also provides inner functionality through actions such as septum division and ascospore assembly (Levin, 2011).

1.7 Glucan

Although β-(1,3)-glucan is most often associated with the outer cell-wall, additional glucans, including β-(1,6)-glucan and α-(1,3)-glucan, exist in *P. marneffei* and also most likely aid in different cell-wall functions (Suwunnakorn et al., 2014). The glucans found in fungi differ based upon their chemical bond linkages, primarily around their glycosidic bonds. Possessing structurally different types of glucan permits specialization. While the exact nature of the cell wall in *P. marneffei*'s remains unknown, the importance of these glucans has been determined in many other fungal species.

Pathogenic fungi including *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* all possess cell-walls that are rich in β-(1,3)-glucan. α-(1,3)-glucan is a major cell-wall polysaccharide for *Aspergillus nidulans* and is required for proper filamentous growth (Beauvais et al., 2013, Yoshimi et al., 2013). In fungal organisms such as *Cryptococcus neoformans* and *Histoplasma capsulatum*, α-(1,3)-glucan has been shown to play a role in virulence through anchoring functions and through masking immunogenic molecules. In *Aspergillus fumigatus*, in addition to its β-(1,3)-glucan-chitin fibrillar core, α-(1,3)-glucan also proved necessary for virulence based on knock-out mutants that proved capable of normal growth *in vitro* but were avirulent *in vivo* (Beauvais et al., 2013; Maubon et al., 2006).

1.8 Genetic Expression of Glucan, Cell-wall Stressors, and Adaptability

Penicillium marneffei has been found to contain at least three separate genes that code for glucan synthases, the enzymes that ultimately produce glucan. These genes include *ags2*, which codes for an α-(1,3)-glucan synthase, *kre6*, which codes for a β-(1,6)-glucan synthetase, and *fksP*, which codes for a β-(1,3)-glucan synthase. The inhibition of any of these synthases or synthetases can significantly impact both the growth and/or the virulence of a fungus (Suwunnakorn et al., 2014). Congo Red (CR), Calcofluor White, sodium dodecyl sulfate (SDS), and caspofungin are all potential cell-wall stress agents that can produce this type of inhibition. Congo Red, once a commercially used dye, is a staining agent, a pH indicator, and a highly toxic compound

that interferes with both chitin and glucan structure through its high affinity for the polymers (Kopecka and Gabriel, 1992). Calcofluor White is a fluorescent dye that can bind to and inhibit proper chitin function, somewhat mirroring the effects of the antifungal drug caspofungin which specifically inhibits β-(1,3)-glucan synthesis (Hill et al., 2006; Roncero and Duran, 1985; Hohl et al., 2008). Finally, SDS is a cell-membrane-perturbing agent that interferes with fungal cell-walls (Suwunnakorn et al., 2014). All three of these agents produce adverse effects on fungal cell-walls, but multiple studies have determined that fungi are capable of defending themselves from both these effects and genetic malformations that replicate these effects. Beauvais et al. (2013) determined that *A. fumigatus* mutations or any type of modifications in the glucan components of the cell-wall were associated with compensatory reactions that seemed developed to counteract external stressors. There is extensive cross-linking throughout pathogenic fungal cell-walls that results in dynamic matrices (Adams, 2004). These matrix-like cell-walls, composed of chitin and glucans, were capable of producing stress response proteins which allowed for extensive manipulation of the cell-wall whenever necessary (Fuchs and Mylonakis, 2009; Latge, 2014). These type of research efforts highlighted the intricate, dynamic nature of the cell-wall components found in fungi and reinforce the concept of genetic manipulation that allows organisms such as *P. marneffei* to defend themselves whether their opposition lies within their own genes or the external environment (Lee et al., 2011).

CHAPTER 2: SPECIFIC AIMS AND HYPOTHESIS

The specific aim of this research study is to assess the gene expression levels of three *Penicillium marneffei* glucan synthases, *ags2*, *kre6*, and *fksP*, under different types of cell-wall stress in a wild-type strain, a Δ*yakA* mutant strain, and a CY21 complement strain at both 25°C and 37°C. The three strains will be identically cultured and subjected to either predetermined concentrations of Congo Red, SDS, or caspofungin, or to a non-stressor control medium for a period of 4 hours. These cultures will have their RNA isolated and will be subjected to qRT-PCR in order to assess gene expression levels relative to the reference gene *benA*. Based on these results, it will be determined whether or not a defective *yakA* gene influences glucan synthase gene expression under "non-stressful" control conditions or under conditions that produce cell-wall stress.

Hypothesis

The Δ*yakA* strain of *Penicillium marneffei* possesses significantly different gene expression levels of the glucan synthase genes *ags2*, *kre6*, and *fksP* when compared to the wild-type strain F4 and the complement strain *CY21* under cell-wall stress conditions. Additionally, these glucan synthase gene expression levels will also vary significantly from the levels exhibited by the wild-type and complement strains under non-stressor conditions, indicating a glucan-based cell-wall malformation in this mutant strain of the fungus.

CHAPTER 3: MATERIALS AND METHODS

3.1 *Penicillium marneffei* Strains and Strain Maintenance

Three strains of *P. marneffei* were used for all experimental procedures: F4 (wild type; CBS 119456), *ΔyakA* (the mutant formerly designated I231), and CY21 (the genetic complement strain of *ΔyakA*). The F4 strain was originally isolated from an AIDS patient of Chiang Mai, Thailand in 1998 (Pongpom et al., 2005). The *ΔyakA* strain was originally derived by Aksarakorn et al (2010) via ATMT, whereas strain CY21 was generated by Suwunnakorn et al. (2014) using the methods of Borneman et al. (2001). All strains were continually cultured on potato dextrose agar (PDA) (BD Difco; Franklin Lakes, New Jersey) in 100x 15mm Petri dishes (FisherScientific; Pittsburgh, Pennsylvania) on a weekly basis throughout the duration of this research. These cultures were maintained at room temperature. Glycerol (15%) stocks of these strains were stored at -80°C in 2.0 ml screw-top culture tubes.

3.2 Culture Preparation for Stress Induction and RNA Isolation

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 1×10^6 conidia/ml.

3.3 Stress Induction

Stress induction experiments were always performed with one strain at a time. This strain being studied, however, would have separate samples subjected to stress induction at either 25°C or 37°C. Two 1000-ml screw-top flasks, each containing 250 ml of Sabouraud Dextrose Broth (BD Difco; Franklin Lakes, New Jersey), were inoculated with the proper volume of conidial suspension to yield a total concentration of 1×10^6 conidia/ml. These cultures were then incubated in water baths for 48 hours with an agitation of 120 rpm and held at a constant temperature of either 25°C or 37°C. After 48 hours, the cultures were quickly taken out of their water baths, divided into four 50 ml aliquots each (within 250 ml Erlenmeyer flasks), had the proper volume of culture removed (that corresponded to the volume of stressor that would be added), inoculated with the desired volume of Congo red (CR; Amresco; Solon, Ohio), SDS (Amresco), or caspofungin (Sigma-Aldrich; St. Louis, Missouri) (or no stressor for the control), and then placed back in the same incubator (in terms of both temperature and agitation) for 4

hours (refer to appendices B and C for stressor volumes and concentrations). After the 4 hour period was complete, the samples were immediately poured into 50 ml conical tubes and centrifuged at 6500 rpm for 15 minutes at 4°C. After centrifugation, the broth in each of the samples was discarded, making sure to preserve the cell pellet. Once all of the samples were successfully pelleted, they were divided into 700 µl aliquots, placed into 1.5 ml microcentirfuge tubes, and stored at either – 20°C (if being used the next day) or – 80°C (if not being used the next day).

3.4 RNA Isolation

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 bashingbead 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.

3.5 Spectrophotometry

The purities and concentrations of all RNA samples were determined using the NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific; Waltham, Massachusetts). All blanks were measured with 2 µl of nuclease-free water and all RNA

samples were analyzed with 2 µl from the previously obtained isolates. Purities were measured in terms of the 260/280 nm wavelength values and the concentrations were measured in the units ng/µl.

3.6 Primer Assessment Using RT-PCR and Gel Electrophoresis

All primers used for this study were previously described by Suwunnakorn et al. (2014) (Appendix A). The quality of all primers was assessed through an initial RT-PCR reaction using a Qiagen OneStep RT-PCR kit 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) 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).

3.7 Primer Assessment Using qRT-PCR Standard Curves

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 recommended one-step qRT-PCR reaction was used (refer to appendix G). The resulting standard curve was produced through the system software (Bio-Rad). Images of the standard curves are available in Appendix I.

3.8 Determining Relative Gene Expression using qRT-PCR

Relative gene expression of the *fksP*, *ags2*, *kre6*, and *benA* genes in each RNA sample was determined using a Bio-Rad iTaq Universal SYBR Green One-step kit to manufacturer specifications (Bio-Rad), the iQ5 Real-Time PCR System Thermocycler (Bio-Rad), and the manufacturer's recommended one-step qRT-PCR program (refer to appendices F and G). All samples were run in triplicate with a series of non-template controls for each gene. All relative gene expressions were determined by normalizing the results to *benA*. This data was exported to Microsoft Excel (Microsoft; Redmond, Washington) for organization and graph generation.

3.9 Data Organization and Analysis

All charts and graphs were produced using Microsoft Excel (Microsoft; Redmond, Washington). Data analysis was carried out using SPSS statistical software (version 17.0; IBM; Armonk, New York). Multivariate data analysis models were used to determine possible relationships between strains, temperatures (phases), conditions, and genes. Based on these results, univariate models and post-hoc tests could be used to find more specific interactions within and among the samples. Interactions were considered significant if analyses produced a p-value of less than 0.05.

CHAPTER 4: RESULTS

4.1 Overview

The relative expression of three glucan synthase gene (*fksP*, *ags2*, and *kre6*) 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, normalized to the expression of beta-tubulin (*benA*), was graphically depicted for each culture and treatment condition. 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 combined results graphs illustrate the general gene expression trends that were seen amongst the three glucan synthase genes and the stressor conditions (Figures 1-6). A composite chart of overall expression patterns is depicted in Figure 7. The wild-type strain, at 25°C and 37°C, exhibited relatively higher fold expression levels of the glucan synthase genes under the control condition when compared to the stressor conditions. The opposite scenario was seen for the *ΔyakA* strain at 25°C and 37°C in which the control condition had relatively low fold expression levels compared to the higher fold expression levels of the stressor conditions. The only notable exception to this trend, amongst both strains, was a tendency for the *fksP* fold expression levels to be higher than *ags2* or *kre6*. Moreover the fold expression levels for the CY21 strain, at both 25°C and 37°C were a "blend" of the results for the wild type and *ΔyakA* mutant with

homogenous expressions across all conditions with the exception of higher *fksP* fold expression levels.

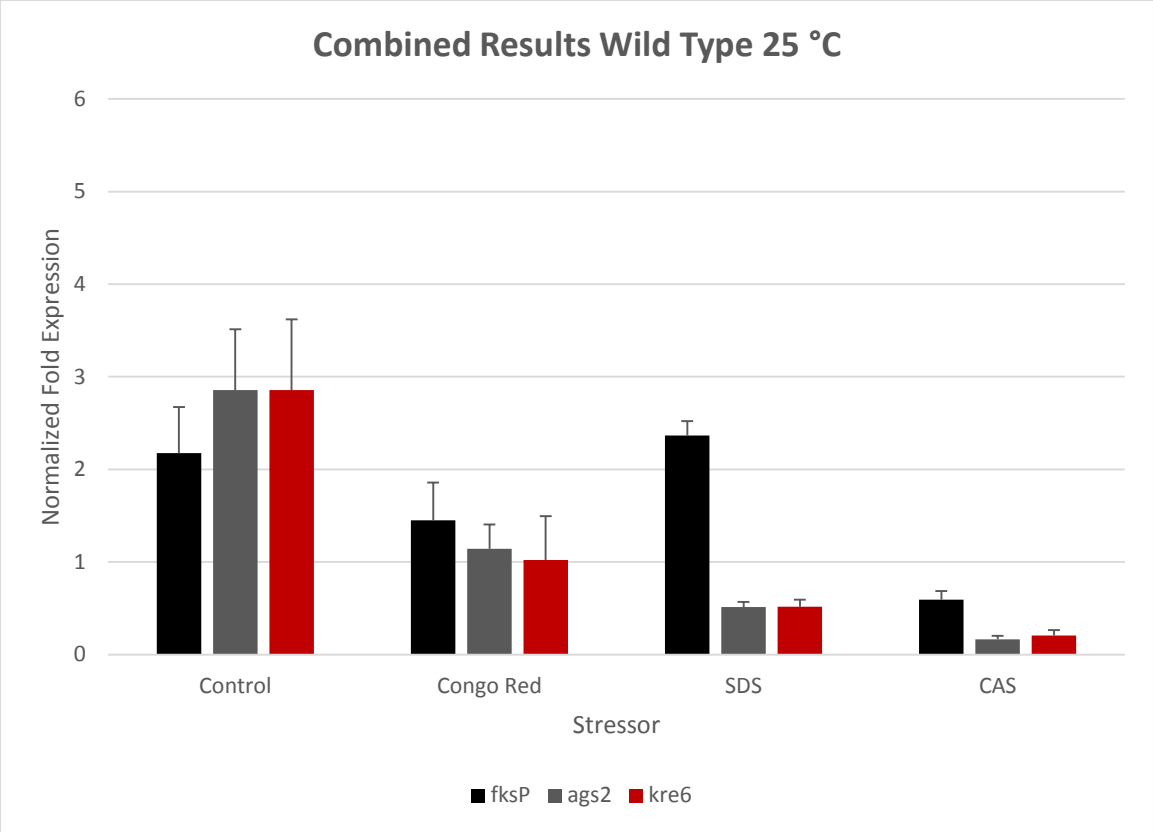


Figure 1: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the wild-type strain of *P. marneffei* incubated at 25°C derived from combined Round 1 and Round 2 data. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis).

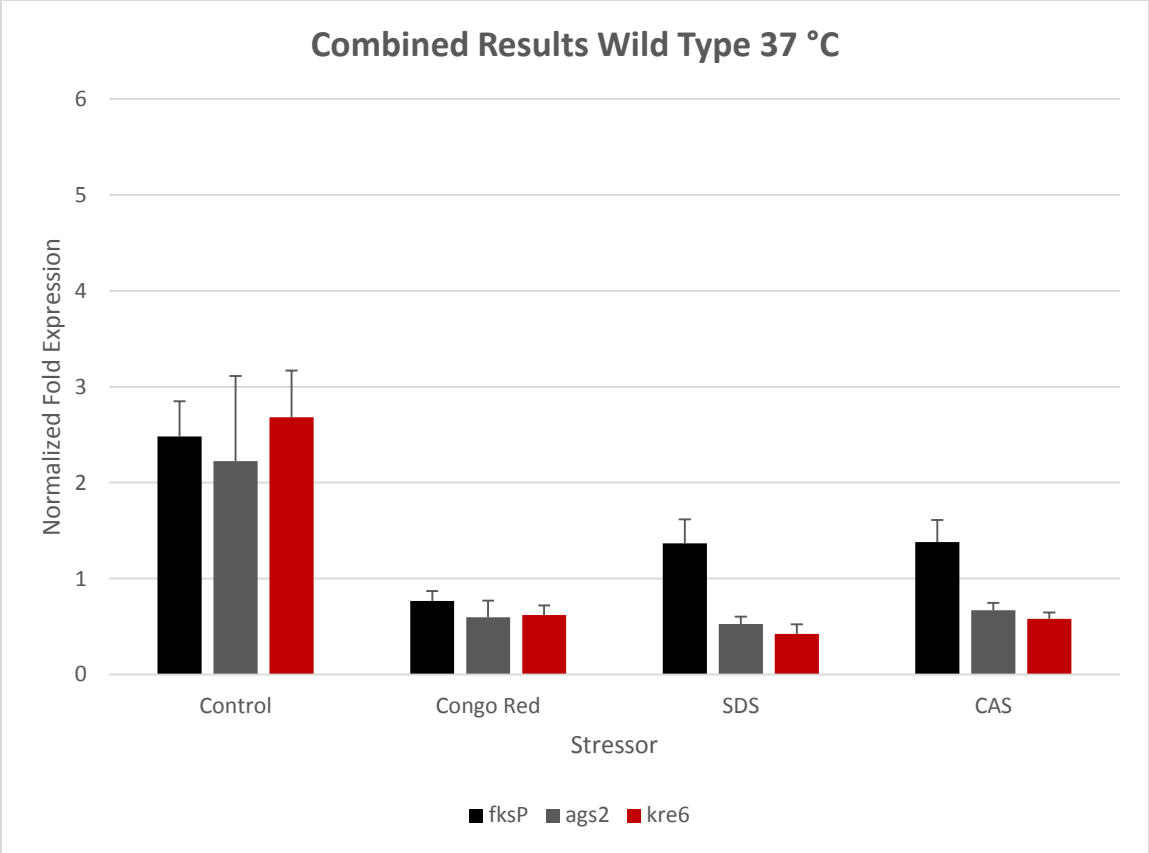


Figure 2: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the wild-type strain of *P. marneffei* incubated at 37°C derived from combined Round 1 and Round 2 data. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis).

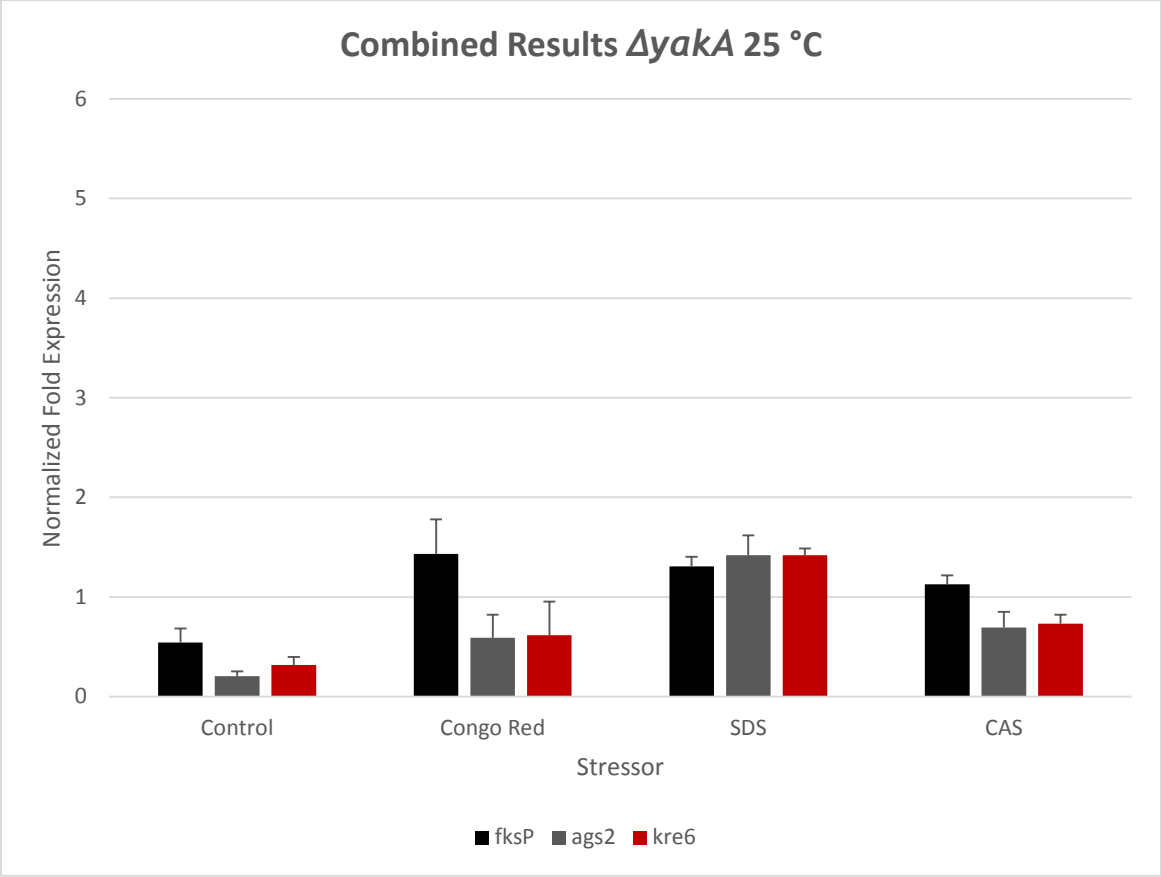


Figure 3: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the *ΔyakA* strain of *P. marneffei* incubated at 25°C from combined Round 1 and Round 2 data. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis).

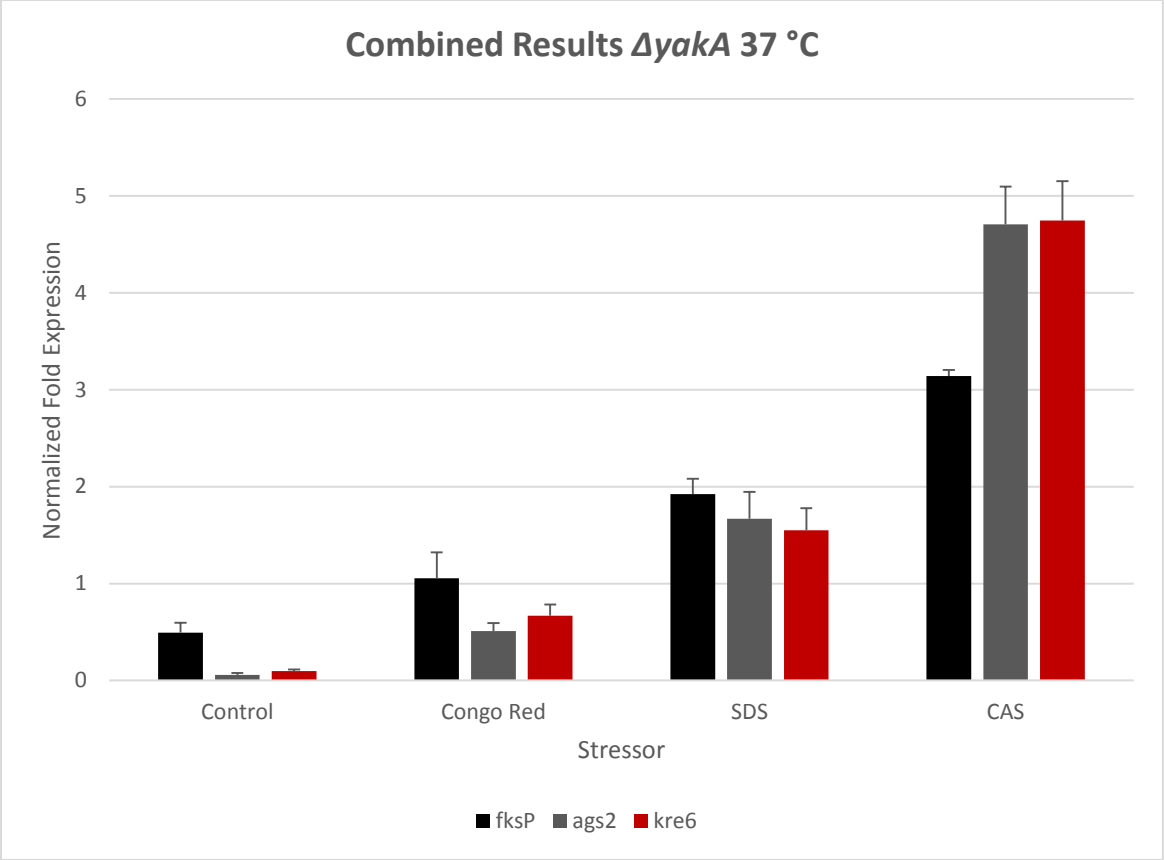


Figure 4: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the *ΔyakA* strain of *P. marneffei* incubated at 37°C from combined Round 1 and Round 2 data. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis).

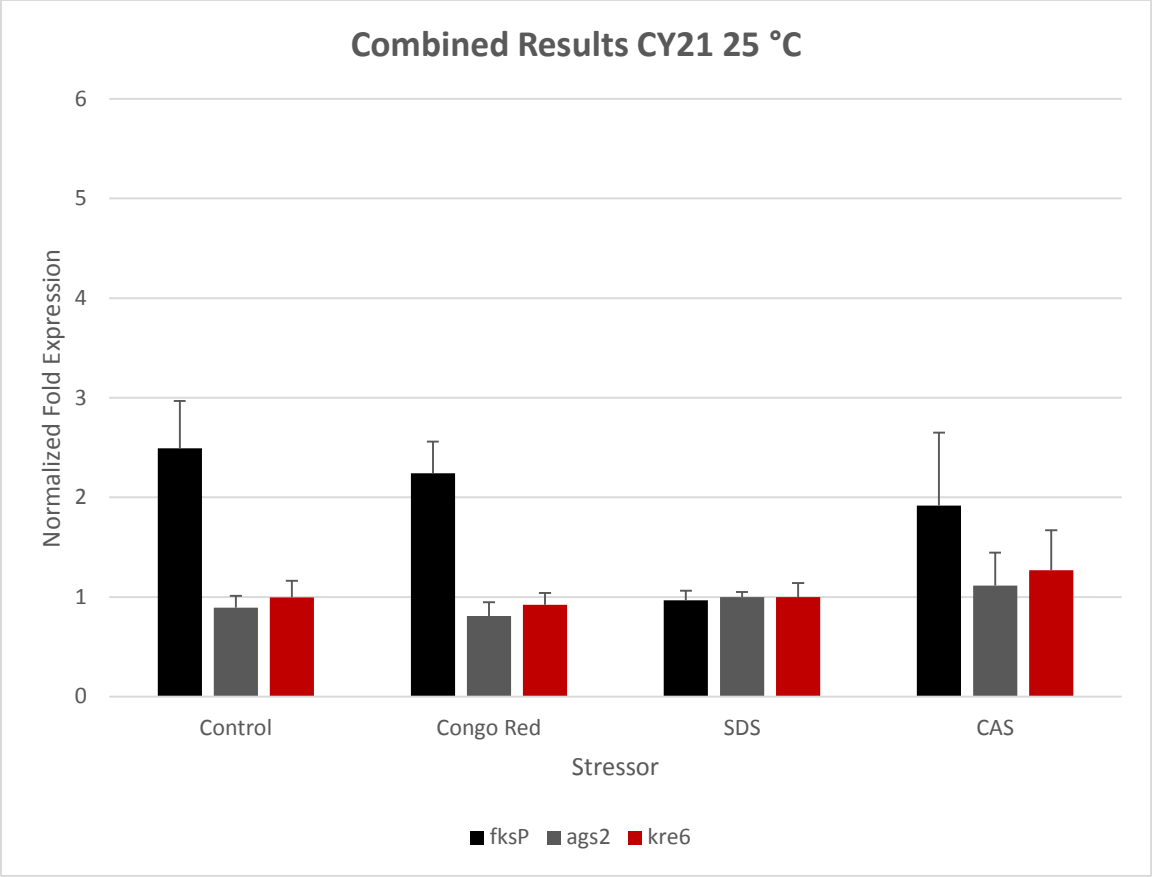


Figure 5: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the CY21 strain of *P. marneffei* incubated at 25°C from combined Round 1 and Round 2 data. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis)

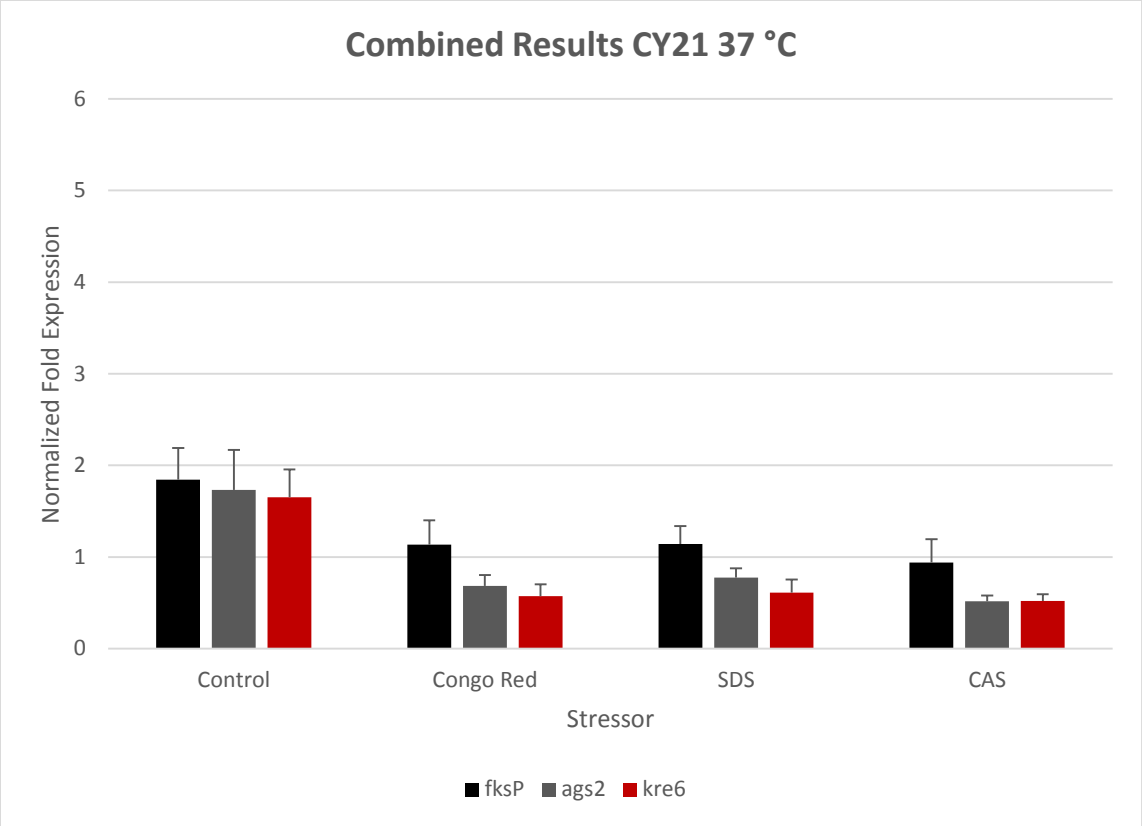


Figure 6: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the CY21 strain of *P. marneffei* incubated at 37°C from combined Round 1 and Round 2 data. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis).

Combined Results Summary

25 C

fksP	Wild Type	*yakA*	CY21
Control	2.1	↓	↑
Congo Red	1.5	↓	↑
SDS	2.4	↓	↓
CAS	0.6	↑	↑

ags2	Wild Type	*yakA*	CY21
Control	2.9	↓	↓
Congo Red	1.1	↓	↓
SDS	0.5	↑	↑
CAS	0.2	↑	↑

kre6	Wild Type	*yakA*	CY21
Control	2.9	↓	↓
Congo Red	1.0	↓	↓
SDS	0.5	↑	↑
CAS	0.2	↑	↑

Combined Results Summary

37 C

fksP	Wild Type	*yakA*	CY21
Control	2.5	↓	↓
Congo Red	0.8	↑	↑
SDS	1.4	↑	↓
CAS	1.4	↑	↓

ags2	Wild Type	*yakA*	CY21
Control	2.2	↓	↓
Congo Red	0.6	↓	↑
SDS	0.5	↑	↑
CAS	0.7	↑	↓

kre6	Wild Type	*yakA*	CY21
Control	2.7	↓	↓
Congo Red	0.6	↑	≈
SDS	0.4	↑	↑
CAS	0.6	↑	↓

Figure 7: Summary chart illustrating the general patterns of gene expression for each glucan synthase gene under each set of possible conditions for the combined Round 1 and Round 2 data. The normalized relative gene expression for each wild type condition is listed. An up arrow (↑) indicates a higher fold gene expression for that strain under that particular condition whereas a down arrow (↓) indicates a lower fold gene expression for that strain under that particular condition. An approximate equal sign indicates that the indicated gene expression is approximately equivalent to that of the wild-type. These data were not subjected to statistical analyses. However, statistical analyses of data from the individual experiments (Rounds 1 and 2) are presented elsewhere (see Sections 4.2 and 4.3 of this thesis).

4.2 Normalized Relative Gene Expression Output from qRT-PCR - Round 1

The wild-type (F4) strain at 25°C under control conditions displayed relative fold expressions of approximately 2.5 for *fksP* and 4.0 for both *ags2* and *kre6*. Under stress conditions, the fold expressions were lower when compared to the control condition. When exposed to CR, all three glucan synthase genes exhibited two-fold expression levels whereas SDS and CAS induced approximately 0.5 fold expression of each gene. Statistically, the expression of both *fksP* and *ags2* under control conditions differed significantly from the SDS and CAS conditions. The expression of *kre6* under control conditions differed significantly from all treatment conditions (see statistical analyses contained in Appendix J).

At 37°C, the wild-type strain exhibited expression patterns under control conditions similar to that observed at 25°C. The relative fold expression for *fksP* was approximately 3.5, 3.0 for *ags2*, and approximately 4.0 for *kre6*. Also, as was observed at 25°C, the gene expression levels under the different stress conditions, with the exception of the CR condition, were all statistically lower than those seen under the control conditions (see statistical analyses contained in Appendix J). In the presence of CR, SDS, or CAS, the fold expression of all three glucan synthase gene fold expressions was approximately 1.0.

For the *ΔyakA* mutant incubated at 25°C under control conditions, expression of *fksP*, *ags2*, and *kre6* was significantly lower (see statistical analyses contained in Appendix J) than that exhibited by the wild-type strain. Specifically, the fold expression

of *fksP* was approximately 1.0, whereas *ags2* and *kre6* exhibited fold expression under 0.5. Under stress by CR, *fksP* was expressed 1.5 fold, but only 1.0 fold in the presence of SDS and CAS. By comparison, the expression of *ags2* and *kre6* under all stress conditions was approximately 1.0.

At 37°C, the *ΔyakA* mutant exhibited significantly decreased expression of the three glucan synthase genes under non-stressed conditions when compared to the control condition. Moreover, *ags2* and *kre6* expression was notably higher than control conditions when cultures were subjected to CR and SDS treatments, and possibly higher when exposed to CAS.

The genetically complemented strain, CY21, had a unique fold expression profile at 25°C when compared to either the wild-type or *ΔyakA* strains (Figure 12). Under the control condition and when exposed to CR, the *fksP* fold expression was relatively high, approximately 2.0 fold, whereas the expression of *ags2* and *kre6* was approcimately 1.0. When exposed to SDS or CAS, the expression levels of all three genes were approximately 1.0.

At 37°C, CY21 exhibited a two-fold level of expression for all three glucan synthase genes (Figure 13). However, when exposed to CR, SDS, or CAS, the expression levels decreased dramatically (0.5 to 1.0 fold) when compared to the control conditions.

An overall summary of the results from Round 1 is depicted in Figure 14. In general, the expression of *fksP*, *ags2*, and *kre6* is decreased in the *ΔyakA* mutant. In the wild-type, exposure to CR, SDS, or CAS results in a 4- to 20- fold decrease in expression of the three glucan synthase genes at 25°C and roughly a 2.5- to 4-fold decrease at 37°C.

In the *ΔyakA* mutant, exposure to CR generally decreases glucan synthase gene expression and mixed results at 25°C and 37°C upon treatment with SDS or CAS. Mixed expression results were observed at 25°C in strain CY21, but glucan synthase gene expression uniformly decreased under all conditions at 37°C when compared to the wild-type.

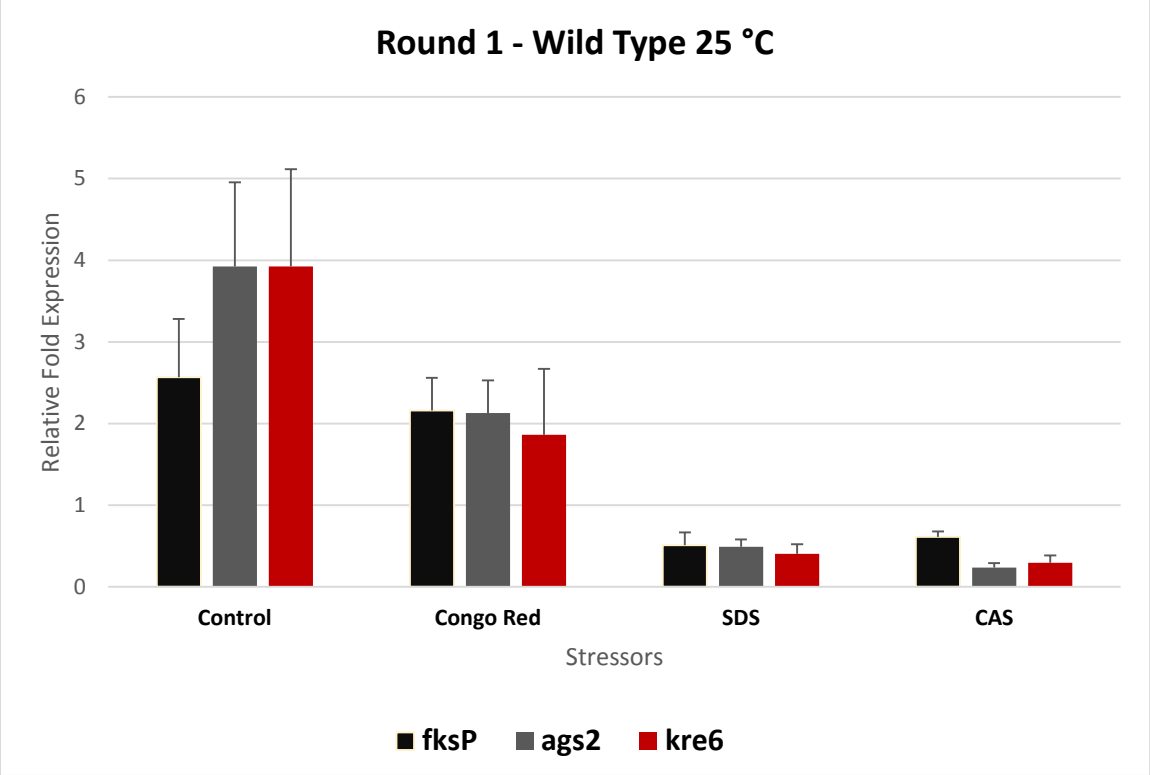


Figure 8: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the wild-type strain of *P. marneffei* incubated at 25°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of all three gene under control conditions differed significantly from all treatment conditions except for *ags2* and *kre6* under the CR condition (see statistical analyses contained in Appendix J).

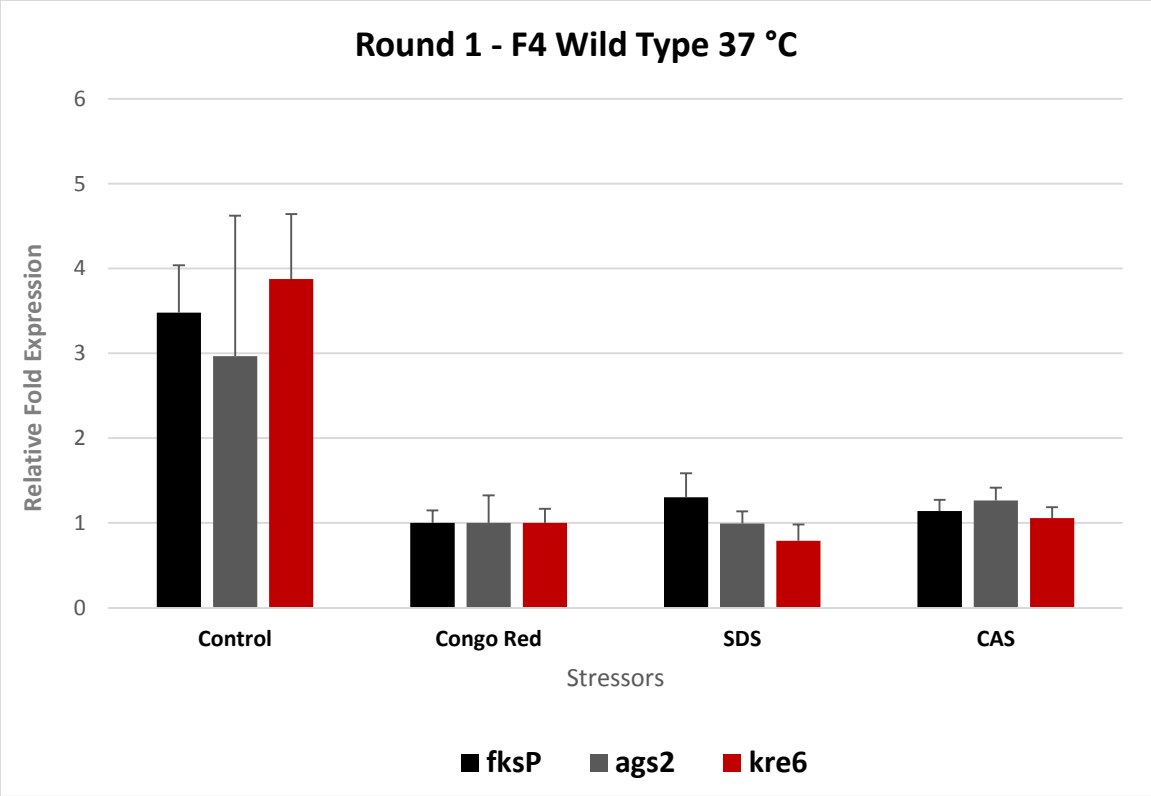


Figure 9: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the wild-type strain of *P. marneffei* incubated at 37°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of all three gene under control conditions differed significantly from all treatment conditions except for *ags2* and *kre6* under the CR condition (see statistical analyses contained in Appendix J).

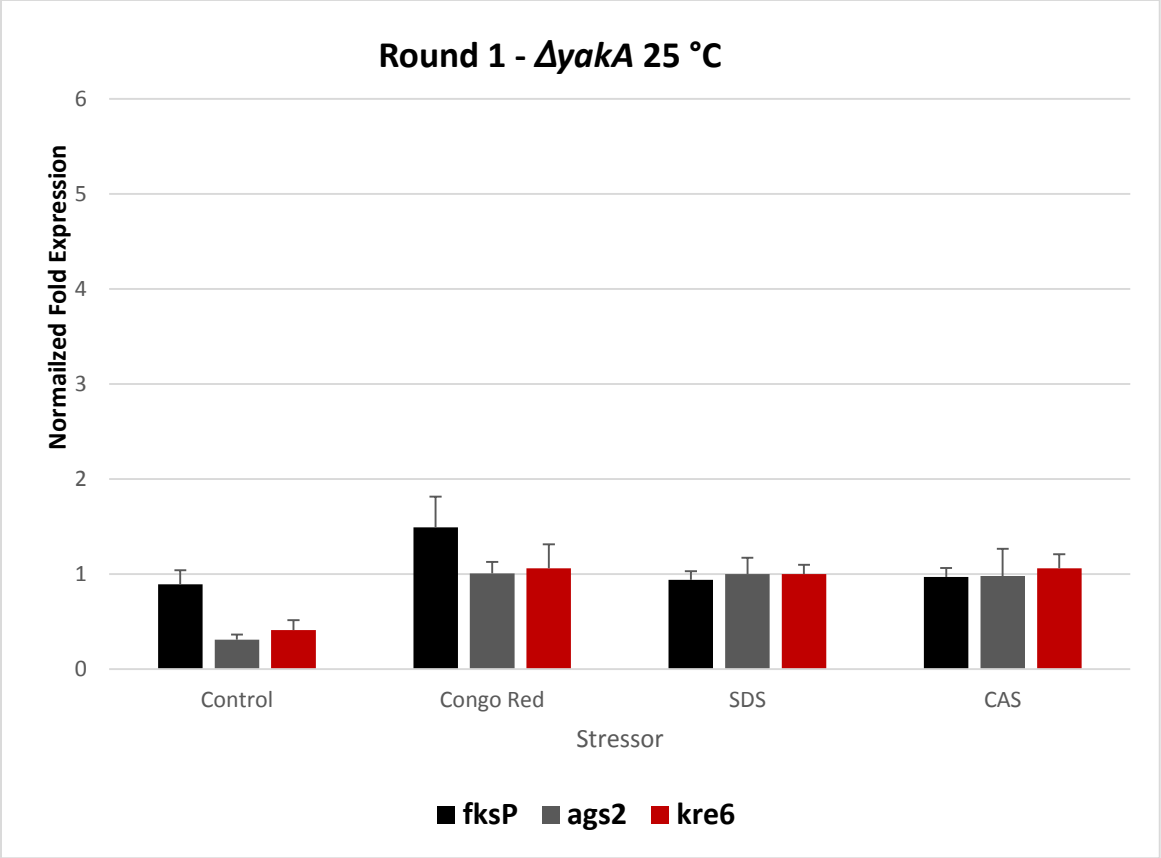


Figure 10: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the *ΔyakA* strain of *P. marneffei* incubated at 25°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of all three gene under control conditions differed significantly from the wild-type under control conditions. Moreover, *ags2* under the CR and CAS conditions and *kre6* under all three treatment conditions also differed significantly from the wild-type under control conditions (see statistical analyses contained in Appendix J).

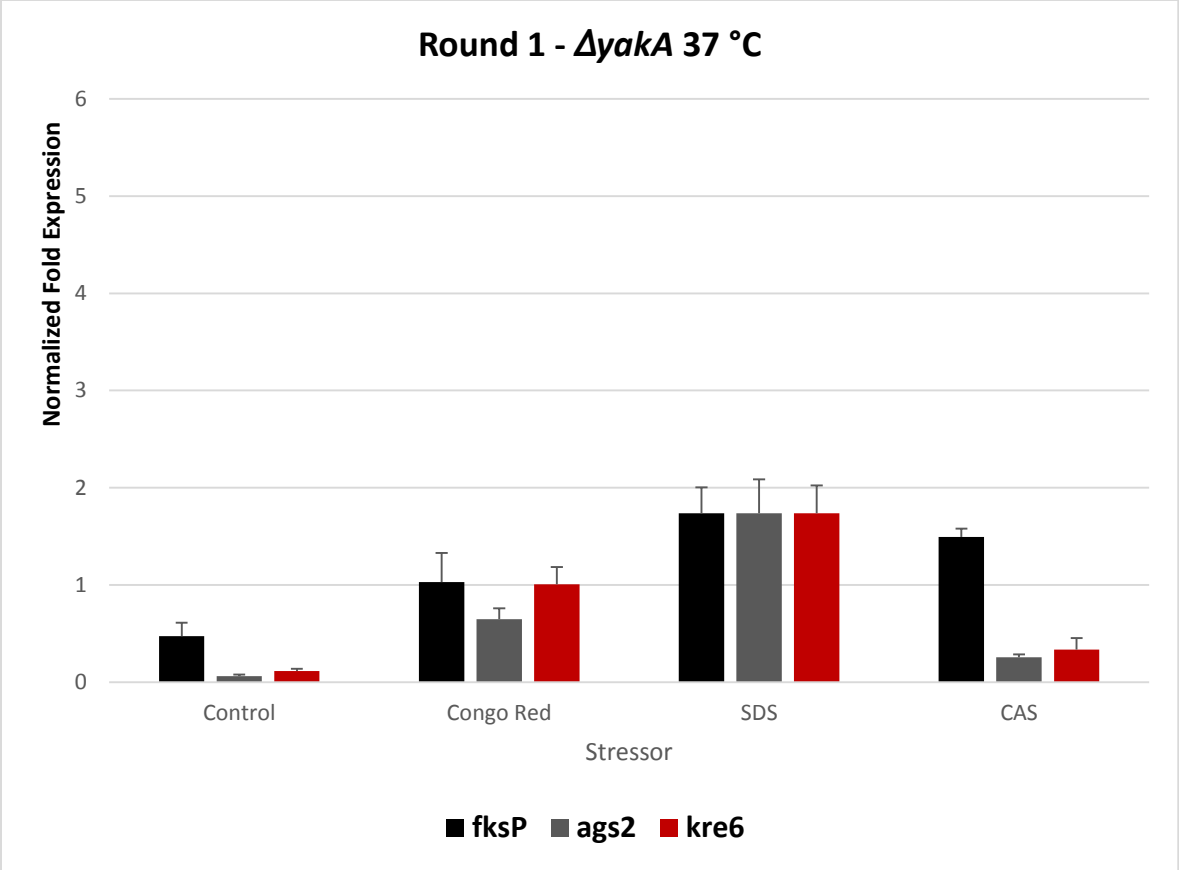


Figure 11: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the *ΔyakA* strain of *P. marneffei* incubated at 37°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of all three gene under control conditions differed significantly from the wild-type under control conditions. Moreover, *ags2* under the CR and CAS conditions and *kre6* under all three treatment conditions also differed significantly from the wild-type under control conditions (see statistical analyses contained in Appendix J).

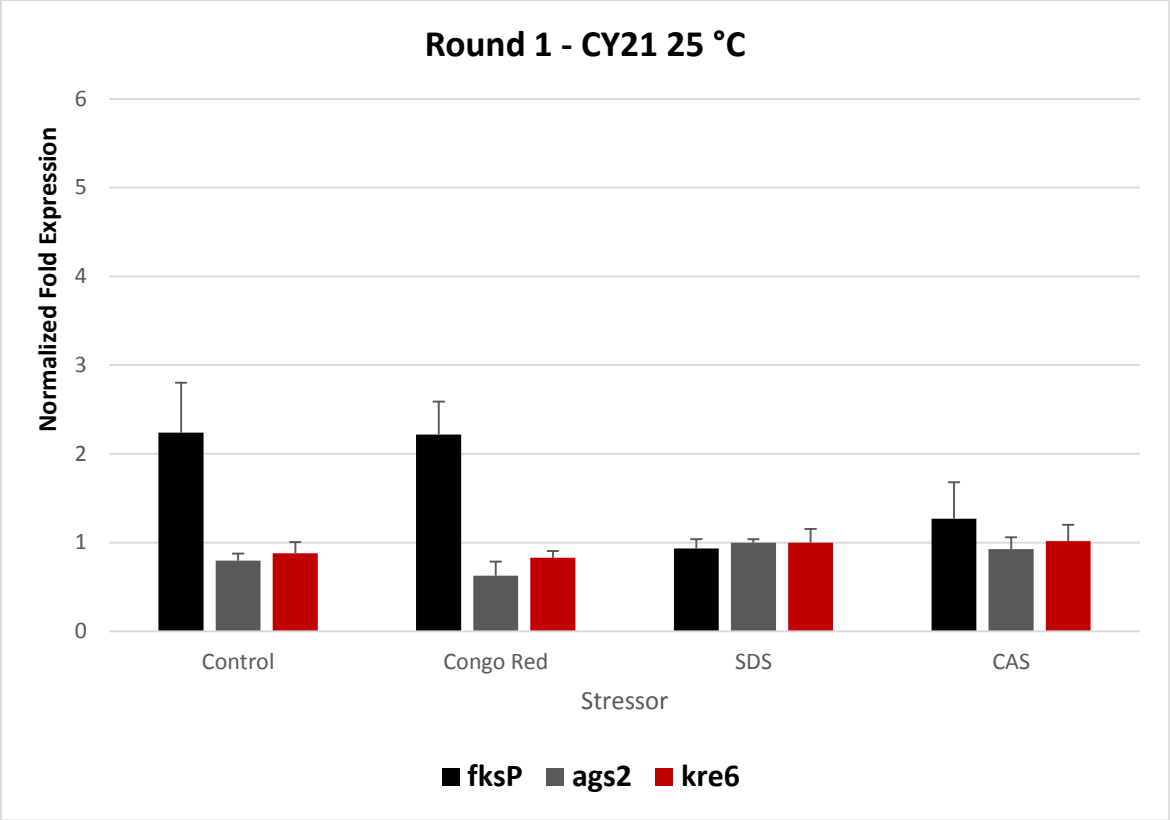


Figure 12: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the CY21 strain of *P. marneffei* incubated at 25°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of *fksP* under the SDS condition differed significantly from the wild-type control condition. The expression of *ags2* under all three treatment conditions differed significantly from the wild-type control conditions. The expression of *kre6* under all tested conditions differed significantly from the wild-type control condition (see statistical analyses contained in Appendix J).

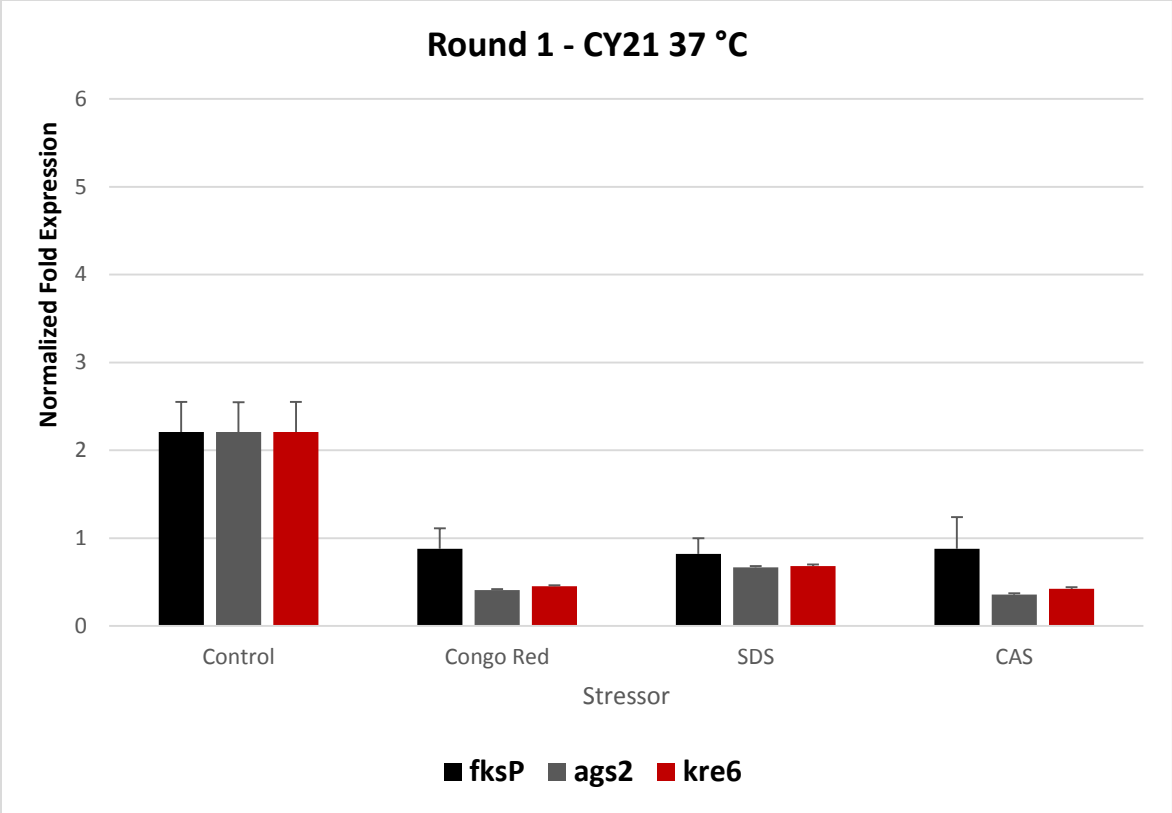


Figure 13: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the CY21 strain of *P. marneffei* incubated at 37°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of *fksP* under the SDS condition differed significantly from the wild-type control condition. The expression of *ags2* under all three treatment conditions differed significantly from the wild-type control conditions. The expression of *kre6* under all tested conditions differed significantly from the wild-type control condition (see statistical analyses contained in Appendix J).

QRT-PCR Round 1 Summary

25 C

fksP	Wild Type	*yakA*	CY21
Control	2.6	↓	↑
Congo Red	2.2	↓	↑
SDS	0.5	↑	↑
CAS	0.6	↑	↑

ags2	Wild Type	*yakA*	CY21
Control	3.9	↓	↓
Congo Red	2.1	↓	↓
SDS	0.5	↑	↑
CAS	0.2	↑	↑

kre6	Wild Type	*yakA*	CY21
Control	3.9	↓	↓
Congo Red	1.9	↓	↓
SDS	0.4	↑	↑
CAS	0.3	↑	↑

QRT-PCR Round 1 Summary

37 C

fksP	Wild Type	*yakA*	CY21
Control	3.5	↓	↓
Congo Red	1.0	≈	↓
SDS	1.3	↑	↓
CAS	1.1	↑	↓

ags2	Wild Type	*yakA*	CY21
Control	3.0	↓	↓
Congo Red	1.0	↓	↓
SDS	1.0	↑	↓
CAS	1.3	↓	↓

kre6	Wild Type	*yakA*	CY21
Control	3.9	↓	↓
Congo Red	1.0	≈	↓
SDS	0.8	↑	↓
CAS	1.1	↓	↓

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

4.3 Normalized Relative Gene Expression Output from qRT-PCR - Round 2

The second round of qRT-PCR data is noticeably similar to the first round with the exception of consistently higher fold expression levels of *fksP* across all strains and conditions. For the wild-type incubated at 25°C under control conditions, the expression of all three glucan synthase genes was higher when compared to the stressor conditions (Figure 15). Under CR treatment, *fksP* expression was approximately 1.3 fold whereas *ags2* and *kre6* were both near 0.5 fold. Although the fold expression differed, the same pattern was observed for cultures treated with SDS and CAS. At 37°C, an overall similar pattern of gene expression was noted for the wild-type under control and stress conditions (Figure 16).

For the *ΔyakA* mutant, the results of Round 2 yielded a similar pattern of gene expression compared to the Round 1 experiments (Figures 17 and 18). Under control conditions at both 25°C and 37°C, glucan synthase gene expression was markedly lower than that expressed by the wild-type strain. Notable difference, however, were observed. At 25°C, expression of *ags2* and *kre6* was lower in Round 2 and noticeably lower than *fksP* expression (Figure 17). At 37°C in the *ΔyakA* mutant, expression of all three genes in the presence of CAS was 2- to 10-fold higher (Figure 18).

For CY21, the Round 2 results were very similar in their patterns of expression to those exhibited in Round 1 (Figures 19 and 20). At 25°C, however, *fksP* expression in the presence of CAS was approximately twice that observed in Round 1 as well as greater than that of *ags2* and *kre6*. At 37°C, the CY21 strain exhibited increased expression of all

three genes under all conditions when compared to the Round 1 results, but the overall expression patters were roughly equivalent.

As stated previously, the overall fold expression patterns seen in the second round of data were very similar to the patterns seen throughout the first round with the exception of numerous instances of relatively higher *fksP* fold expression levels. Despite this, once again the wild-type strain had higher fold expression levels in the control conditions when compared to the stressor conditions at both 25°C and 37°C. Essentially the opposite was seen in the *ΔyakA* where the control conditions revealed lower fold expression of the three glucan synthase genes. Figure 21 summarizes the overall expression patterns under the different experimental conditions. Again, the expression of *fksP*, *ags2*, and *kre6* is decreased in the *ΔyakA* mutant under control conditions. In the wild-type, exposure to CR, SDS, or CAS results in a decrease in expression of the three glucan synthase genes at 25°C and 37°C. Curiously, in the *ΔyakA* mutant, exposure to stressors at 37°C increases gene expression, whereas strain CY21 exhibits increased expression when exposed to CR, SDS, or CAS under any experimental conditions.

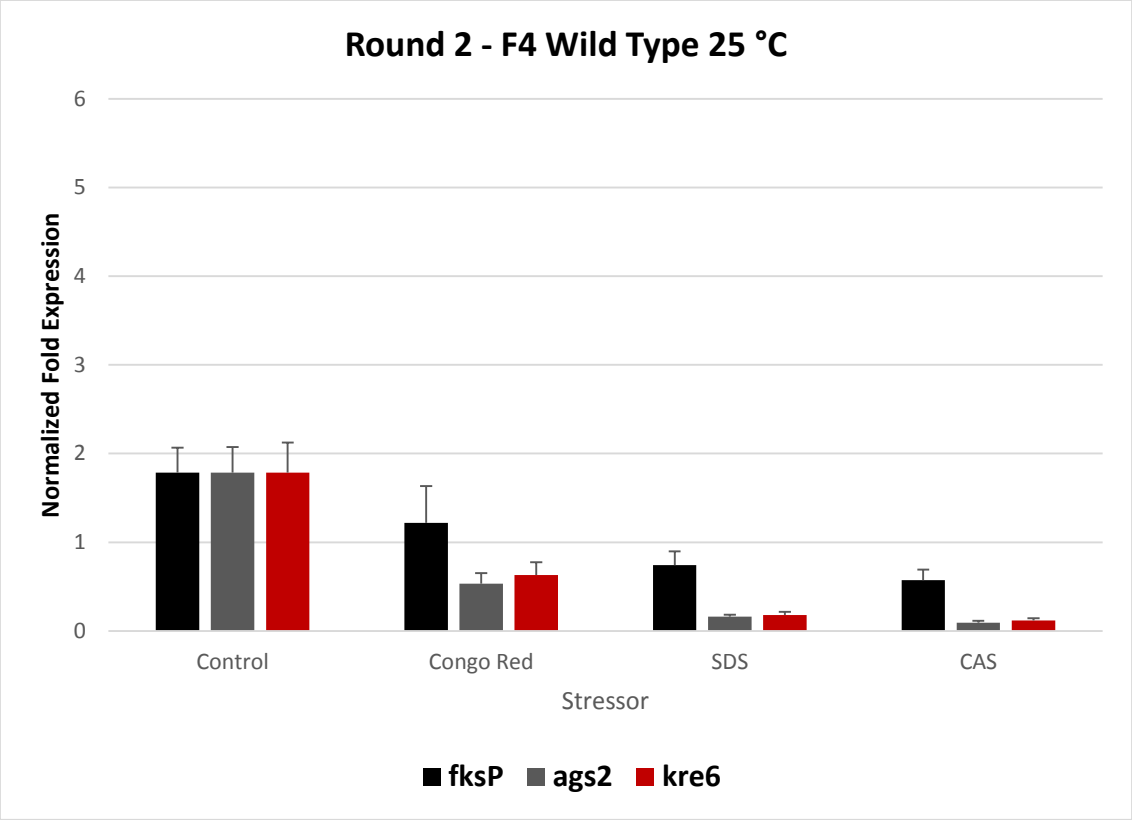


Figure 15: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the wild-type strain of *P. marneffei* incubated at 25°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of *ags2* and *kre6* under the three treatment conditions differed significantly from the *ΔyakA* strain under the CAS condition (see statistical analyses contained in Appendix K).

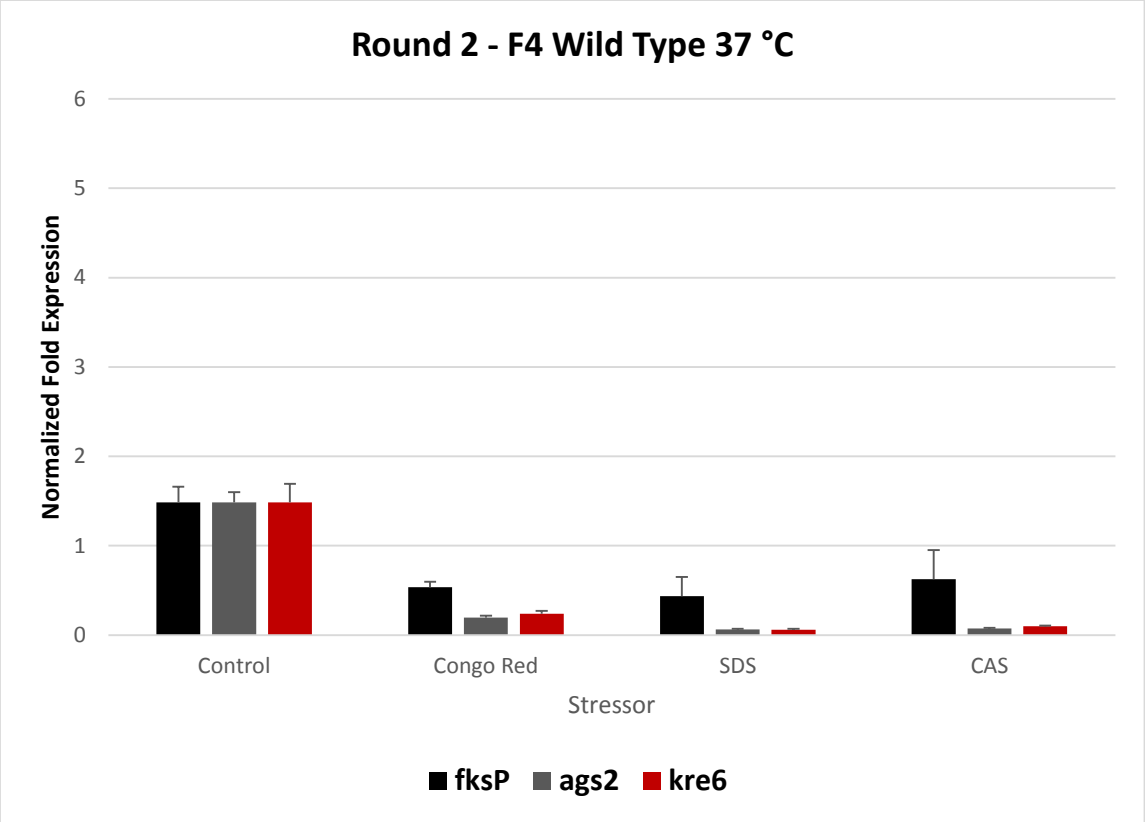


Figure 16: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the wild-type strain of *P. marneffei* incubated at 37°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of *ags2* and *kre6* under the three treatment conditions differed significantly from the *ΔyakA* strain under the CAS condition (see statistical analyses contained in Appendix K).

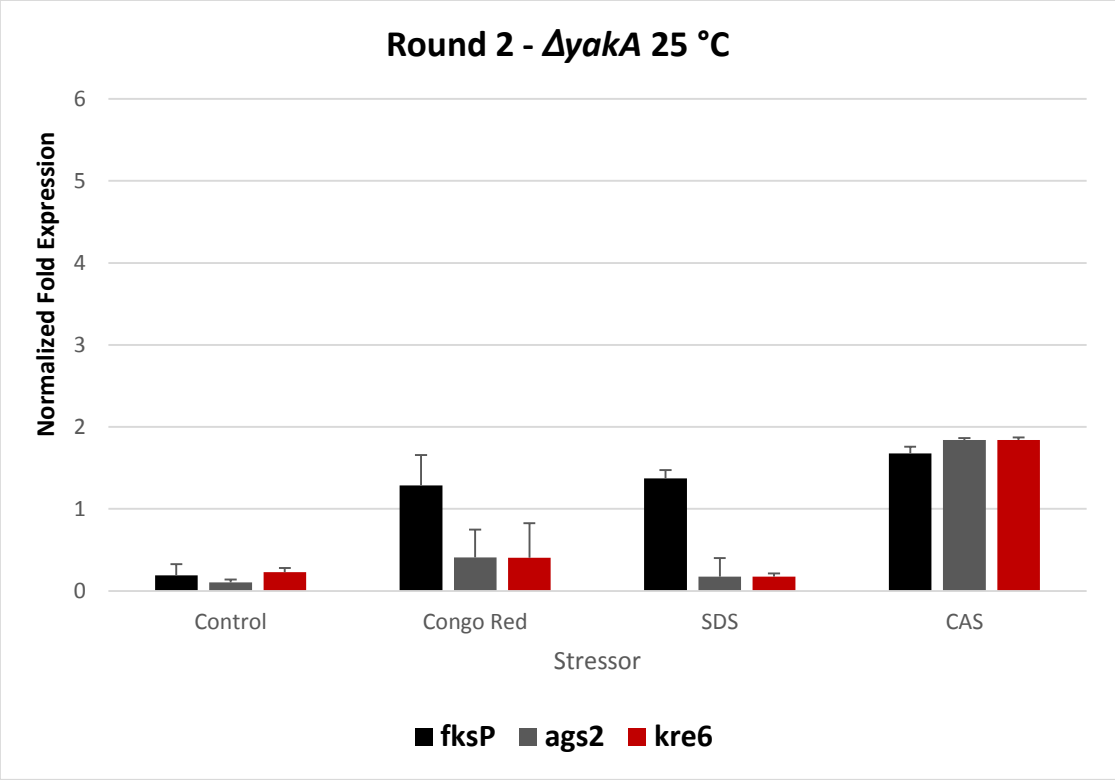


Figure 17: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the *ΔyakA* strain of *P. marneffei* incubated at 25°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of *ags2* and *kre6* under the control condition and the CR condition differed significantly from the CAS condition (see statistical analyses contained in Appendix K).

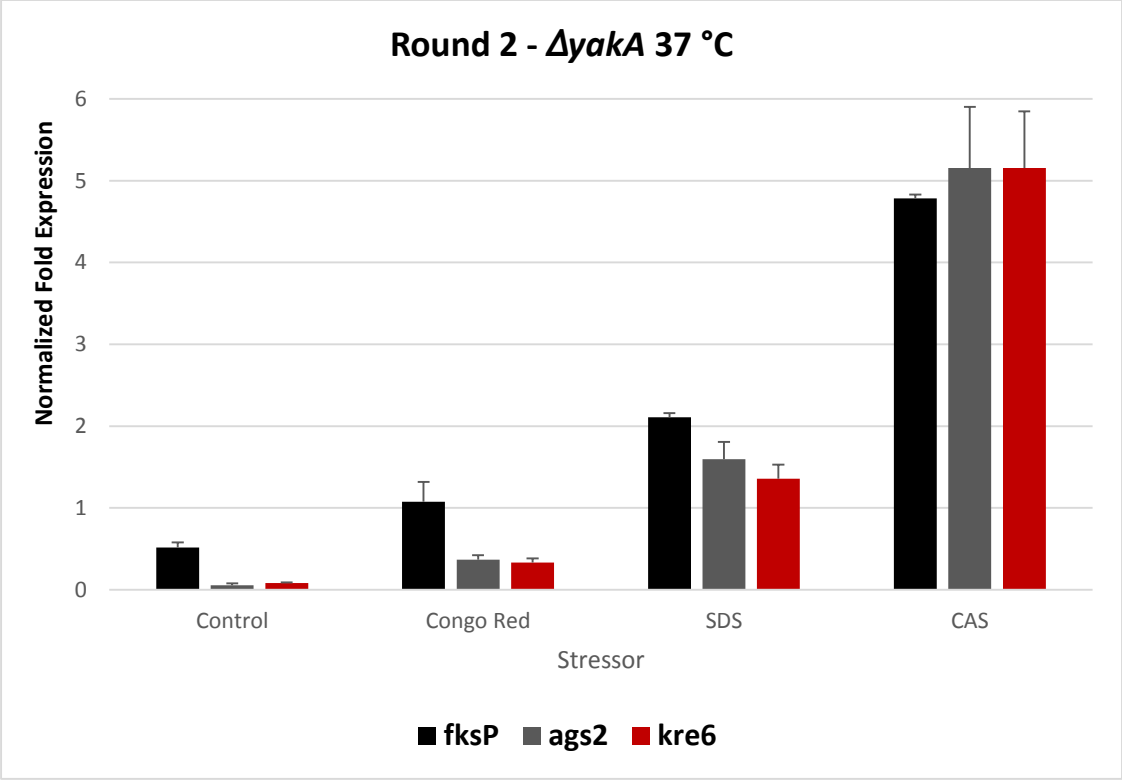


Figure 18: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the *ΔyakA* strain of *P. marneffei* incubated at 37°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. The expression of *ags2* and *kre6* under the control condition and the CR condition differed significantly from the CAS condition (see statistical analyses contained in Appendix K).

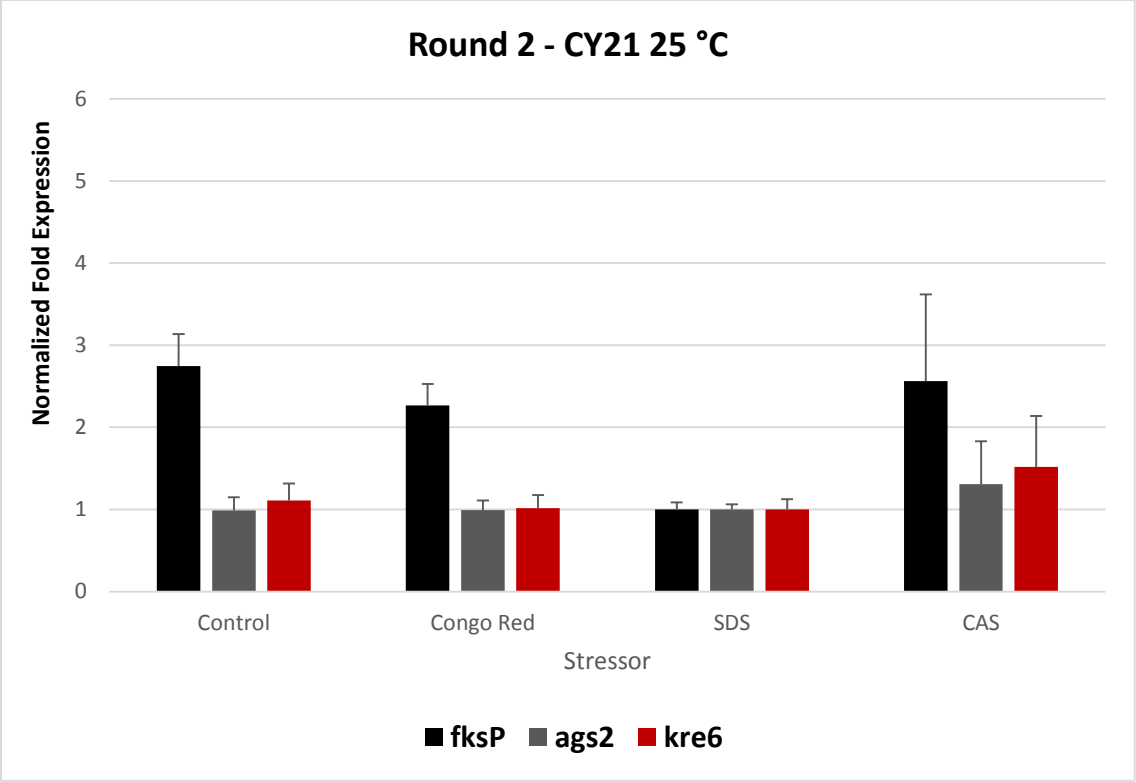


Figure 19: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the CY21 strain of *P. marneffei* incubated at 25°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. There were no significant interactions found for the CY21 strain in Round 2 (see statistical analyses contained in Appendix K).

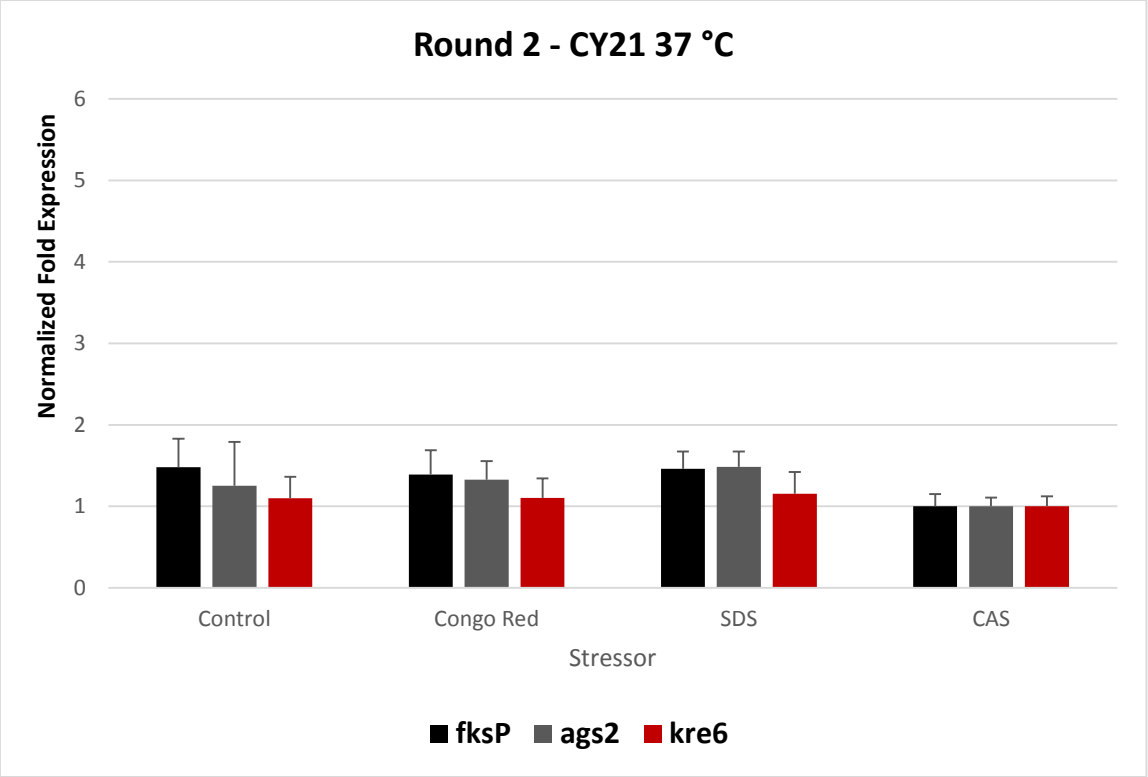


Figure 20: Normalized relative gene expression of the *fksP*, *ags2*, and *kre6* genes in the CY21 strain of *P. marneffei* incubated at 37°C. The expression of each gene was normalized to the housekeeping gene *benA*. The experimental conditions included subjecting cultures to a continuous 4-hour period of no stressor (control), Congo Red, sodium dodecyl sulfate (SDS), or caspofungin (CAS). All error bars represent one standard deviation. There were no significant interactions found for the *CY21* strain in Round 2 (see statistical analyses contained in Appendix K).

QRT-PCR Round 2 Summary

25 C

fksP	Wild Type	*yakA*	CY21
Control	1.8	↓	↑
Congo Red	1.2	↑	↑
SDS	0.7	↑	↑
CAS	0.6	↑	↑

ags2	Wild Type	*yakA*	CY21
Control	1.8	↓	↓
Congo Red	0.5	↓	↑
SDS	0.2	↓	↑
CAS	0.1	↑	↑

kre6	Wild Type	*yakA*	CY21
Control	1.8	↓	↓
Congo Red	0.6	↓	↑
SDS	0.2	↓	↑
CAS	0.1	↑	↑

QRT-PCR Round 2 Summary

37 C

fksP	Wild Type	*yakA*	CY21
Control	1.5	↓	≈
Congo Red	0.5	↑	↑
SDS	0.4	↑	↑
CAS	0.6	↑	↑

ags2	Wild Type	*yakA*	CY21
Control	1.5	↓	↓
Congo Red	0.2	↑	↑
SDS	0.1	↑	↑
CAS	0.1	↑	↑

kre6	Wild Type	*yakA*	CY21
Control	1.5	↓	↓
Congo Red	0.2	↑	↑
SDS	0.1	↑	↑
CAS	0.1	↑	↑

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

CHAPTER 5: DISCUSSION

Based on all statistical tests that were performed, there was a significant difference in normalized gene expression between the three different strains under the different conditions that were tested. For the first round of data, this was true for all three glucan synthase genes. For the second round of data, this was true for *ags2* and *kre6*. This indicates that an interrupted *yakA* gene and even a reinserted complementary *yakA* gene will influence glucan synthase gene expression in both control conditions and stress conditions. With a functional, properly located *yakA* gene, the wild-type at 25°C exhibited higher control, "non-stressed" expression levels of the glucan synthases. When placed in any of the stress conditions, the glucan synthase expression levels tended to decrease, indicating a diminished response either through direct inhibition by the stressors themselves or possibly through destruction of fungal cells, leading to diminished levels. Compared to the wild-type, *ΔyakA* at 25°C strain tended to have significantly lower expression levels under control conditions but elicited higher overall stress responses, especially in the case of the critically important glucan produced by the enzyme encoded by *fksP*. The complement strain CY21 at 25°C had a more balanced response, with most expression levels falling around one fold, except for multiple instances of higher *fksP* levels again. Similar overall patterns were exhibited at 37°C amongst the three strains. The wild-type strain had higher expression levels under the control conditions whereas the stress conditions yielded lower levels. The *ΔyakA* strain essentially responded in the reverse, once again eliciting a significantly higher stress response, and the complement CY21 strain maintained a more balanced model with

instances of higher *fksP* levels. These outcomes, and the statistical analyses behind them, point to several possible conclusion that will be discussed further.

First, it is important to keep in mind that all multivariate analyses that were performed only indicate significance when the different strains were analyzed in terms of the different conditions (stressed or non-stressed). Looking at phase versus condition and phase versus strain yielded no significance. As stated in the above paragraph, the first round of data found significance in the strain versus condition model for all three genes while the second round of data found significance for *ags2* and *kre6*. For the first round of data, the majority of possible strain-condition combinations had a significant interaction with the wild-type strain under control conditions. Based on the fact that the wild-type control had a consistently high expression, it's not surprising that it interacted significantly with the other possible conditions. In the second round of data, however, significant interactions were all found with the strain-condition combination of *ΔyakA* in caspofungin-supplemented media. It's possible that this was primarily influenced by the high stress response present at 37°C for *ΔyakA* in round 2. Additionally, the consistently higher expression of *fksP* in round 2 may have contributed to less overall variation in glucan synthase expression amongst all of the strains and conditions, which therefore caused a variation in the significance that was found through the post hoc tests. Still, the overall indication is that a manipulated *yakA* gene causes variability in the expression of these glucan synthases under stressed and non-stressed conditions.

yakA, and its orthologues in other fungi, has been shown to be important for growth regulation and stress responses. Based on the results of this study, it is possible that an interrupted *yakA* gene in *P. marneffei* causes deviations in the regulation and

expression of the glucan synthases *fksP*, *ags2*, and *kre6*. Each of these genes produces a highly specific type of glucan, each of which has a highly specific role in the maintenance and functionality of the cell-wall. Their proper regulation is essential to proper cell-wall integrity. If a dysfunctional *yakA* gene did cause lower levels of glucan synthase gene expression as seen in the results, it would help explain the weakened, more pliable cell-walls that Suwunnakorn et al. (2014) found in their study. Additionally, the increased chitin content they found in the *ΔyakA* strain is a classic symptom of fungi that are attempting to compensate for some time of cell-wall malformation. Normally this chitin synthase compensatory response is not directly caused by the interrupted gene, but is rather a natural reaction that is handled by the numerous and somewhat redundant chitin synthases. This exact type of response was observed by the work of another graduate student (Joshua Engle, unpublished data). This study indicates that an overall heightened chitin synthase gene expression at least in the *ΔyakA* mutant at 25°C and 37°C. This would be typical of a mutant fungal strain that had a cell-wall issue such as a *yakA* gene that was interrupted and potentially hampering proper expression of the critical glucan synthases.

As previously noted, *yakA* is not only responsible for growth regulation, but it is also responsible for numerous, unspecified stress responses. In other fungi, this stress response functionality is governed through the *yakA* gene product's ability to phosphorylate the transcription factors *hsf1* and *msn2/4* (Sadeh et al., 2012; Sadeh et al., 2011; Elfving et al., 2014; Hartley et al., 1994; Lee et al., 2008; Liu et al., 2013). The transcription factor *hsf1* is known to play roles in the regulation of thermotolerance of the fungus while *msn2/4* are responsible for general, usually unspecified stress responses to

cell-wall damage. The *yakA* gene is known to regulate *msn2/4* through inhibition. If the *yakA* gene was not functioning properly, as in the case of the mutant studied here, it may not properly down regulate *msn2/4* and therefore cause an unregulated, irregular stress response to any number of conditions. This may explain the significantly higher stress response gene expression levels that are seen in the *yakA* strain at both 25°C and 37°C despite the normally lower non-stressed control levels.

In summary, *yakA* potentially plays a significant role in the regulation of glucan synthase gene expression both in terms of normal growth regulation and in terms of stress response regulation most likely through the transcription factor *msn2/4*. This is supported by the characteristic chitin synthase up regulation and the higher overall levels of chitin that are present in this mutant. Understanding these levels of regulation, especially in terms of cell-wall stress, is critical to understanding how this fungus deals with its outside environment. A better understanding of this is one of the first steps in understanding how to treat an organism like *P. marneffei* which is often difficult to deal with due to the normally immunocompromised nature of those individuals that it infects.

CHAPTER 6: APPENDICES

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

Gene	qRT-pcr Primer Sequence	Description
fksP-F	CACGGTGATGCCTATTACCA	1,3-β-glucan ynthase
fksP-R	GCGTGTTGTTCACCGTAATG	1,3-β-glucan synthase
ags2-F	TGGGGCTTTGACGGTTACTCA	α-1,3-glucan synthase
ags2-R	TGTTCCTGGTGGATGAATGGA	α-1,3-glucan synthase
kre6-F	GAGATGCAGGATCGGATTGG	β-1,6-glucan synthase
kre6-R	TGCCGTCGTACCAGTTGTTG	β-1,6-glucan synthase
benA-F	GCTCCGGTGTCTACAATGGC	β-tubulin (used for normalization)
benA-R	AGTTGTTACCAGCACCGGAC	β-tubulin (used for normalization)

Appendix B: Stressor Concentrations and Inoculation volumes for Cell-wall Stress Experiments at 25°C:

Stressor	Inoculation Volume	Concentration in Culture
Congo Red	350 µl	70 µg ml^{-1}
Sodium Dodecyl Sulfate (SDS)	250 µl	0.02%
caspofungin (CAS)	1.2 ml	120 µg ml^{-1}

Appendix C: Stressor Concentrations and Inoculation volumes for Cell-wall Stress Experiments at 37°C:

Stressor	Inoculation Volume	Concentration in Culture
Congo Red	200 µl	40 µg ml^{-1}
Sodium Dodecyl Sulfate (SDS)	220 µl	0.0175%
caspofungin (CAS)	700 µl	70 µg ml^{-1}

Appendix D: Qiagen One-Step RT-PCR Protocol used for primer assessment in conjunction with agarose gel electrophoresis:

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 for primer assessment in conjunction with agarose gel electrophoresis:

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

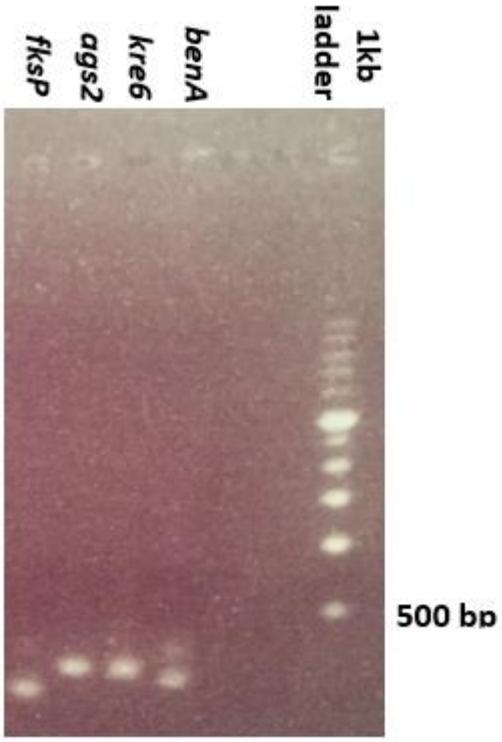
Appendix F: iTaq Universal SYBR Green One-Step kit protocol used in conjunction with the Bio-Rad iQ5 Real-Time PCR System Thermocycler for assessment of normalized gene expression.

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 in conjunction with the Bio-Rad iQ5 Real-Time PCR System Thermocycler for assessment of normalized gene expression.

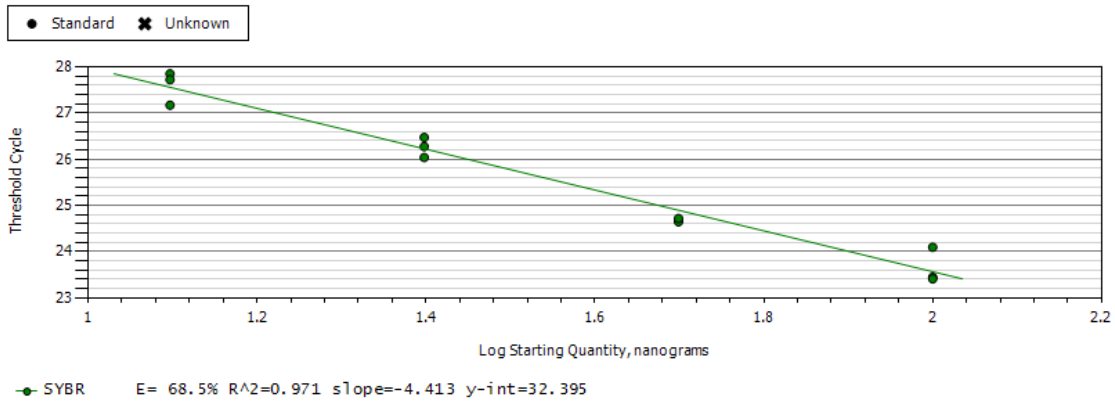
iTaq Universal SYBR Green One-Step Kit Thermal Cycler Program	**Time/Cycles**	**Temperature**
Reverse Transcription Reaction	10 min	50 °C
Polymerase Activation and DNA Denaturation	5 min	95 °C
Cycle: 2 steps		
Denaturation	10 sec	95 °C
Annealing/Extension + Plate Read	30 sec	60 °C
Number of Cycles	45	
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 generated through reverse transcription and amplification of F4 wild-type, non-stressed control RNA samples. Lane 7 contains a 1 kilobase molecular weight ladder. All resulting bands were betweem 200 and 400 kilobases as expected

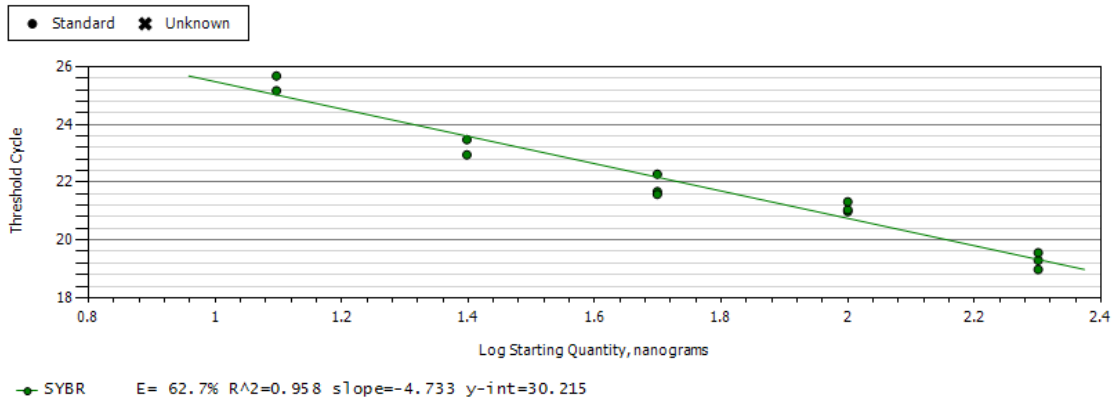


Appendix I: Standard curves generated in order to assess primer quality using the Bio-Rad iQ5 Real-Time PCR System software for each of the genes analyzed in this study. All standard curves based off dilution series of each gene as described in the materials and methods section:

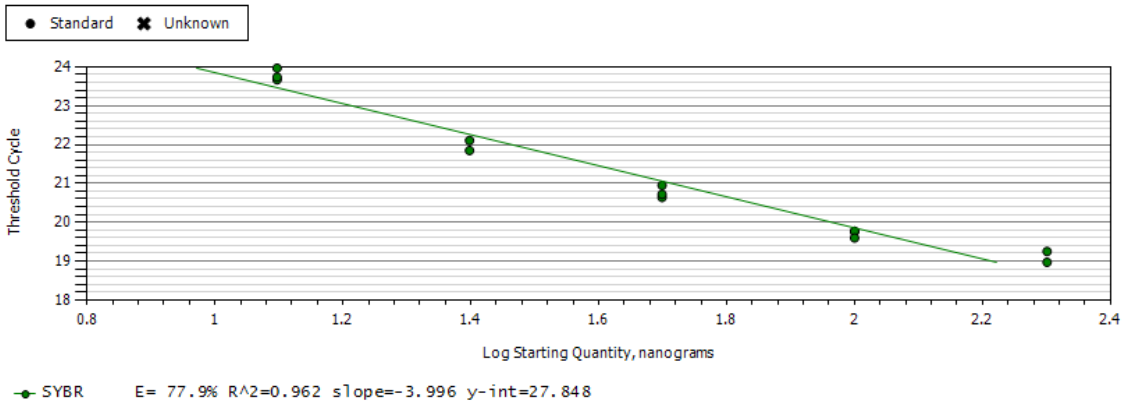
fksP



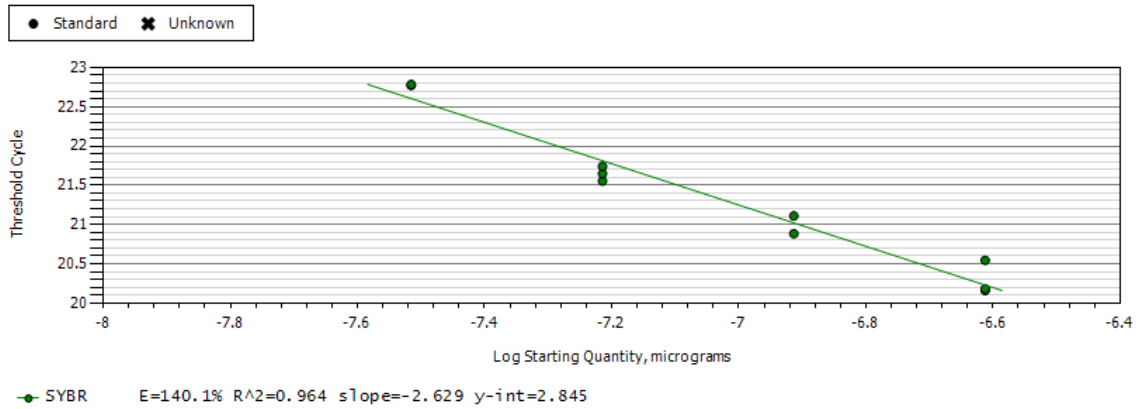
ags2



kre6


Standard
Unknown
Threshold Cycle
24
23
22
21
20
19
18
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
Log Starting Quantity, nanograms
SYBR
E= 77.9% R^2=0.962 slope=-3.996 y-int=27.848

benA


Standard
Unknown
Threshold Cycle
23
22.5
22
21.5
21
20.5
20
-8
-7.8
-7.6
-7.4
-7.2
-7
-6.8
-6.6
-6.4
Log Starting Quantity, micrograms
SYBR
E=140.1% R^2=0.964 slope=-2.629 y-int=2.845

Appendix J: Statistical analyses for the first round of qRT-PCR data. For Post-Hoc test results, only significant outcomes ($p < .05$) have been listed. Refer to the key immediately below this legend:

Term	Meaning
Expression1	*fksP* Normalized Fold Expression
Expression2	*ags2* Normalized Fold Expression
Expression3	*kre6* Normalized Fold Expression
StrainCond 1	F4 WT Control
StrainCond 2	F4 WT Congo Red
StrainCond 3	F4 WT SDS
StrainCond 4	F4 WT CAS
StrainCond 5	*yakA* Control
StrainCond 6	*yakA* Congo Red
StrainCond 7	*yakA* SDS
StrainCond 8	*yakA* CAS
StrainCond 9	*CY21* Control
StrainCond 10	*CY21* Congo Red
StrainCond 11	*CY21* SDS
StrainCond 12	*CY21* CAS

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.851	30.394	3.000	16.000	0.000
	Wilks' Lambda	0.149	30.394	3.000	16.000	0.000
	Hotelling's Trace	5.699	30.394	3.000	16.000	0.000
	Roy's Largest Root	5.699	30.394	3.000	16.000	0.000
Strain	Pillai's Trace	0.427	1.537	6.000	34.000	0.196
	Wilks' Lambda	0.613	1.480	6.000	32.000	0.216
	Hotelling's Trace	0.568	1.420	6.000	30.000	0.240
	Roy's Largest Root	0.412	2.334	6.000	17.000	0.110
Phase	Pillai's Trace	0.044	0.243	3.000	16.000	0.865
	Wilks' Lambda	0.956	0.243	3.000	16.000	0.865
	Hotelling's Trace	0.046	0.243	3.000	16.000	0.865
	Roy's Largest Root	0.046	0.243	3.000	16.000	0.865
Strain * Phase	Pillai's Trace	0.135	0.411	6.000	34.000	0.867
	Wilks' Lambda	0.866	0.399	6.000	32.000	0.874
	Hotelling's Trace	0.154	0.386	6.000	30.000	0.882
	Roy's Largest Root	0.148	0.839	6.000	17.000	0.491

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Expression 1	1.509	5.000	0.302	0.479	0.788
	Expression 2	4.490	5.000	0.898	0.946	0.476
	Expression 3	4.130	5.000	0.826	0.725	0.614
Intercept	Expression 1	46.037	1.000	46.037	73.001	0.000
	Expression 2	25.482	1.000	25.482	26.840	0.000
	Expression 3	28.297	1.000	28.297	24.822	0.000
Strain	Expression 1	0.905	2.000	0.452	0.717	0.502
	Expression 2	4.283	2.000	2.142	2.256	0.134
	Expression 3	3.679	2.000	1.939	1.701	0.210
Phase	Expression 1	0.005	1.000	0.005	0.009	0.927
	Expression 2	0.193	1.000	0.193	0.203	0.658
	Expression 3	0.086	1.000	0.086	0.076	0.786
Strain * Phase	Expression 1	0.599	2.000	0.300	0.475	0.630
	Expression 2	0.014	2.000	0.007	0.007	0.993
	Expression 3	0.165	2.000	0.082	0.072	0.931
Error	Expression 1	11.352	18.000	0.631		
	Expression 2	17.089	18.000	0.949		
	Expression 3	20.519	18.000	1.140		
Total	Expression 1	58.898	24.000			
	Expression 2	47.062	24.000			
	Expression 3	52.946	24.000			
Corrected Total	Expression 1	12.861	23.000			
	Expression 2	21.579	23.000			
	Expression 3	24.650	23.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.939	51.738	3.000	10.000	0.000
	Wilks' Lambda	0.061	51.738	3.000	10.000	0.000
	Hotelling's Trace	15.521	51.738	3.000	10.000	0.000
	Roy's Largest Root	15.521	51.738	3.000	10.000	0.000
Strain	Pillai's Trace	0.749	2.197	6.000	22.000	0.082
	Wilks' Lambda	0.361	2.214	6.000	20.000	0.084
	Hotelling's Trace	1.463	2.195	6.000	18.000	0.092
	Roy's Largest Root	1.210	4.438	3.000	11.000	0.028
Phase	Pillai's Trace	0.825	1.516	9.000	36.000	0.180
	Wilks' Lambda	0.270	1.941	9.000	24.488	0.093
	Hotelling's Trace	2.365	2.278	9.000	26.000	0.049
	Roy's Largest Root	2.214	8.858	3.000	12.000	0.002
Strain * Condition	Pillai's Trace	1.242	1.412	18.000	36.000	0.185
	Wilks' Lambda	0.115	1.838	18.000	28.770	0.070
	Hotelling's Trace	4.855	2.338	18.000	26.000	0.024
	Roy's Largest Root	4.254	8.508	6.000	12.000	0.001

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Expression 1	9.692	11.000	0.881	3.336	0.250
	Expression 2	17.201	11.000	1.564	4.285	0.009
	Expression 3	21.236	11.000	1.931	6.787	0.001
Intercept	Expression 1	46.037	1.000	46.037	174.335	0.000
	Expression 2	25.482	1.000	25.482	69.834	0.000
	Expression 3	28.297	1.000	28.297	99.476	0.000
Strain	Expression 1	0.905	2.000	0.452	1.713	0.222
	Expression 2	4.283	2.000	2.142	5.869	0.017
	Expression 3	3.879	2.000	1.939	6.818	0.011
Phase	Expression 1	3.485	3.000	1.162	4.399	0.026
	Expression 2	4.045	3.000	1.348	3.695	0.043
	Expression 3	5.702	3.000	1.901	6.682	0.007
Strain * Condition	Expression 1	5.302	6.000	0.884	3.346	0.036
	Expression 2	8.872	6.000	1.479	4.052	0.019
	Expression 3	11.655	6.000	1.943	6.829	0.002
Error	Expression 1	3.169	12.000	0.264		
	Expression 2	4.379	12.000	0.365		
	Expression 3	3.414	12.000	0.284		
Total	Expression 1	58.898	24.000			
	Expression 2	47.062	24.000			
	Expression 3	52.946	24.000			
Corrected Total	Expression 1	12.861	23.000			
	Expression 2	21.579	23.000			
	Expression 3	24.650	23.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.912	48.407	3.000	14.000	0.000
	Wilks' Lambda	0.088	48.407	3.000	14.000	0.000
	Hotelling's Trace	10.373	48.407	3.000	14.000	0.000
	Roy's Largest Root	10.373	48.407	3.000	14.000	0.000
Strain	Pillai's Trace	0.436	0.906	9.000	48.000	0.528
	Wilks' Lambda	0.601	0.886	9.000	34.223	0.547
	Hotelling's Trace	0.606	0.852	9.000	38.000	0.574
	Roy's Largest Root	0.491	2.617	3.000	16.000	0.087
Phase	Pillai's Trace	0.041	0.197	3.000	14.000	0.896
	Wilks' Lambda	0.959	0.197	3.000	14.000	0.896
	Hotelling's Trace	0.042	0.197	3.000	14.000	0.896
	Roy's Largest Root	0.042	0.197	3.000	14.000	0.896
Condition * Phase	Pillai's Trace	0.601	1.337	9.000	48.000	0.243
	Wilks' Lambda	0.440	1.527	9.000	34.223	0.178
	Hotelling's Trace	1.181	1.662	9.000	38.000	0.133
	Roy's Largest Root	1.096	5.844	3.000	16.000	0.007

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Expression 1	5.418	7.000	0.774	1.664	0.188
	Expression 2	4.845	7.000	0.692	0.662	0.701
	Expression 3	6.495	7.000	0.928	0.818	0.586
Intercept	Expression 1	46.037	1.000	46.037	98.975	0.000
	Expression 2	25.482	1.000	25.482	24.364	0.000
	Expression 3	28.297	1.000	28.297	24.939	0.000
Strain	Expression 1	3.485	3.000	1.162	2.498	0.097
	Expression 2	4.045	3.000	1.348	1.289	0.312
	Expression 3	5.702	3.000	1.901	1.675	0.212
Phase	Expression 1	0.005	1.000	0.005	0.012	0.916
	Expression 2	0.193	1.000	0.193	0.184	0.674
	Expression 3	0.086	1.000	0.086	0.076	0.786
Condition * Phase	Expression 1	1.928	3.000	0.643	1.382	0.284
	Expression 2	0.607	3.000	0.202	0.194	0.899
	Expression 3	0.707	3.000	0.236	0.208	0.890
Error	Expression 1	7.442	16.000	0.465		
	Expression 2	16.735	16.000	1.046		
	Expression 3	18.154	16.000	1.135		
Total	Expression 1	58.898	24.000			
	Expression 2	47.062	24.000			
	Expression 3	52.946	24.000			
Corrected Total	Expression 1	12.861	23.000			
	Expression 2	21.579	23.000			
	Expression 3	24.650	23.000			

Tests of Between-Subjects Effects

Dependent Variable: Expression 1					
Source	**Type III Sum of Squares**	**df**	**Mean Suare**	**F**	**Sig.**
Corrected Model	9.692	11.000	0.881	3.336	0.025
Intercept	46.037	1.000	46.037	174.335	0.000
Strain Condition	9.692	11.000	0.881	3.336	0.025
Error	3.169	12.000	0.264		
Total	58.898	24.000			
Corrected Total	12.861	23.000			

R Squared =.754
Adjusted R Squared = .528

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Expression 1	Tukey HSD	1	3	0.039
			4	0.036
			5	0.02
			11	0.036
		3	1	0.039
		4	1	0.036
		5	1	0.02
		11	1	0.036
	Bonferroni	1	5	0.043
		5	1	0.043

Tests of Between-Subjects Effects

Dependent Variable: Expression 2					
Source	**Type III Sum of Squares**	**df**	**Mean Suare**	**F**	**Sig.**
Corrected Model	17.201	11.000	1.564	4.285	0.009
Intercept	25.482	1.000	25.482	69.834	0.000
Strain Condition	17.201	11.000	1.564	4.285	0.009
Error	4.379	12.000	0.365		
Total	47.062	24.000			
Corrected Total	21.579	23.000			

R Squared =.797
Adjusted R Squared = .611

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Expression 2	Tukey HSD	1	3	0.022
			4	0.023
			5	0.005
			6	0.028
			8	0.016
			10	0.008
			11	0.013
			12	0.011
		3	1	0.022
		4	1	0.023
		5	1	0.005
		6	1	0.028
		8	1	0.016
		10	1	0.008
		11	1	0.013
		12	1	0.011
	Bonferroni	1	3	0.049
.			5	0.01
			8	0.034
			10	0.016
			11	0.027
			12	0.023
		3	1	0.049
		5	1	0.01
		8	1	0.034
		10	1	0.016
		11	1	0.027

		12	1	0.023

Tests of Between-Subjects Effects

Dependent Variable: Expression 3					
Source	Type III Sum of Squares	df	Mean Suare	F	Sig.
Corrected Model	21.236	11.000	1.931	6.787	0.001
Intercept	28.297	1.000	28.297	99.476	0.000
Strain Condition	21.236	11.000	1.931	6.787	0.001
Error	3.414	12.000	0.284		
Total	52.946	24.000			
Corrected Total	24.650	23.000			

R Squared =.862
Adjusted R Squared = .735

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
Expression 3	Tukey HSD	1	2	0.017
			3	0.002
			4	0.002
			5	0.001
			6	0.005
			7	0.015
			8	0.002
			9	0.024
			10	0.001
			11	0.001
			12	0.001
		2	1	0.017
		3	1	0.002
		4	1	0.002
		5	1	0.001
		6	1	0.005
		7	1	0.015
		8	1	0.002
		9	1	0.024
		10	1	0.001
		11	1	0.001
		12	1	0.001
	Bonferroni	1	2	0.038
			3	0.003
			4	0.004
			5	0.001
			6	0.011

			7	0.031
			8	0.004
			10	0.002
			11	0.003
			12	0.002
		2	1	0.038
		3	1	0.003
		4	1	0.004
		5	1	0.001
		6	1	0.011
		7	1	0.031
		8	1	0.004
		10	1	0.002
		11	1	0.003
		12	1	0.002

Appendix K: Statistical analyses for the first round of qRT-PCR data. For Post-Hoc test results, only significant outcomes ($p < .05$) have been listed:

Term	Meaning
EXP1	*fksP* Normalized Fold Expression
EXP2	*ags2* Normalized Fold Expression
EXP3	*kre6* Normalized Fold Expression
StrainCond 1	F4 WT Control
StrainCond 2	F4 WT Congo Red
StrainCond 3	F4 WT SDS
StrainCond 4	F4 WT CAS
StrainCond 5	*yakA* Control
StrainCond 6	*yakA* Congo Red
StrainCond 7	*yakA* SDS
StrainCond 8	*yakA* CAS
StrainCond 9	*CY21* Control
StrainCond 10	*CY21* Congo Red
StrainCond 11	*CY21* SDS
StrainCond 12	*CY21* CAS

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.833	26.603	3.000	16.000	0.000
	Wilks' Lambda	0.167	26.603	3.000	16.000	0.000
	Hotelling's Trace	4.988	26.603	3.000	16.000	0.000
	Roy's Largest Root	4.988	26.603	3.000	16.000	0.000
Strain	Pillai's Trace	0.295	0.980	6.000	34.000	0.454
	Wilks' Lambda	0.710	0.996	6.000	32.000	0.445
	Hotelling's Trace	0.401	1.003	6.000	30.000	0.442
	Roy's Largest Root	0.383	2.170	3.000	17.000	0.129
Phase	Pillai's Trace	0.465	4.637	3.000	16.000	0.016
	Wilks' Lambda	0.535	4.637	3.000	16.000	0.016
	Hotelling's Trace	0.869	4.637	3.000	16.000	0.016
	Roy's Largest Root	0.869	4.637	3.000	16.000	0.016
Strain * Phase	Pillai's Trace	0.561	2.207	6.000	34.000	0.066
	Wilks' Lambda	0.494	2.254	6.000	32.000	0.063
	Hotelling's Trace	0.913	2.283	6.000	30.000	0.062
	Roy's Largest Root	0.769	4.359	3.000	17.000	0.019

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Expression 1	6.574	5.000	1.315	1.514	0.235
	Expression 2	5.058	5.000	1.012	0.833	0.543
	Expression 3	4.240	5.000	0.848	0.701	0.630
Intercept	Expression 1	49.052	1.000	49.052	56.471	0.000
	Expression 2	22.892	1.000	22.892	18.861	0.000
	Expression 3	22.360	1.000	22.360	18.485	0.000
Strain	Expression 1	3.112	2.000	1.556	1.791	0.195
	Expression 2	2.207	2.000	1.103	0.909	0.421
	Expression 3	1.857	2.000	0.928	0.768	0.479
Phase	Expression 1	0.012	1.000	0.012	0.013	0.909
	Expression 2	0.910	1.000	0.910	0.750	0.398
	Expression 3	0.417	1.000	0.417	0.344	0.565
Strain * Phase	Expression 1	3.450	2.000	1.725	1.986	0.166
	Expression 2	1.941	2.000	0.971	0.800	0.465
	Expression 3	1.966	2.000	0.983	0.813	0.459
Error	Expression 1	15.635	18.000	0.869		
	Expression 2	21.847	18.000	1.214		
	Expression 3	21.773	18.000	1.210		
Total	Expression 1	71.260	24.000			
	Expression 2	49.797	24.000			
	Expression 3	48.373	24.000			
Corrected Total	Expression 1	22.209	23.000			
	Expression 2	26.905	23.000			
	Expression 3	26.013	23.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.890	27.092	3.000	10.000	0.000
	Wilks' Lambda	0.110	27.092	3.000	10.000	0.000
	Hotelling's Trace	8.128	27.092	3.000	10.000	0.000
	Roy's Largest Root	8.128	27.092	3.000	10.000	0.000
Strain	Pillai's Trace	0.538	1.350	6.000	22.000	0.278
	Wilks' Lambda	0.501	1.378	6.000	20.000	0.271
	Hotelling's Trace	0.920	1.380	6.000	18.000	0.276
	Roy's Largest Root	0.826	3.027	3.000	11.000	0.075
Condition	Pillai's Trace	0.768	1.377	9.000	36.000	0.235
	Wilks' Lambda	0.304	1.719	9.000	24.488	0.138
	Hotelling's Trace	2.061	1.984	9.000	26.000	0.083
	Roy's Largest Root	1.945	7.780	3.000	12.000	0.004
Strain * Condition	Pillai's Trace	1.473	1.930	18.000	26.000	0.046
	Wilks' Lambda	0.094	2.086	18.000	28.770	0.038
	Hotelling's Trace	4.347	2.093	28.000	26.000	0.042
	Roy's Largest Root	2.868	5.736	6.000	12.000	0.005

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Expression 1	14.201	11.000	1.291	1.935	0.136
	Expression 2	20.034	11.000	1.821	3.181	0.029
	Expression 3	19.536	11.000	1.776	3.291	0.026
Intercept	Expression 1	49.052	1.000	49.052	73.507	0.000
	Expression 2	22.892	1.000	22.892	39.983	0.000
	Expression 3	22.360	1.000	22.360	41.428	0.000
Strain	Expression 1	3.112	2.000	1.556	2.332	0.139
	Expression 2	2.207	2.000	1.103	1.927	0.188
	Expression 3	1.857	2.000	0.928	1.720	0.220
Condition	Expression 1	1.657	3.000	0.552	0.828	0.504
	Expression 2	3.183	3.000	1.061	1.853	0.191
	Expression 3	3.879	3.000	1.293	2.395	0.119
Strain * Condition	Expression 1	9.433	6.000	1.572	2.356	0.097
	Expression 2	14.645	6.000	2.441	4.263	0.016
	Expression 3	13.801	6.000	2.300	4.262	0.106
Error	Expression 1	8.008	12.000	0.667		
	Expression 2	6.871	12.000	0.573		
	Expression 3	6.477	12.000	0.540		
Total	Expression 1	71.260	24.000			
	Expression 2	49.797	24.000			
	Expression 3	48.373	24.000			
Corrected Total	Expression 1	22.209	23.000			
	Expression 2	26.905	23.000			
	Expression 3	26.013	23.000			

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	0.767	15.378	3.000	14.000	0.000
	Wilks' Lambda	0.233	15.378	3.000	14.000	0.000
	Hotelling's Trace	3.295	15.378	3.000	14.000	0.000
	Roy's Largest Root	3.295	15.378	3.000	14.000	0.000
Condition	Pillai's Trace	0.517	1.110	9.000	48.000	0.374
	Wilks' Lambda	0.516	1.188	9.000	34.223	0.333
	Hotelling's Trace	0.875	1.232	9.000	38.000	0.305
	Roy's Largest Root	0.799	0.426	3.000	16.000	0.022
Phase	Pillai's Trace	0.397	3.071	3.000	14.000	0.063
	Wilks' Lambda	0.603	3.071	3.000	14.000	0.063
	Hotelling's Trace	0.658	3.071	3.000	14.000	0.063
	Roy's Largest Root	0.658	3.071	3.000	14.000	0.063
Condition * Phase	Pillai's Trace	0.190	0.360	9.000	48.000	0.948
	Wilks' Lambda	0.819	0.326	9.000	34.223	0.960
	Hotelling's Trace	0.211	0.298	9.000	38.000	0.971
	Roy's Largest Root	0.140	0.745	3.000	16.000	0.541

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Expression 1	2.994	7.000	0.428	0.356	0.915
	Expression 2	5.217	7.000	0.745	0.550	0.785
	Expression 3	5.467	7.000	0.781	0.608	0.741
Intercept	Expression 1	49.052	1.000	49.052	40.846	0.000
	Expression 2	22.829	1.000	22.892	16.889	0.001
	Expression 3	22.360	1.000	22.360	17.414	0.001
Condition	Expression 1	1.657	3.000	0.552	0.460	0.714
	Expression 2	3.183	3.000	1.061	0.783	0.521
	Expression 3	3.879	3.000	1.293	1.007	0.415
Phase	Expression 1	0.012	1.000	0.012	0.010	0.923
	Expression 2	0.910	1.000	0.910	0.671	0.425
	Expression 3	0.417	1.000	0.417	0.324	0.577
Condition * Phase	Expression 1	1.326	3.000	0.442	0.368	0.777
	Expression 2	1.124	3.000	0.375	0.276	0.842
	Expression 3	1.172	3.000	0.391	0.304	0.822
Error	Expression 1	19.214	16.000	1.201		
	Expression 2	21.688	16.000	1.355		
	Expression 3	20.546	16.000	1.284		
Total	Expression 1	71.260	24.000			
	Expression 2	49.797	24.000			
	Expression 3	48.373	24.000			
Corrected Total	Expression 1	22.209	23.000			
	Expression 2	26.905	23.000			
	Expression 3	26.013	23.000			

Tests of Between-Subjects Effects

Dependent Variable: Expression 2					
Source	**Type III Sum of Squares**	**df**	**Mean Suare**	**F**	**Sig.**
Corrected Model	20.034	11.000	1.821	3.181	0.029
Intercept	22.892	1.000	22.892	39.983	0.000
Strain Condition	20.034	11.000	1.821	3.181	0.029
Error	6.871	12.000	0.573		
Total	49.797	24.000			
Corrected Total	26.905	23.000			

R Squared =.754
Adjusted R Squared = .511

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
EXP2	Tukey HSD	2	8	0.038
		3	8	0.023
		4	8	0.021
		5	8	0.021
		6	8	0.04
		8	2	0.038
			3	0.023
			4	0.021
			5	0.021
			6	0.04
	Bonferroni	4	8	0.047
		5	8	0.047
		8	4	0.047
			5	0.047

Tests of Between-Subjects Effects

Dependent Variable: Expression 3					
Source	**Type III Sum of Squares**	**df**	**Mean Suare**	**F**	**Sig.**
Corrected Model	19.536	11.000	1.776	3.291	0.026
Intercept	22.360	1.000	22.360	41.428	0.000
Strain Condition	19.536	11.000	1.776	3.291	0.026
Error	6.477	12.000	0.540		
Total	48.373	24.000			
Corrected Total	26.013	23.000			

R Squared =.751
Adjusted R Squared = .523

Expression	Post-Hoc Test	Strain-Condition	Strain-Condition	Significance
EXP3	Tukey HSD	2	8	0.037
		3	8	0.019
		4	8	0.018
		5	8	0.02
		6	8	0.032
		8	2	0.037
			3	0.019
			4	0.018
			5	0.02
			6	0.032
	Bonferroni	3	8	0.04
		4	8	0.04
		5	8	0.044
		8	3	0.04
			4	0.04
			5	0.044

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