APPLICATION OF PROTEOMICS IN THE INVESTIGATION OF MORPHOGENESIS IN *WANGIELLA DERMATITIDIS*

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ABSTRACT

Wangiella dermatitidis is a melanized, pathogenic, polymorphic fungus that *in vitro* grows primarily in a yeast form, but is capable of morphological changes resulting in growth as either hyphae (filaments) or swollen, multicellular bodies. The latter morphology strongly resembles the *in vivo* form of tissues infected with *W. dermatitidis* and other closely-related fungi. Each of these three morphologies can be readily induced through varying growth conditions of the wild-type strain or by culture of developmental mutants. Of particular interest is the conversion of the yeast phase to the multicellular growth form, which is the focus of the present study. Whole-cell, protein profiles of the wild-type strain and the temperature-sensitive mutant, Mc3, were developed following incubation at both 25°C and 37°C (body temperature). At both temperatures, the wild type grew as yeast cells, whereas strain Mc3 also grew as a yeast at 25°C, but formed the multicellular growth form at 37°C. Protein profiles showed consistency in landmark proteins found within all four study groups, as well as temperature dependent and strain dependent proteins. Fifty protein spots were excised and sequenced by capillary-liquid chromatography-nanospray tandem mass spectrometry. A Mascot search of established databases revealed putative identities of these proteins. The identified proteins included: heat shock protein 60 mitochondrial precursor, NADP-dependent mannitol dehydrogenase, and copper-zinc superoxide dismutase (Cu-Zn SOD). The results presented in this study indicate the relevance of proteomics as a tool in interpreting the morphological and physiological conditions of phase changes exhibited by *W. dermatitidis.*

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Finally, I would like to thank my family. My son, Toft, was a constant calming force of encouragement. My husband, Mark, was always a source of knowledge and assistance. Mark provided the financial stability that allowed me to take time away from my teaching career to complete this project.

TABLE OF CONTENTS

FIGURES AND TABLES

Chapter 3 Tables **Page**

INTRODUCTION

Classification of *Wangiella dermatitidis*

Wangiella dermatitidis is a melanized, pathogenic, polymorphic fungus which grows primarily in the yeast form, but is capable of various morphological phase changes (Szaniszlo 2002; Liu et al. 2004; Szaniszlo 2006). It is classified in the division Deuteromycota, class Hyphomycetes and, therefore, has no known sexual cycle (Schell 2003). In the past, the ability of *W. dermatitidis* to appear in different morphologies has created confusion with regard to its taxonomy and binomial nomenclature. This confusion has resulted in several synonyms making a historical review of this organism complicated (Geis and Jacobs 1985). Although this organism is often referred to as *Exophiala dermatitidis* by many present day investigators, for the purposes of this thesis, the fungus used in the studies described herein, specifically strain 8656 (ATCC 34100) and it derivatives, the epithet *W. dermatitidis* will be employed.

Why *Wangiella dermatitidis* is an important research species

Wangiella dermatitidis is a member of a group of fungi commonly called the black yeasts, known for their highly pigmented cell wall material (Cooper and Szaniszlo 1993). *Wangiella dermatitidis* is considered a dematiaceous fungus, which is a term used to characterize the presence of melanin in the cell walls and or spores. It is thought that this cell wall pigmentation is a virulent factor which aids these fungi in pathogenesis (Ye, Feng and Sazaniszlo 1999). Black yeasts, in particular *W. dermatitidis*, have become a model for the study of cell wall related virulence factors (Szaniszlo 2002; 2006).

Decoding and making sense of these cell wall virulence factors is vital to developing new fungal control with the black yeast.

Wangiella dermatitidis is considered a saprobe that is abundant in the environment and has been isolated from natural sources including soil, wood, and plant material. It has also been isolated from sewage, drainpipe sludge and humidifiers (Diemert 2001). Despite its saprobic nature, it is the most frequently implicated causal agent of human infections within the black yeasts (De Hoog 2005). The mycoses caused by *W. dermatitidis* are rare but potentially lethal (Brush 1999). For example, *W. dermatitidis* has been isolated from human infections involving skin, brain, lung, eye, joints and endocardium (Diemet 2001). Infections of this nature are thought to be increasing in frequency (Brush 1999).

Clinical infections with pigmented fungi, including *W. dermatitid*is, are referred to as phaeohyphomycoses (Figure 1.1) (Peng, Cooper, and Szaniszlo 1994). Phaeohyphomycoses begin with fungal cells entering tissue via inhalation or injury. The fungus is found in the tissue in one or more of the following forms: yeastlike cells, pseudohyphae, or hyphae. At least some pigmentation is found in most phaeohyphomycoses and the few that do not show pigmentation *in vivo* reveal their dematiaceous nature when grown in culture. There are more than 100 documented species of fungi implicated in phaeohyphomycoses; due to their similarities in infected tissue they must be grown in culture for identification purposes (Schell 2003).

While *W. dermatitidis* primarily grows as yeast, it can be induced to grow in three distinct forms (Szaniszlo 2002; Liu et al. 2004; Szaniszlo 2006). The three morphological stages are: i) single celled yeasts that grow via budding, ii) hyphae with

apical growth, and iii) an isotropic multicellular form that grows by swelling and subsequent development of internal septa. The polymorphic nature of *W. dermatitidis* makes it an ideal species for investigation of phaeohyphomycoses as well as the similar condition, chromoblastomycosis, caused by related dematiaceous fungi. The latter pathology is defined by the presence of multicellular (muriform) bodies often referred to as "sclerotic bodies" (McGinnis, 1983). Because *W. dermatitidis* can be induced to undergo phase change, all three morphologies can be investigated. Thus, *W. dermatitidis* is an excellent experimental organism for investigations in developmental phase changes related to pathogenicity and virulence (Szaniszlo 2002; 2006).

Morphological phases of *Wangiella dermatitidis*

The vegetative morphologies of *W. dermatitidis* are separated by distinct characteristics. Because the yeast form of *W. dermatitidis* is the most common morphological state, it has been examined more extensively. Yeasts grow by means of a process termed budding. This process begins with a polarized, site-specific, cell-wall synthesis that is followed by isotropic growth resulting in an increased cell volume. Yeast cells range in shape from globose to egg-shaped and are capable of budding at either pole. Figure 1.2A displays an electron micrograph of the yeast cell shape. Also pictured in this figure, are examples of budding yeasts and bud scars from the process. Yeasts have thin cell walls and numerous cytoplasmic inclusions. Each yeast cell contains one nucleus; cell cleavage follows nuclear division producing a population composed of unicellular yeast. However, in a colony, it is common to see two or three yeasts together that have not entirely cleaved from one another.

Reproduction in yeast cells produces one bud each cell cycle. The nucleus of the mother cell is shared and the cell wall is continuous between the mother cell and the bud during the budding process. When the bud has grown to a diameter approximately two thirds the size of the mother cell, the nucleus travels to the area between the mother cell and forms a bud. Mitosis occurs quickly. Following mitosis, the daughter nuclei migrate to the bud and mother cell. At the neck between the mother and budding cell, a septum is formed and the two then disassociate (Geis and Jacobs 1985, Brown 2004) leaving the mother cell with a bud scar.

The filamentous form of *W. dermatitidis* grows primarily by apical extension. The cell walls of hyphae are thin and relatively consistent in diameter. Simple septa with septal pores develop from the inner layers of the cell wall; pores are often closed by pore plugs. Growth can also take place via budding in which lateral hyphal buds emerge from the inner layers of the cell wall. Conidia are produced from both the apices of undifferentiated conidiophores derived from hyphal branches or directly from hyphal walls (Geis and Jacobs 1985, Brown 2004). Figure 1.2B displays an electron micrograph of the filamentous form of *W. dermatitidis*. Several septa are visible in this figure.

The isotropic multicellular form of *W. dermatitidis* is divided into two stages of growth. In stage 1, cell wall growth is isotropic. The cell wall thickens and the cell grows, enlarges in diameter and becomes multinucleated. Stage 1 growth is void of budding and apical growth. In stage 2, cell growth continues; the cell wall becomes thicker with multiple layers and highly pigmented. The multicellular form is reached via the formation of septa. Two types of septa are formed: simple septa with septal pores and pore plugs, and complete septa composed of several layers. Figure 1.2C displays an

electron micrograph of a cross-section of the isotropic multicellular form of *W. dermatitidis.*

The Mc3 Strain

While *W. dermatitidis* is a polymorphic fungus with three distinct phases, the focus of this research was to investigate the conversion of the yeast form to the multicellular phenotype using the wild-type and Mc3 strains. The Mc3 strain was developed from the 8656 strain. The Mc3 strain is a temperature sensitive mutant that grows in the yeast form at 25 °C and becomes multicellular at 37 °C (Jacobs and Szaniszlo 1985; Roberts and Szaniszlo 1978). While the Mc3 strain becomes multicellular at 37 °C (body temperature) the 8656 (wild type) strain continues to grow in the yeast from. This research utilized proteomics to investigate protein changes in the 8656 and Mc3 strains at the distinct temperatures of 25 °C and 37 °C.

Proteomics as a tool in the investigation

While biochemists have dabbled in the investigation of proteins for nearly a century, the use of "proteomics" began in 1975 with the introduction of two-dimensional gel electrophoresis (Graves and Haystead, 2002). Proteomics is the study of all the proteins expressed by the genome of a cell and is a logical extension of genomics (Twyman 2004). There are many questions that can not be answered through the study of genetics alone. The protein content of all organisms is constantly changing in response to disease, aging and various environmental factors (Graves and Haystead 2002). Proteomics can be employed to understand and identify components critical to normal

cellular function as well as those responsible for disease. This information can then be used to exploit these components as targets in the development of new methods to prevent and or treat diseases.

The term “proteome” refers to the entire set of proteins produced by an organism or cell under specified conditions (Twyman 2004). This research compared and contrasted four defined proteomes of *W. dermatitidis*: 8656 and Mc3strains grown at both 25°C and 37°C. All other conditions were constant between the four groups.

Research Goals

These studied sought to identify proteins involved in transitions between yeast and isotropic multicellular phases of *W. dermatitidis*. The goals were as follows:

1. Develop protein profiles of the wild-type and Mc3 strains of *W. dermatitidis* at both 25 ˚C and 37 ˚C by two-dimensional gel electrophoresis.
2. Examine protein profiles to determine which proteins should be extracted and sequenced by mass spectrometry.
3. Analyze protein sequences from the wild-type and Mc3 strains of *W. dermatitidis* determined by mass spectrometry and compare with known fungal sequences.

Decoding protein profiles of these strains might lead to a deeper understanding of which proteins play crucial roles related to morphogenesis and virulence in *W. dermatitidis* as well as other black yeasts. This is critical for the continued development of new and better methods to prevent and treat fungal diseases.

Figure 1.1 *Wangiella dermatitidis* skin infections: Left) Ungual phaeohyphomycosis; Right) Cutaneous phaeohyphomycosis. **http://www.sbs.utexas.edu/mycology/sza_faqs_about_research.htm#3.0_Phaeohyphomycosis**

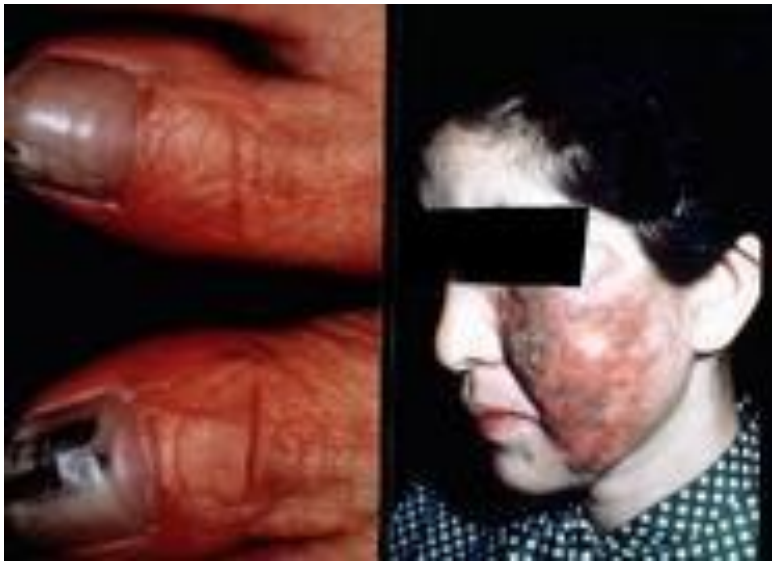
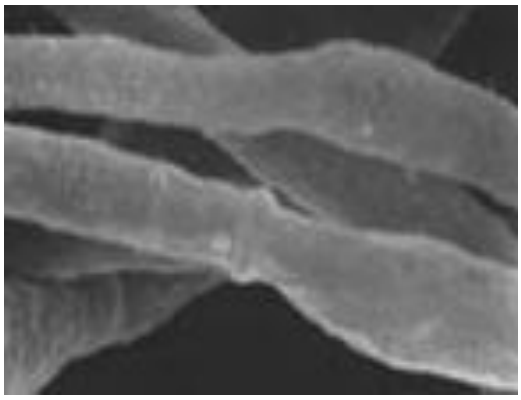


Figure 1.2 Electron micrographs of morphological phases of *Wangiella dermatitidis*: A) yeast from in which budding yeast and bud scars are visible; B) filamentous form; C) isotropic multicellular form. ***www.sbs.utexas.edu/ mycology/sza_images_SEM.htm***

A.

B.



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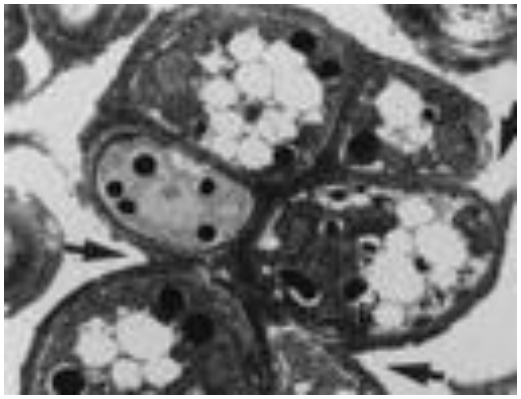


MATERIALS AND METHODS

Chemicals, reagents, and media

Unless otherwise noted, all chemicals, reagents, and media were obtained from Amresco, Inc. (Solon, OH), Bio-Rad, Inc. (Hercules, CA), Fisher Scientific (Pittsburgh, PA), or Sigma Chemical Co. (St. Louis, MO). All solutions and media were prepared in distilled-deionized water (ddH_2O).

Media and Fungal Strains

Cultures of *W. dermatitidis* strains 8656 (ATCC 34100) and Mc3 (38716) were maintained on yeast peptone dextrose (YPD) agar (1% yeast extract, 2% peptone, 2% glucose, and 5% agar) slants. These strains were obtained from Paul Szaniszlo, University of Texas at Austin.

In preparation for experimental studies, colonies grown on slants were transferred to 50 ml of YPD broth (1% yeast extract, 2% peptone, and 2% glucose) and cultured at room temperature on a shaker operating at 120 rpm for 4-7 days. After the 4-7 days, 1 ml of this broth was transferred to 50 ml of fresh YPD broth and incubated in a water bath at 25°C with the shaker operating at 120 rpm. The culture was transferred every 48 hours by first determining cell concentrations using a hemocytometer, then using this data to inoculate 50 ml of fresh YPD broth to a final concentration of 10^6 cells/ml.

Primary inoculum concentrations for all experiments were calculated utilizing a cell count from the 48-hour cultures. Experimental cultures containing 10^6 cells/ml in a total of 25 ml of YPD broth were grown for 24 hours at 25°C and 37°C with the shaker

operating at 120 rpm. The YPD medium was pre-warmed to the experimental temperature before inoculation.

Hemocytometer Count

In order to determine the quantity of inoculum needed to obtain a concentration of 10^6 cells/ml, liquid cell cultures to be used as inoculum were diluted 1:10, 1:100, and 1:1000 in 4% p-formaldehyde. The final dilution chosen for the cell count depended on the concentration of cells in the culture. Generally, the 1:100 dilution was most appropriate for the cell count given the allowed 48 hour period of growth. A micropipette was used to draw up 15 µl of the dilution which was inserted under the cover slip of the two sides of the hemocytometer slide. Each side of the hemocytometer contains a grid with four large squares each which contain 16 smaller squares. In order to count the cells, the top grid was observed using a light microscope at a magnification of 400x. The cells within the four corner squares of each larger square were counted and totaled. This was repeated for the bottom grid. The grid pair totals were averaged and multiplied by the dilution factor of the hemocytometer slide of 10^4 (cells/ml) and by the dilution factor of the original cell dilution (10^1 for the 1:10 dilution and 10^2 for the 1:100 dilution). The resulting product describes the number of cells per ml in a 48 hour cell culture and was used to determine the volume of original culture for inoculating the next 48 hour culture and/or 24 hour experimental cultures at 10^6 cells per ml.

Protocols for Protein Isolation

Cells from experimental cultures were collected and washed prior to cell disruption and protein extraction. Materials were kept on ice throughout the procedure and all transfers were conducted aseptically to minimize the likelihood of contamination. The following is a detailed description of this process**.**

The 24 hour cell cultures consisting of 25 ml of yeast cells in YPD broth were transferred to a pre-chilled (-80°C), sterile, 50 ml screw-capped conical tubes. Cells were collected as a pellet by centrifugation at 3000 x g for 20 minutes at 4°C. The supernatant was discarded. The conical tubes containing the cellular pellets were stored at -80°C for future protein isolation.

Conical tubes were removed from freezer and thawed on ice. The resulting cell pellets were suspended in 1 ml of ice-cold 20 mM Tris (pH 7.6). Suspensions were mixed thoroughly with a vortex and transferred to pre-weighed, pre-chilled microfuge tubes. Cells were immediately collected from suspension by centrifugation at 4000 x g (6600 rpm) for 5 minutes at 4°C. The supernatant was discarded. Once dry, pellet weight was determined by subtracting the initial weight of the microfuge tube from the final weight.

The cell pellets were resuspended in ice-cold lysis buffer (20mM Tris-HCl, pH 7.6, 10mM NaCl, 0.5 nM deoxycholate, 40 µl/ml protease inhibitor cocktail) to a final concentration of approximately 500 mg cells/ml. The suspensions were mixed thoroughly. From these suspensions, 800 µl were transferred to a pre-chilled, 2.0 ml screw-capped microfuge tubes containing 0.8 g of acid-washed 0.5 mm diameter glass beads. Cells were mechanically disrupted using a Mini-Beadbeater-1 operating at

5000 rpm. Cells were disrupted for 30 seconds and chilled on ice for a minimum of 30 seconds. This procedure was repeated six times. The remaining debris was collected via centrifugation at 6000 x g (8000rpm) for 10 minute at 4°C. The resulting supernatant was transferred to pre-chilled, pre-weighed 1.5 ml microfuge tubes. To the supernatant, ice-cold trichloroacetic acid was added to a final concentration of 20% (vol:vol) and mixed thoroughly. The solution was held on ice for 20 minutes allowing a precipitant to form. The precipitated protein was collected by centrifugation at 2000 x g (4600 rpm) for 20 minutes at 4 °C. The supernatant was discarded and the resulting pellet was washed three times with 500 µl of ice-cold acetone. Between each wash, the protein was centrifuged for 1 minute at 900 x g (3000 rpm). The remaining acetone was removed by placing the open tubes under a hood in aseptic conditions. Once pellets were dry, pellet weight was determined by subtracting the initial weight of the microfuge tube from the final weight.

These protein pellets were then resuspended in Modified Sample Buffer (MSB) (2 M thiourea, 7 M urea, 4% w/v 3-[(3-cholamidoproply) dimethylammonio]propanesulfonic acid, (CHAPS), 1% dithiothreitol). The amount of MSB used depended on the weight of the pellet. For pellets weighing 5-15 mg, 500 µl of MSB was added and for those 16-30 mg, 750 µl was added. The pellets were placed on a platform shaker for several hours at room temperature to resuspend pellets. Once pellets had dissolved, aliquots of 20 µl were placed into separate microfuge tubes to be used for the modified Bradford assay. These protein samples were stored at -80 °C prior to modified Bradford assays and two-dimensional gel electrophoresis.

Modified Bradford Assay

In order to determine the concentration of protein extracted from experimental samples, a modified Bradford assay was performed before the protein was used in 2-dimensional electrophoresis (2-DE) (Bradford 1976). A standard curve for the assay was prepared using bovine serum albumin (BSA). This standard was prepared using microgram amounts of 0 (blank), 10, 15, 20, 25, 30, 35, and 40. The test tubes of each standard contained 80 µl of ddH20 10 µl of HCl, 0.1 µl 2-DE buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, mM spermine base, 50 mM dithiothreitol), the appropriate amount of BSA and 4 ml of Bradford Dye (0.01% Coomassie Billiant Blue G-250, 8.5% [v/v] phosphoric acid). The ingredients were mixed thoroughly and allowed to sit for five minutes before the addition of the Bradford dye. The contents of each test tube were poured into a cuvette and placed into a spectrophotometer; absorbance was recorded at 595 nm and standards generated. Experimental samples, were handled identically with the exception that 10 µl of experimental protein replaced BSA. Generated standard coefficients had an R-value of 0.98 or higher. The volume of experimental protein sample needed for the 2-DE procedure was calculated from the standard curve.

Protein Separation by 2-Dimensional Gel Electrophoresis

This procedure allowed the protein profiles of the wild-type and Mc3 *W. dermatitidis* strains (hereafter *Wd* 8656, *Wd* Mc3) to be evaluated following growth at two distinct temperatures (25 °C and 37 °C). A two step method in which proteins were first separated by pH and secondly by molecular weight was used (Beranova-Giorgianni 2003). This procedure was first conducted with 7 cm IPG strips and 7 cm gels using

Coommassie stain (Twyman 2004). Once consistent results were achieved with small gels, the procedure was conducted with 17 cm IPG strips and 17 cm gels stained with SYPRO-Ruby stain.

Active Rehydration and IEF

IPG strips were reyhydrated actively with protein and rehydration buffer added at the same time. A pH range of 5-8 was used throughout this research. It was a 24 hour process when running 7 cm strips and several hours longer when running 17 cm strips. When running 7 cm strips, 100 µg of protein were loaded. The Bradford standard was used to determine this equivalent in µl. To determine the amount of rehydration buffer used, the necessary volume (µl) of protein was subtracted by 125 µl. For 17 cm strips, 250 µg were loaded and the necessary volume (µl) of protein was subtracted by 300 µl.

Strips were placed face down into the IEF tray with the loaded protein solution and rehydration buffer with the "+" end of the strip on the anode. Care was taken to insure there were no bubbles and that the strips were in contact with both electrodes. The strips were overlaid with mineral oil. The focusing tray was placed into the Bio-Rad Protein IEF System and programmed for active rehydration at 50V, 20ºC. The program was set to rehydrate for 12 hours, isoelectric focus proteins for 12-16 hours, and hold at 500 volts until the IEF strips were removed.

SDS-Polyacrylamide Gel Electrophoresis

In preparation for the second dimension, IPG strips were rinsed in two equilibration buffers and then held in 1X TGS buffer before they were placed on the SDS-polyacrylamide gel. Strips were removed from the IEF tray using forceps and blotted, gel side up, on a Kimwipe to remove excess mineral oil. Strips were placed in a new tray containing equilibration buffer I (6 M urea, 0.375 M Tris, pH 8.8, 2% sodium dodecyl sulfide (SDS), 20% glycerol, 2% (w.v) dithiothreitol) and agitated for ten minutes. After 10 minutes, the same process was followed transferring the IPG strips to equilibration buffer II (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w.v) iodoacetamide) and shaking for 10 minutes. Finally, strips were placed in 1X Tris-glycine-SDS (TGS) running buffer (25 mM Tris Base, 250 mM glycine, 0.1% SDS). The IPG strips were placed at the top of the SDS-PAGE gels with the gel side of the strip facing out. The SDS-page gels were prepared fresh in the lab and stored in 1/2X TGS solution at 10°C prior to use. Strips were covered with warm overlay agarose (0.5% w/v low melting agarose, 1X TGS, trace bromophenol blue) and care was taken to ensure there were no bubbles. For preliminary gels, the 7 cm gels were run at 10-16mA/gel and the 17 cm gels were run at 24mA/gel. Gels used in the generation of match sets or for mass spectrometry (MS) preparation were processed in the Dodecacell box capable of processing 12 gels simultaneously. These gels were subject to electrophoresis at a constant 200 volts. All gels ran until the dye (from the overlay agarose) reached approximately 1cm from the bottom of the gel.

Staining and Fixing Gels

When finished, gels were removed from gel plates, placed in plastic containers with lids, stained, fixed, placed in deionized water for 15 minutes and finally placed in a 5% solution of acetic acid. The staining and fixing process varied depending on the stain used and the purpose of the gels (preliminary research, match sets, or mass spectrometry).

Preliminary Research Gels

When processing gels (7 cm and 11 cm) for preliminary research, gels were removed from gel plates and placed into plastic containers and immersed in Coomassie stain. Gels were placed on an orbital shaker overnight. The next day the stain was removed and the gels were immersed in destain/fixing solution (on an orbital shaker) for one hour. High destain was then removed and replaced with low destain (on an orbital shaker) for a period of time until the background was clear and protein spots became distinct. The low destain was then removed and replaced with water. After 15 minutes, gels were ready to be imaged.

Match Set Gels

Gels that were used to create match sets followed the protocol for SYPRO Ruby staining. Gels were removed from gel plates, placed into plastic containers, immersed in high destain/fixing solution and placed on an orbital shaker for one hour. The fixing solution was then removed and gels were immersed in SYPRO stain (on an orbital shaker) overnight. Next, the SYPRO was removed and gels were covered with deionized water (on an orbital shaker) for 15 minutes. Water was removed and replaced with fresh

deionized water. After 15 minutes in fresh deionized water, gels were ready to be imaged.

Overload Gels for Mass Spectrometry (MS)

Avoiding contamination and maintaining aseptic technique was essential in each of the procedures described above, but particular attention was given to protein samples intended to be sent to Ohio State for mass spectrometry analysis. This involved using all freshly prepared or purchased chemical for the making of SDS-polyacrylamide gels, the prepping of IEF strips and SDS-polyacrylamide gels, as well as the staining and fixing process. The following describes the staining and fixing process.

Gels were removed from plates, placed in sterile plastic containers containing 500 mL of freshly prepared gel-fixing solution (50% ethanol, 10% acetic acid, 40% deionized water), and gently agitated on an orbital shaker for 1 hour. After 1 hour, the gel-fixing solution was removed via aspiration. The gels were immersed in 500 mL of gel-washing solution (50% methanol, 10% acetic acid, 40% water) and gently agitated on an orbital shaker overnight. The following morning, the gel-washing solution was removed via aspiration; gels were then covered with new SYPRO Ruby stain and agitated on an orbital shaker for at least 3 hours. A serological pipette was used to remove stain; gels were then covered with ample deionized water and agitated on an orbital shaker for 15 minutes. Gels were placed in storage solution (5% acetic acid, 95% water). After 3 hours, they were imaged.

Image analysis.

This section follows the protocols established in Chandler (2008). Gels were visualized under UV illumination at 365 nm using a Molecular Imager ChemiDoc XRS system (Bio-Rad). Digital images of 2DGE gels from given resolution parameters (i.e., acrylamide concentration and pI range) were generated for each of three or more independent cultures per incubation strain (8656 and Mc3) and temperature (25°C or 37°C). The digital images of these three gels were used in conjunction with PDQuest 2-D Analysis Software (Bio-Rad) to create one match set per experimental condition. The master of each match set resulting from different experimental conditions was then subjected to further analysis using PDQuest.

Differences in levels of expression, founded upon spot staining intensity, were assessed using three-dimensional contour maps. For data presentation, all digital images and contour maps were processed using PowerPoint and Windows Photo Gallery and Paint software (Microsoft Corp. Seattle, WA).

Protein isolation and preparation for mass spectrometry

This section follows the protocols established in Chandler (2008). Based upon image analysis results, proteins of interest were isolated for sequencing by MS. Protein spots were excised directly by hand from illuminated SYPRO Ruby-stained gels using a sterile Pasteur pipette or a 2500 µl pipette tip. The gel piece was then expelled into a sterile microfuge tube and covered with 5% (v/v) acetic acid. Samples were submitted to the Ohio State University Mass Spectrometry and Proteomics Facility (Columbus, OH; http://www.ccic.ohiostate.edu/MS/proteomics.htm) for sequencing by MS.

The acrylamide-embedded protein samples submitted for MS sequencing were processed by the following procedure. First, samples were digested with sequencing grade trypsin (Promega, Madison, WI) or sequencing grade chymotrypsin (Roche,Indianapolis, IN) using the Montage In-Gel Digestion Kit (Millipore, Bedford, MA) following the recommended protocols. Briefly, samples were trimmed as close as possible to minimize background polyacrylamide material, then washed for one hour in a methanol/acetic acid solution (50% methanol: 5% acetic acid; v/v). The wash step was repeated once before gel pieces were dehydrated in acetonitrile. Subsequently, the protein/acrylamide samples were rehydrated in a DTT solution (5 mg/ml in 100 mM ammonium bicarbonate) for 30 minutes prior to the addition of iodoacetamide (15 mg/ml in 100 mM ammonium bicarbonate). The samples were incubated in the dark for 30 min before sequential 5 min washes in acetonitrile and 100 mM ammonium bicarbonate.

Again, the samples were vacuum dried, then rehydrated in 50 µl of 50 mM ammonium bicarbonate containing either sequencing grade modified trypsin or chymotrypsin at 20 µg/ml. After 10 min of incubation, an additional 20 µL of 50 mM ammonium bicarbonate was added to the samples that were then incubated overnight at room temperature. Finally, the peptides were extracted several times from the polyacrylamide using an acetonitrile/formic acid solution (50% acetonitrile: 5% formic acid; v/v). The extracts were pooled, then concentrated under vacuum to a final volume of approximately 25 µl.

Mass spectrometry

This section follows the protocols established in Chandler (2008). Capillary-liquid chromatography-nanospray tandem MS (Nano-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was a UltiMate™ Plus system (Dionex, Sunnyvale, CA) with a Famos autosampler and Switchos column switcher. Solvent A was water containing 50 mM acetic acid and solvent B was acetonitrile. Five microliters of each sample was first injected on to the trapping column, then washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off of the trap onto the column. A 5 cm 75 μm ID ProteoPep II C18 column (New Objective, Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80% B over 50 minutes, with a flow rate of 300 nl/min. A total run time was 60 minutes. The scan sequence of the mass spectrometer was programmed for a full scan, a zoom scan to determine the charge of the peptide, and a MS/MS scan of the most abundant peak in the spectrum. Dynamic exclusion was used to exclude multiple MS/MS of the same peptide.

Data Processing and Analysis

This section follows the protocols established in Chandler (2008). Sequence information from the MS/MS data was processed by converting the raw data files into a merged file (.mgf) using MGF creator (merge.pl, a Perl script) with first scan number, last scan number, number of intermediate scans, minimum number of grouped scans and minimum number of ions set to blank, blank, 1, 0, and 8, respectively. The resulting

.mgf files were searched using Mascot Daemon (version 2.2.1; Matrix Science, Boston, MA) against the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) databases (NCBI nr). These databases contained in excess of 6 x 106 sequences comprised of more than 2 x 109 residues. Selected files were subjected to a more narrow search using the NCBI nr database limited to the taxon Fungi (>3 x 105 sequences). These databases were employed because a completely sequenced and annotated genome of *W. dermatitidis* has yet to be publicly released. The mass accuracy of the precursor ions were set to 2.0 Da given that the data was acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.5 Da. Considered modifications (variable) were methionine oxidation and carbamidomethyl cysteine. Two missed cleavages for the enzyme were permitted. Peptides with a score less than 20 were filtered and proteins were identified having a significance threshold of $p<0.05$. Protein identifications were checked manually and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a –b or –y ion sequence tag of five residues or better were accepted.

RESULTS

Wild-type (*Wd* 8656) and mutant (*Wd* Mc3) cultures of *W. dermatitidis* were grown in YPD broth at both 25°C and 37°C. Mutant cultures incubated at 37°C showed morphological changes within 24 hours. These cells appeared swollen and spherical compared to the slightly ellipsoidal shape of the wild type and 25°C-grown mutant cells. Additionally, mutant cells grown at 37°C began to form internal septa, growing in a multicellular cluster with a decrease in budding. Cells grown at higher temperatures were slightly larger in both cultures. Figure 3.1 shows micrographs of these cell cultures.

During liquid cell culture, the growth media became darker in color when cells were grown at 37°C compared to those incubated at 25°C. Of the four variables, the Mc3 culture grown at 37°C was the darkest. Likewise, this same variation in color was seen throughout the cell lysing and protein extraction process. The color variation between the four variables is shown in Figure 3.2.

In our first trials, cells were collected at both 24 hours and 48 hours. Gels produced from these preliminary trials were prepared using IPG strips with a pI range 4-7 and 10%, 7 cm gels. Preliminary results from 48 hour samples showed some distinct spot differences between variables (Figure 3.3). However, this study sought to pinpoint proteins that initiate morphological shifts between yeast and multicellular forms. The core of the project, therefore, focused on collecting cells at the onset of the morphological shift at 24 hours of growth.

In order to establish uniformity in colony growth, preparation of cellular material, electrophoresis techniques (IPG strip range of 5-8 and 12% gel), staining, and imaging,

24 preliminary gels (twelve 7 cm gels and six 17 cm gels) were processed and reviewed before the onset of the following series of lettered trials.

All protein profile images presented in the following figures were derived from cells that were grown for 24 hours and processed using an IPG strip range of 5-8 and 12%, 17 cm gels. To further institute consistency, samples were prepared in several trials (Harder et at. 1999). All four variables (wild, mutant, low and high temperatures) were replicated in separate trials labeled: A, B, C, D, E, F, G, and H. Two-dimensional electrophoresis (2DGE) was conducted with each trial except for E, which was discarded due to suspected contamination before the protein extraction was complete. Trials A, B and C were used to create a match set, which is defined as a series of protein extractions subjected to replicate separations by 2DGE. Protein spots from overload trials F and H were analyzed using mass spectrometry (MS). Trial D was processed with A, B, and C but not used in the match set. Trial G was processed as an overload set that was deemed unsatisfactory for MS analysis.

Bradford assays were conducted to determine the amount of protein that would be loaded for every gel processed. The Bradford assays that were used to determine loaded protein concentrations for A and B are shown in Figure 3.4. Similar Bradford assay results were obtained for subsequent trials.

The variables analyzed in this research are labeled 3, 4, 5, and 6 and apply to each trial. Three refers to the wild-type 8656 strain grown at 25 °C, 4 refers to the Mc3 strain grown at 25 °C, 5 refers to wild type 8656 grown at 37 °C, and 6 refers to the mutant MC3 grown at 37 °C. Variables 3 and 5 were started from a culture of wild type 8656

grown at 25 °C grown for 48 hours referred to as culture 1. Variables 4 and 6 were started from a culture of strain Mc3 grown at 25 °C for 48 hours referred to as culture 2.

Cultures 1 and 2 served as controls and as starting points to addition of the temperature variable. This insured that each variable was only different by one factor. Furthermore, since cultures 1 and 2 were grown at 25°C, culture 1 and variable 3 should have the same protein profile, as should culture 2 and variable 4. In order to check for this consistency, cultures 1 and 2 were collected, prepared, imaged and analyzed along with variables 3, 4, 5,and 6 for trials A, B, C and D. An example of this established consistency is shown in Figure 3.5 which presents the protein profiles of A1 and A3. Similar results are observed when comparing protein profiles of B1 and B3 as well as C1 and C3. Since consistency was established, cultures 1 and 2 were no longer collected and processed in subsequent trials.

Protein profiles for all variables in trial A are shown in Figure 3.6. Similar results were obtained between variables for trials B, C, D, F and H. Protein profiles for the wild type *Wd* 8656 grown at 25°C (variable 3) for trials A, B, and C are shown in Figure 3.7. Similar results were obtained between trials for variables 4, 5, and 6. Results obtained from the match set between trials A, B and C are shown in Table 1. This match set was referenced when choosing protein spots for MS submission. Trials F, G, and H were prepared for MS. Trial F and G gels were prepared with the standard trial overload rate of 400 µg. Protein profiles and numbered protein spots for trial F were extracted and analyzed for MS (Figure 3.8). Due to difficulties with MS, F trial spots were analyzed after trial H and are, therefore, numbered 32-53. Table 3.2 shows details and groupings of protein spots from trial F. Trial H was loaded at protein load rates of 250 and 325 µg.

The resolution was clearer for gels ran with a lower protein load. The protein profiles and number spots that were extracted and analyzed by MS for trial H are presented in Figure 3.9 with the spots numbered 1 - 31. For protein profiles H3, H4, and H5, gels with a protein load rate of 250 µg were used and for H6 the 325µg protein load rate was used. Table 3.3 shows the detailing and grouping of protein spots for trial H.

In Tables 3.2 and 3.3, the first column gives the spot identification number, the second column gives the gel (trial and variable) the spot came from, and the third column gives the culture type (wild/mutant). The fourth column describes the protein spot and associated proteins found in other variables. Protein descriptions were distinguished as: landmark – meaning that the protein intensity was similar within all trial variables; down-regulated – meaning that the protein intensity in that spot is less than that of related spots; up-regulated – indicating the protein intensity of a given spot was greater than that of related spots; and up-regulated (solo) – meaning a spot was found only in one variable within the trial.

Fifty-three spots from 8 gels, representing two trials of the 4 variables were selected for MS sequencing. These spots included those common to both strain 8686 and Mc3 cultures at both temperatures as well as those at comparable levels. The putative identifications of these spots were determined by a Mascot database search (Perkins et al. 1999). The results of these analyses are represented in Table 3.4. This table details the key protein detected for each protein spot analyzed. Results are presented for 47 of the 53 protein spots analyzed by MS. These 47 protein spot records are further grouped by protein name and protein spot numbers for trials H and F in Table 3.5. This figure identifies twelve proteins from the MS sequencing, the NCBI Accession number, the

protein spot numbers and species from which the detected protein is known. Three dimensional views of protein spots, grouped by common protein name are shown in figures 3.10-15.

The sequence coverage for each protein ranged from 2 to 28% of the identified ortholog. The Mascot scores ranged from 64 to 1188. The putative functions identified for these proteins can be divided into the following groups: cellular protection from oxygen (Superoxide dismutase [spots: 6, 12, 21, 31]); general metabolism and energy production (NADP-dependent mannitol dehydrogenase [spots: 5, 11, 19, 20, 28, 29, 34, 39, 44, 50]), (alcohol dehydrogenase zinc-containing [spots: 3, 9, 17, 26, 35, 40, 45, 51]), and (aldehyde dehydrogenase [spots 52]); heat shock proteins (heat shock protein 60, mitochondrial precursor [spots: 1,7,13,22]), and formation of septa (septin Nesartorya fischeri NRRL 181 [spot 15]; and five hypothetical or unnamed proteins which include the remaining 19 spots.

Of the twelve proteins, five were present in all four variables (wild and mutant cultures grown at 25°C and 37°C). Two of these proteins, superoxide dismutase and hypothetical protein AN1 were detected from protein spots that were collected as landmark spots in which proteins appeared to be expressed at nearly equal intensities (Figure 3.10). Superoxide dismutase (spots: 6, 12, 21, 31) was detected in all four variables of the trial H with a sequence coverage range of 11-19% and Mascot scores that ranged from 80 to 141. The theoretical and experimental isoelectric points and masses of these spots were 6.36, 16.0 kDa and 6.9, 32.5 kDa. Hypothetical protein AN1 (spots: 2, 8, 14, 23, 32, 36, 41, 47) was detected in all four variables in both trial H and F with a sequence range of 8 to 15% and a mass range 192 to 270. The theoretical and

experimental isoelectric points and masses of these spots were 5.56, 54.5 kDa, 5.9, 59.5 kDA for Trial H and 5.9 and 57.5 kDa for trial F. Interestingly, this protein was also detected in group protein spots (spots: 33, 37, 42, 48) at a lower intensity from trial F in all 4 variables, located near spots 32, 36, 41, and 47, respectively (Figures 3.8, 3.10). The sequence coverage range of these spots was 6 to 9%, the Mascot score range was 126 to 205 and the experimental isoelectric point and mass was 6.1 and 58 kDa.

Two of the five proteins found in all four variables, NADP-dependent mannitol dehydrogenase and alcohol deydrogenase zinc-containing, were detected from protein spots that appeared to be expressed at varying intensities based on temperature with increased spot intensity in gels grown at 37°C (Figure 3.11). Both of these proteins were detected in spots collected from trials H and F. NADP-dependent mannitol dehydrogenase (spots: 5, 11, 19, 28, 34, 39, 44, 50) had a sequence coverage range of 7-13% and Mascot scores that ranged from 136 to 262. The theoretical and experimental isoelectric points and masses of these spots were 5.86, 29.1 kDa, 6.8, 47 kDa for trial H and 6.9, 32.5 kDa and 6.8, 42 kDa for trial F. Again, this protein was detected in two less intense protein spots (20, 29) from gels of the 37°C variable in trial H, located close to spots 19 and 28. While the alcohol dehydrogenase zinc-containing protein was detected in all 4 variables for both trials H and F, there were two separate NCBI accession numbers: gi: 121702865 (spots: 3, 9) and gi: 70990724 (spots: 17, 26, 35, 40, 45, 51). The sequence range for this protein was of 4 to 12% and mass range 92 to 205. The theoretical and experimental isoelectric points and masses of these spots were 6.21, 38.4 kDa, and 6.85, 53.5 kDA for gi: 121702865 (spots: 3, 9) of trial H. For gi: 70990724

(spots: 17, 26, 35, 40, 45, 51) they were 6.21, 38.4 kDa, 6.85, 53.5 kDa for trial H and 6.9, 50 kDa for trial F.

The heat shock protein (spots: 1, 7, 13, 22) was detected from protein spots in trial H, in which the spot from the mutant at 37°C variable was up-regulated and noticeably larger than the spots collected from gels of other variables (Figures 3.9, 3.14). The sequence coverage range was 22 to 28% and mass range was 805 to 1188; the theoretical and experimental isoelectric points and masses of these spots were 5.56, 62.6 kDa and 5.6, 61.5 kDa.

The remaining proteins were detected from protein spots that were either present only at one temperature, only in one culture type, or present only in one of the four variables. The protein of unknown function DUF651, gi: 91773981 (4, 10), from trial H was detected from protein spots found at 25°C in both the wild-type and the mutant (Figures 3.9, 3.12). The sequence coverage for both spots was 2%, the Mascot scores were 67 and 69, the theoretical and experimental isoelectric points and masses of these spots were 6.49, 45.8 kDa and 6.7, 52 kDa. The hypothetical protein CIMG_06727, gi: 90301617 (spots: 38, 49), from trial F was detected from protein spots found at 25°C and 37°C in *Wd* Mc3 (Figure 3.8, 3.13). The sequence coverage for these spots were 8 and 4%, the Mascot scores were 150 and 89, the theoretical and experimental isoelectric points and masses of these spots were 6.37, 41.2 kDa and 6.55, 49 kDa. The septin [Neosartorya fischeri NRRL181] protein was detected from a spot present only in the wild-type at 37°C variable in trial H (Figures 3.9, 3.14). The unnamed protein product gi:833772901 was detected in trials H and F from spots present only in the mutant-type at 37°C (Figures 3.8, 3.9, 3.15). The aldeyde deydrogenase (NAD+) and the unnamed

protein product [*Aspergillus oryae*] were detected from protein spots found only in the mutant-type at 37°C in trial H (Figures 3.9).

Figure 3.1: Micrographs of *Wd* 8656 and *Wd* MC3 at 25°C and 37°C

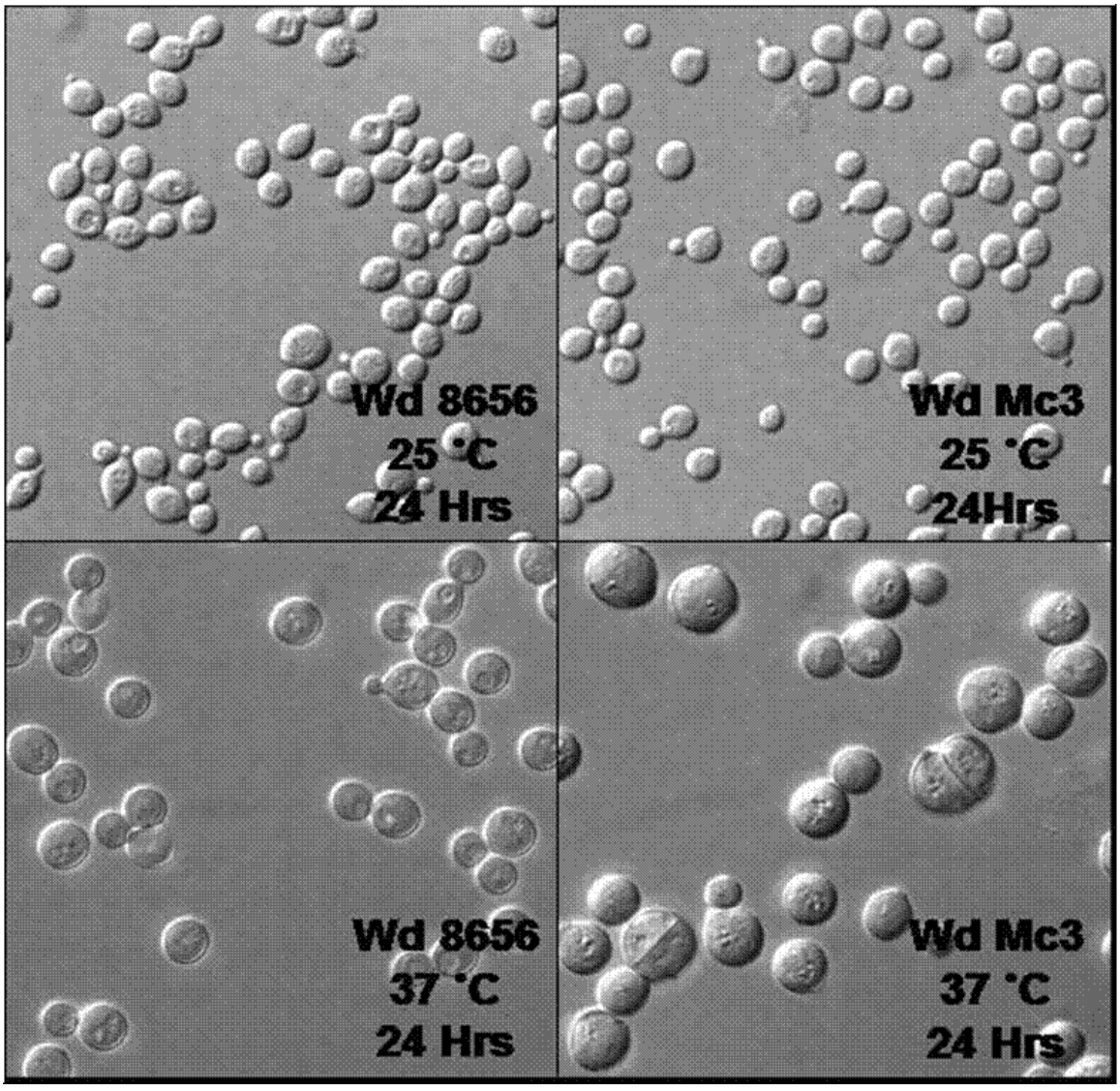


Figure 3.2: Experimental cultures of *Wd* 8656 and *Wd* Mc3 grown at 37°C and 25°C showing the color variation between variables.

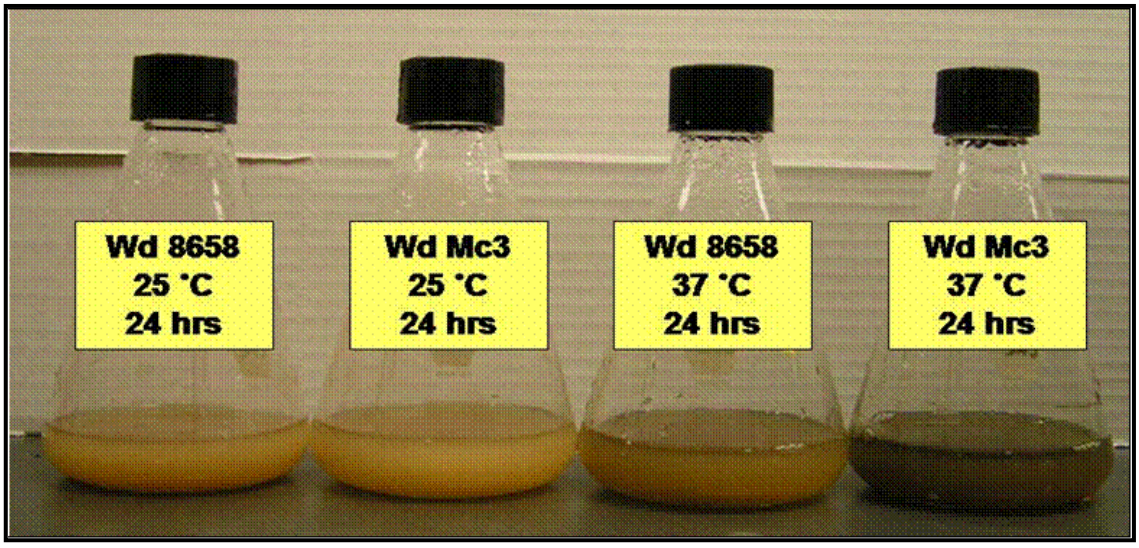


Figure 3.3: Preliminary two dimensional gel electrophoresis results of 48 hour cultures of *Wangiella dermatitidis*. These preliminary gels were processed using a 4-7 pI strips for the first dimension and 7cm (10% gels) for the second dimension. Note that only protein spots C and D are present in culture Wd Mc3 48hrs 37°C, while protein spots A-F are present in the other three cultures.

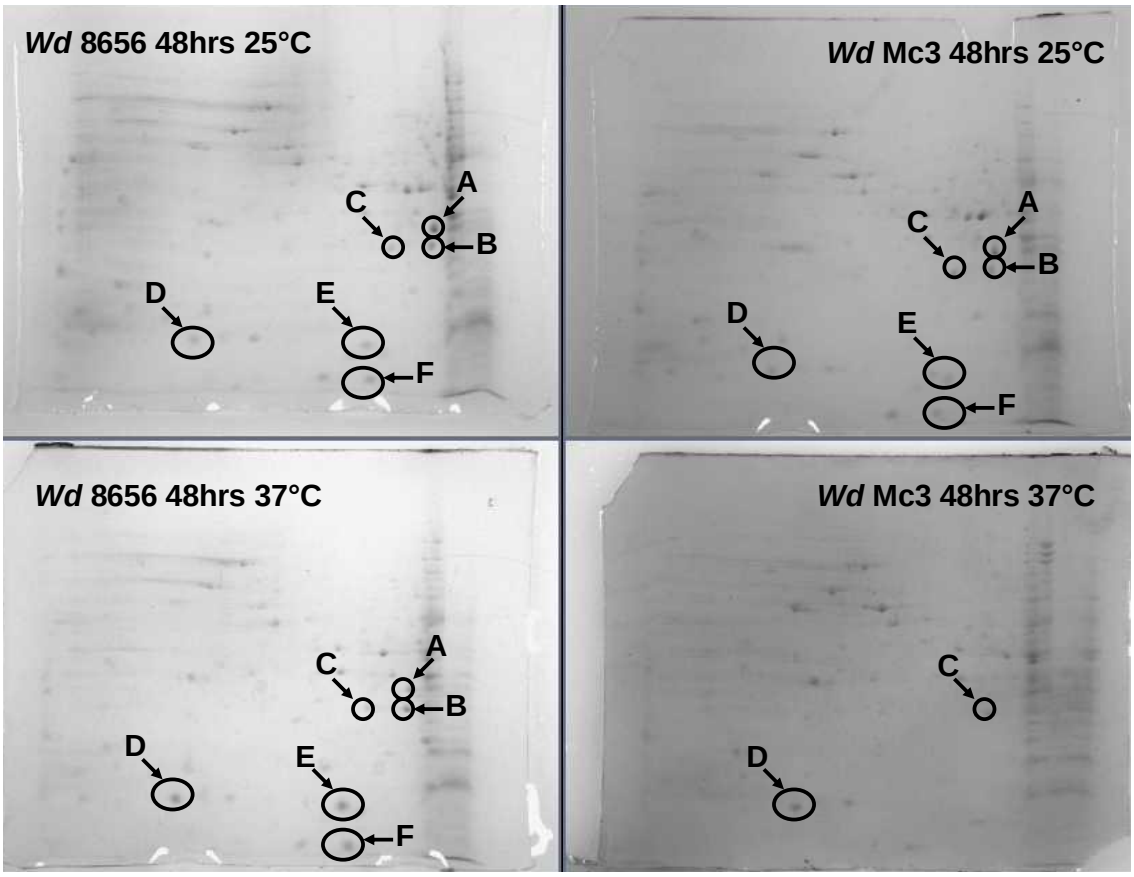


Figure 3.4: Standard curve used for trials A and B.

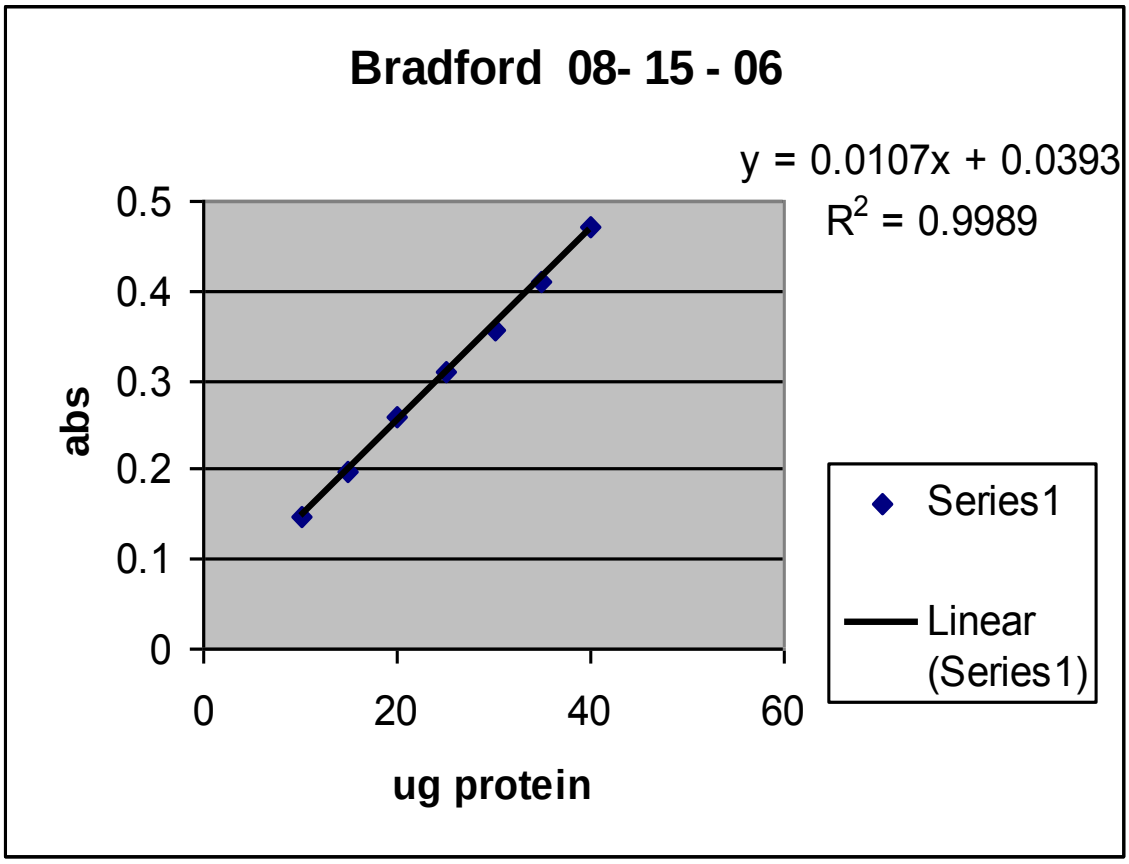


Figure 3.5: Protein profiles of A1 and A3 showing the consistency between the two protein profiles. A1) *Wd* 8656 at 25°C grown for 48 hours; A3) *Wd* 8656 at 25°C grown for 24 hours.

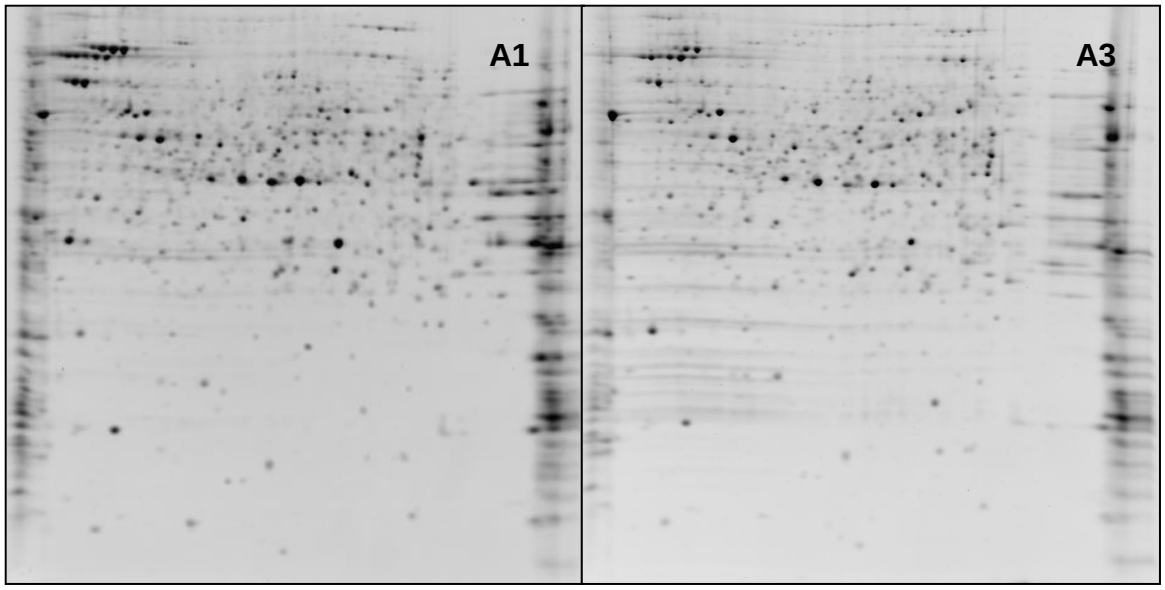


Figure 3.6: Protein profiles of all four variables of trial A showing consistency between variables. A3) *Wd* 8656 at 25˚C; A4) *Wd* MC3 at 25˚C; A5) *Wd* 8656 at 37˚C; A6) *Wd* MC3 at 37˚C.

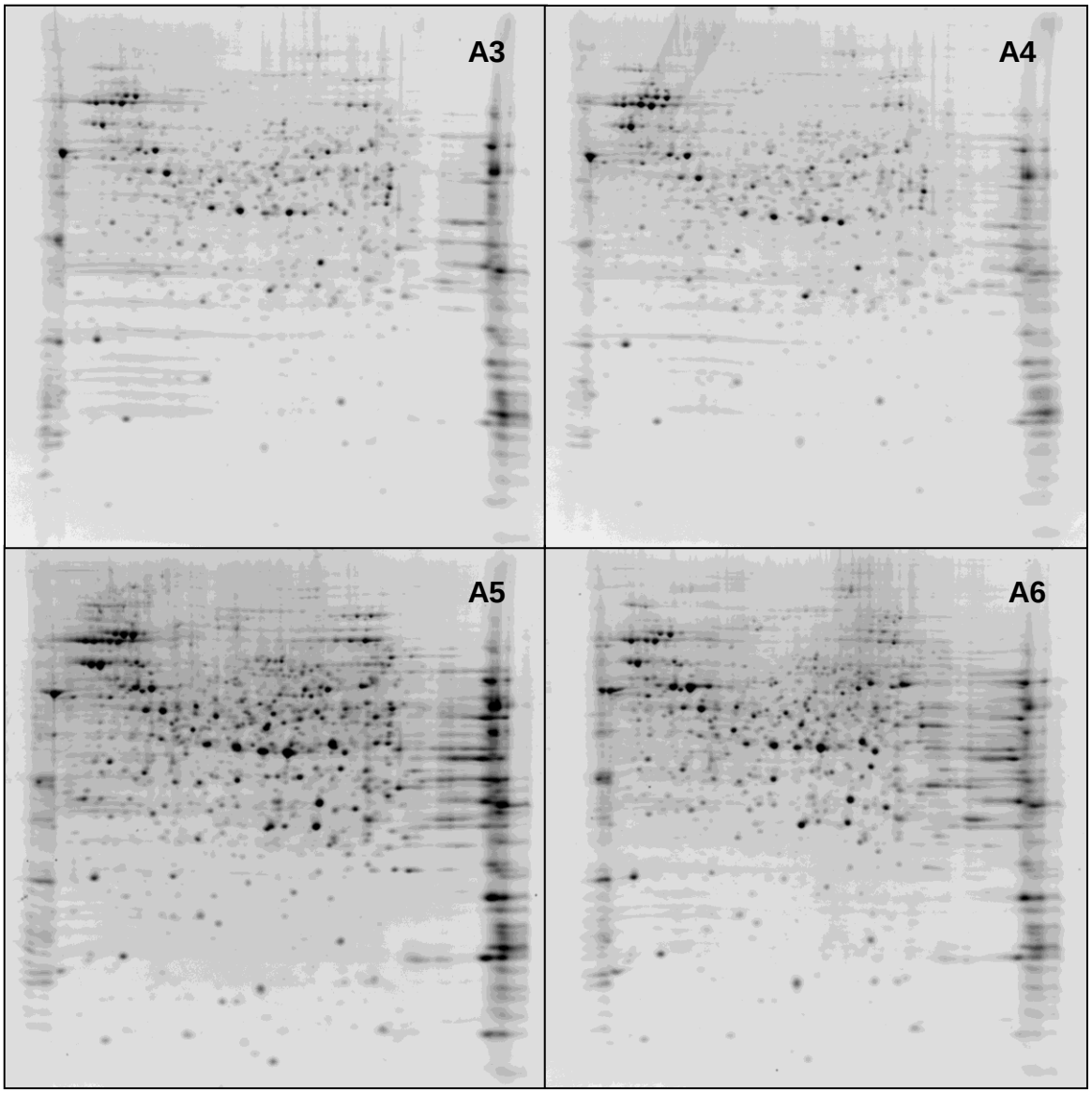


Figure 3.7: Protein profiles of A3, B3, and C3 showing the consistency between three different trials. A3) *Wd* 8656 at 25˚C; B3) *Wd* 8656 at 25˚C; C3) *Wd* 8656 at 25˚C. These gels were also used in the match set of this variable. In each of the 4 match sets, A3 was designated the master gel.

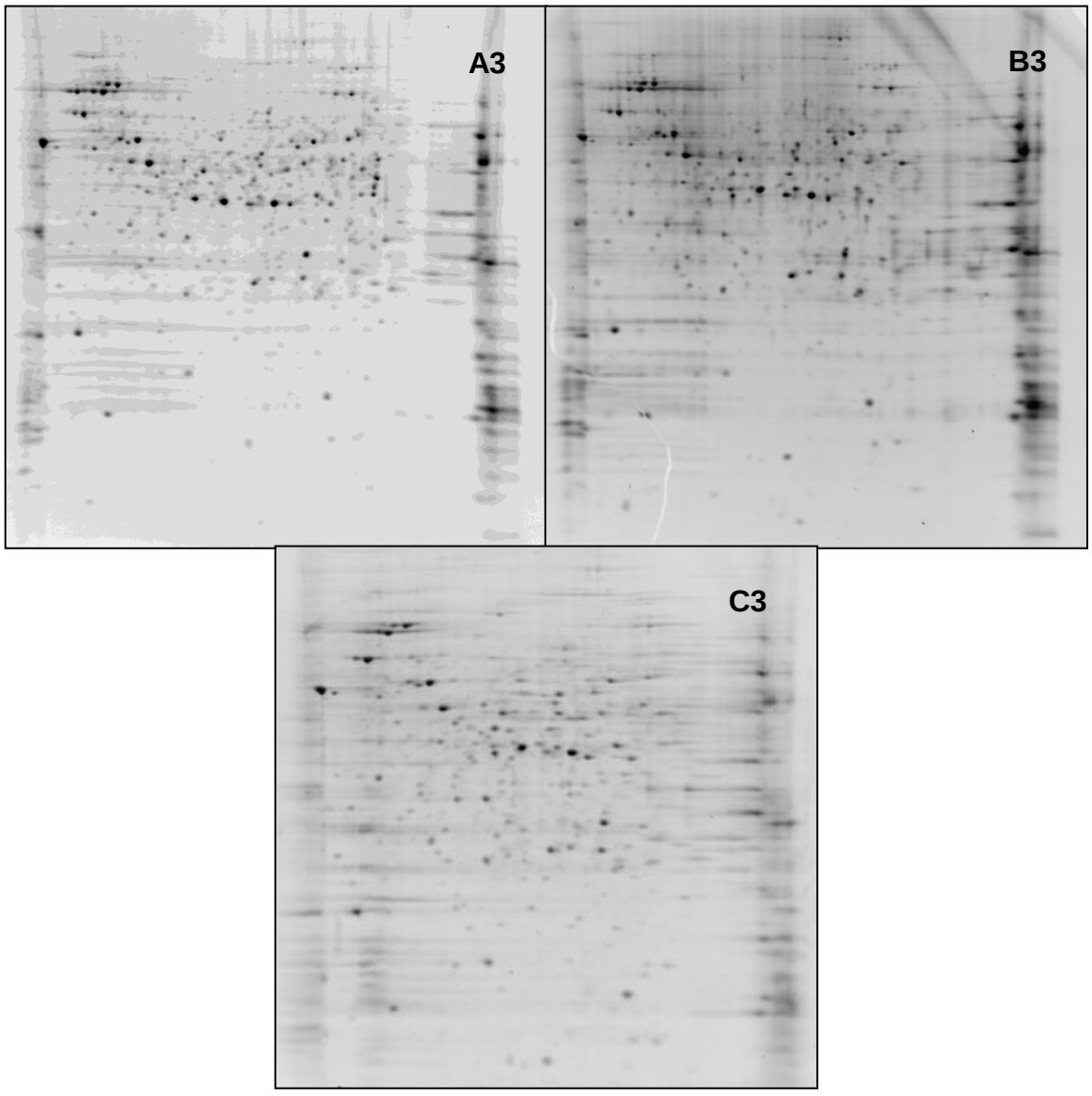


Figure 3.8: Protein profiles for all four variables of trial F. F3) *Wd* 8656 at 25˚C; F4) *Wd* MC3 at 25˚C; F5) *Wd* 8656 at 37˚C; F6) *Wd* MC3 at 37˚C. The numbered arrows point to the protein spots that were excised and analyzed by Mass spectrometry. Spots are designated as either landmark, up-regulated, down-regulated, or up-regulated solo protein spots. Spots 32, 36, 41, 47 and spots 34, 39, 44, 50 were landmark protein spots. Spots 33, 35, 36 and 40 were down-regulated, spots 38, 42, 45, 48, 49, and 50 were up-regulated, and spots 43, 46, 52, and 53 were up-regulated solo protein spots.

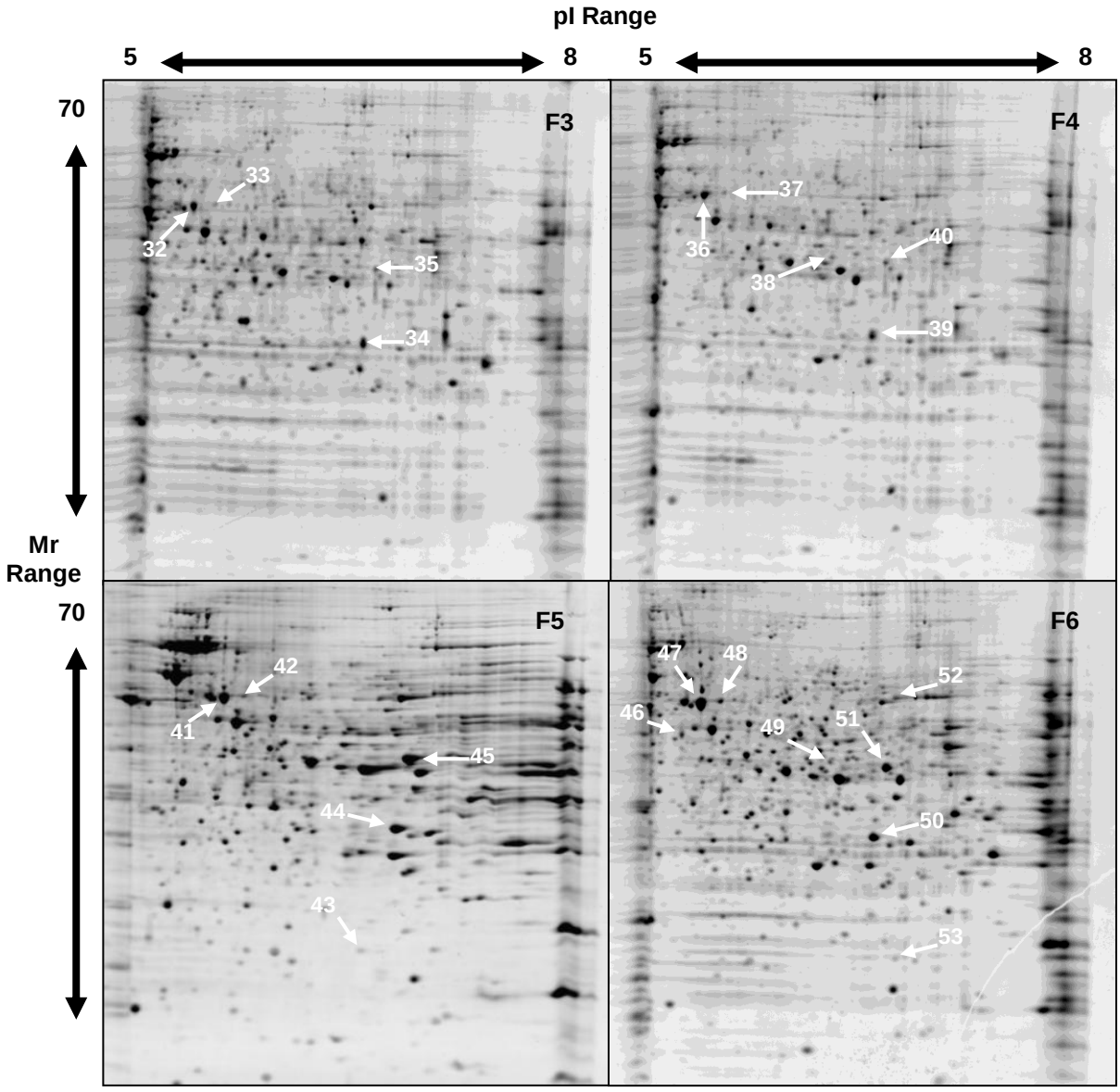


Figure 3.9: Protein profiles for all four variables of trial H. H3) *Wd* 8656 at 25°C; H4) *Wd* MC3 at 25°C; H5) *Wd* 8656 at 37°C; H6) *Wd* MC3 at 37°C. The numbered arrows point to the protein spots that were excised and analyzed by Mass spectrometry. Spots are designated as either landmark, up-regulated, down-regulated, or up-regulated solo protein spots. Spots 2, 8, 14, 23 and spots 6, 12, 21, 31 were landmark protein spots. Spots 1, 3, 5, 7, 9, 11, and 22 were down-regulated, spots 4, 10, 13, 16, 17, 18, 19, 20, 25, 26, 27, 28, and 29 were up-regulated, and spots 15, 24, and 30 were up-regulated solo protein spots.

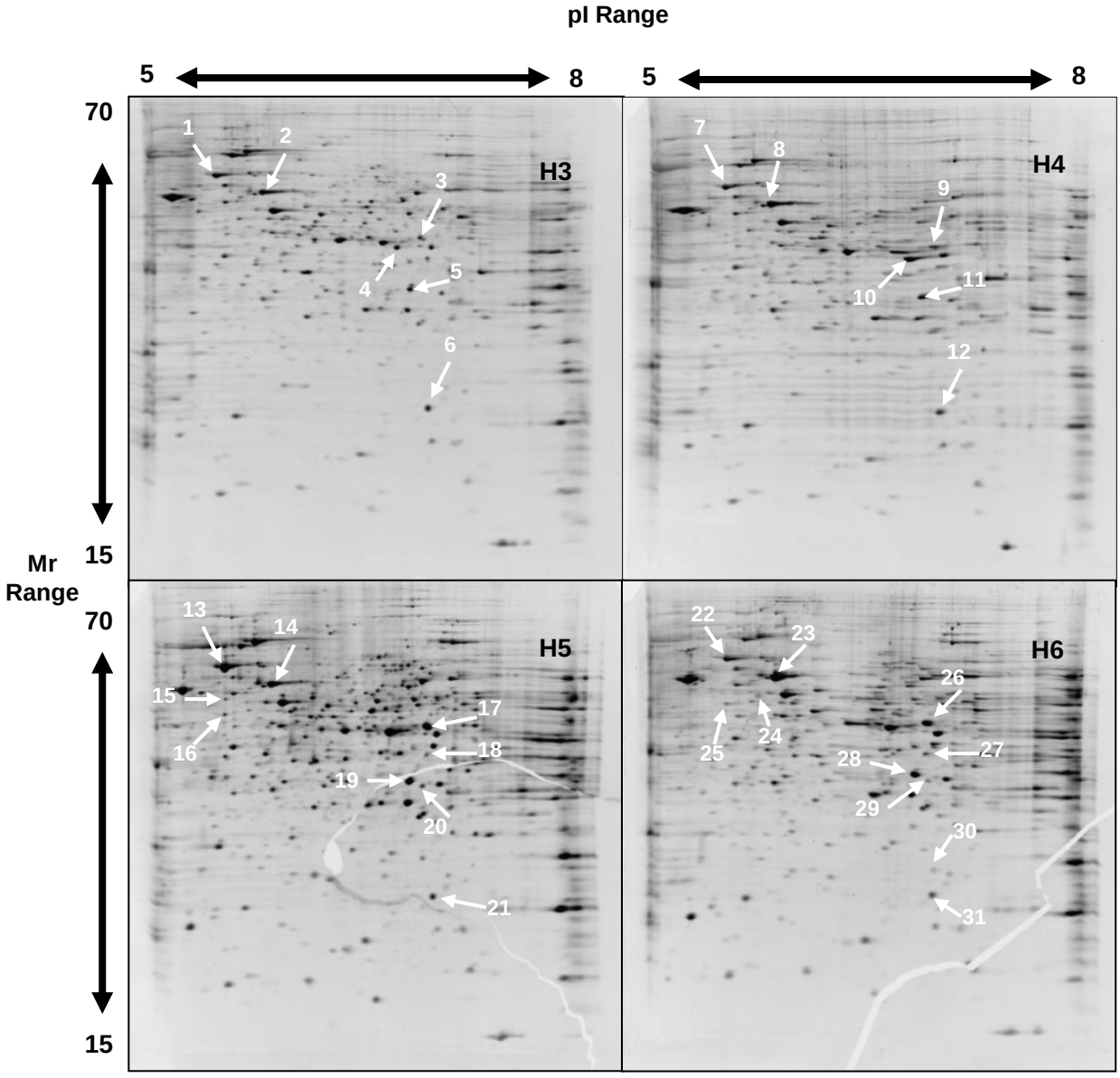


Figure 3.10: Three dimensional views of landmark portein spots found in all four variables

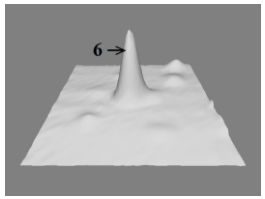
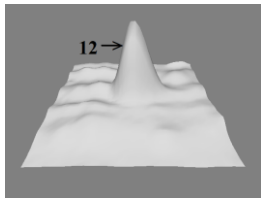
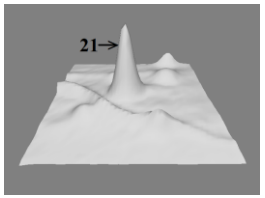
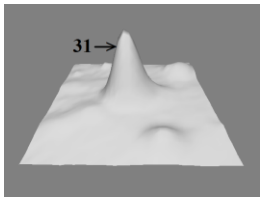
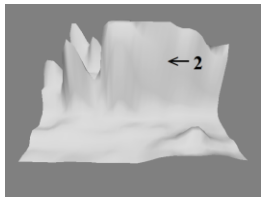
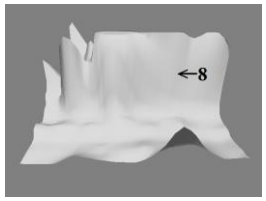
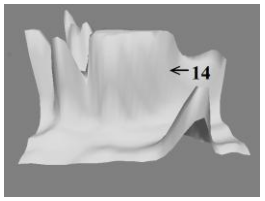
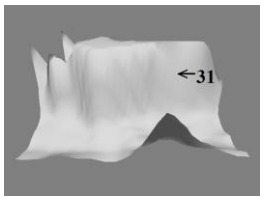
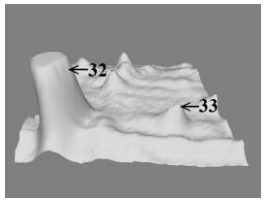
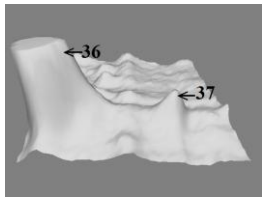
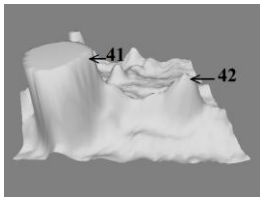
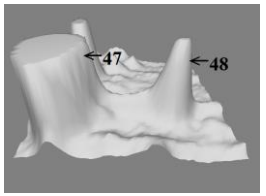
Protein Name	Wild @ 25°C	Mutant @ 25°C	Wild @ 37°C	Mutant @ 37°C
Superoxide dismutase [Cu-Zn] (HISDO) gi|122064579 Trial H				
Hypothetical protein AN1689.2 gi|67522465 Trial H				
Hypothetical protein AN1689.2 gi|67522465 Trial F				

Figure 3.11: Three dimensional views of temperture dependent portein spots found in all four variables yet upregulated at 37°C

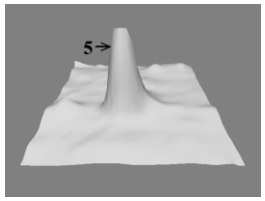
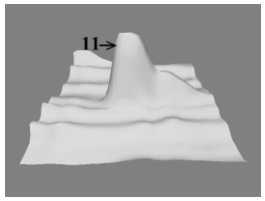
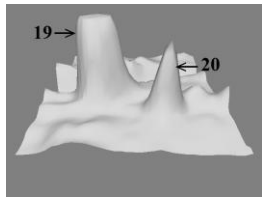
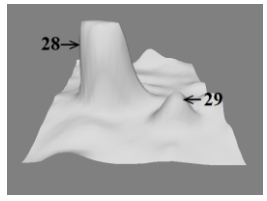
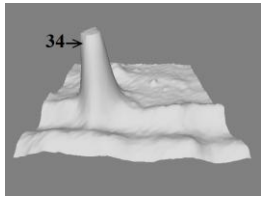
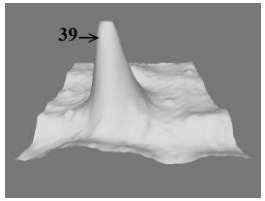
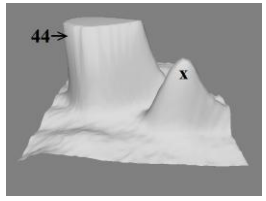
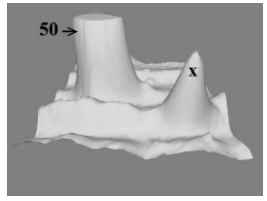
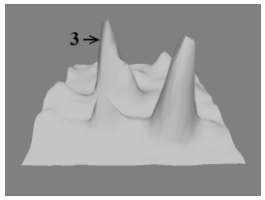
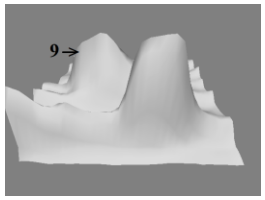
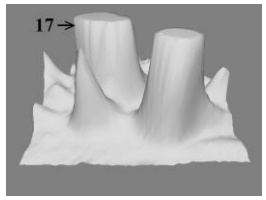
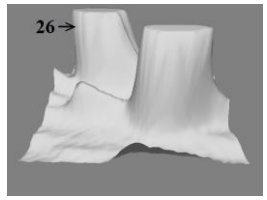
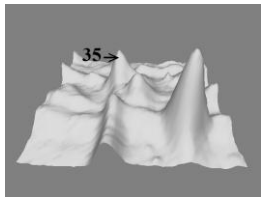
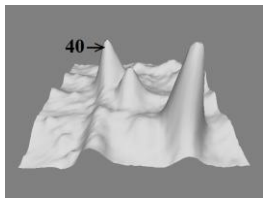
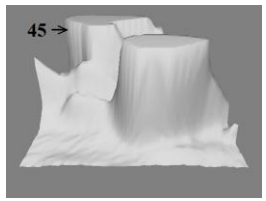
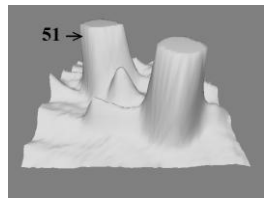
Protein Name	Wild @ 25°C	Mutant @ 25°C	Wild @ 37°C	Mutant @ 37°C
NAPD-deptendent Manital deyhydrogenas gi|37780013 Trial H				
NAPD-dependent mannitol dehydrogenase gi|37780013 Trial F				
Alcohol dehydrogenase, zinc-containing gi|121702865, gi|70990724 Trial H				
Alcohol dehydrogenase, zinc-containing gi|70990724 Trial F				

Figure 3.12: Three dimensional views of temperature dependent protein spots, present only at 25°C

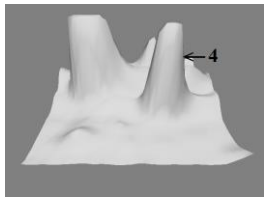
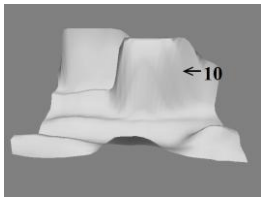
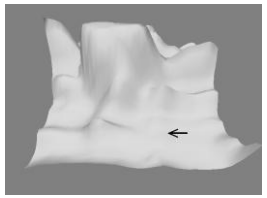
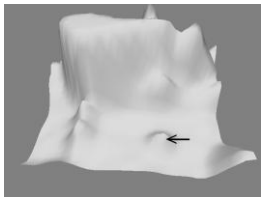
Protein Name	Wild @ 25°C	Mutant @ 25°C	Wild @ 37°C	Mutant @ 37°C
Protein of unknown function DUF65 gi|91773981 Trial H				

Figure 3.13: Three dimensional views of strain dependent protein spots, present only in mutant strian, *Wd* Mc3.

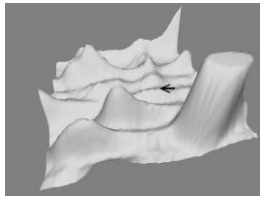
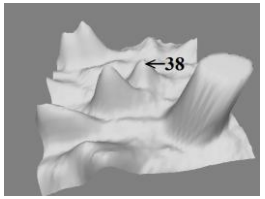
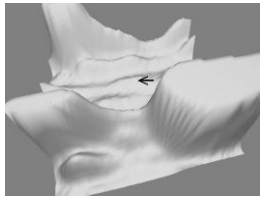
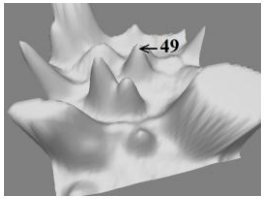
Protein Name	Wild @ 25°C	Mutant @ 25°C	Wild @ 37°C	Mutant @ 37°C
Hypothetical protein CIMG_06727 gi|90301617 Trial F				

Figure 3.14: Three dimensional views of portein spots upregulated in the wild type, *Wd* 8656, at 37°C

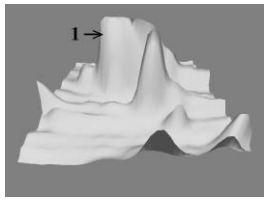
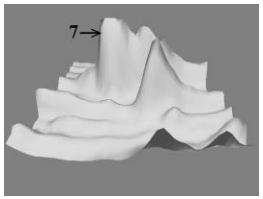
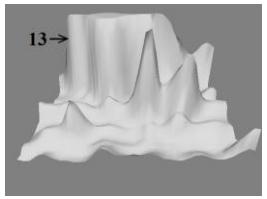
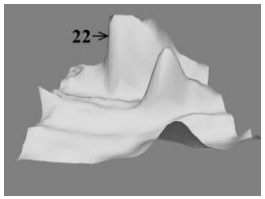
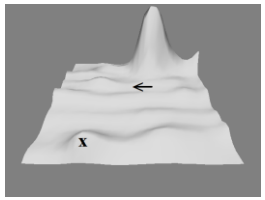
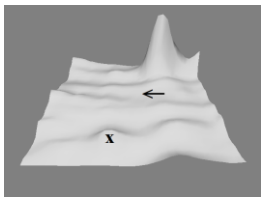
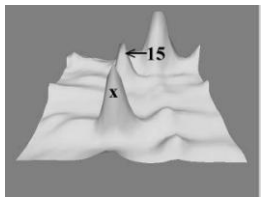
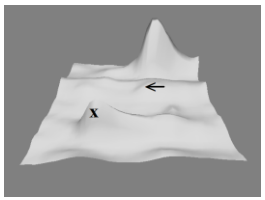
Protein Name	Wild @ 25°C	Mutant @ 25°C	Wild @ 37°C	Mutant @ 37°C
Heat shock protein 60, mitochondrial precursor gi|119182507 Trial H				
Septin [Neosartorya fischeri NRRL181] gi|119479605 Trial H				

Figure 3.15: Three dimensional views of portein spots upregulated in the mutant strain, *Wd*. Mc3, at 37°C

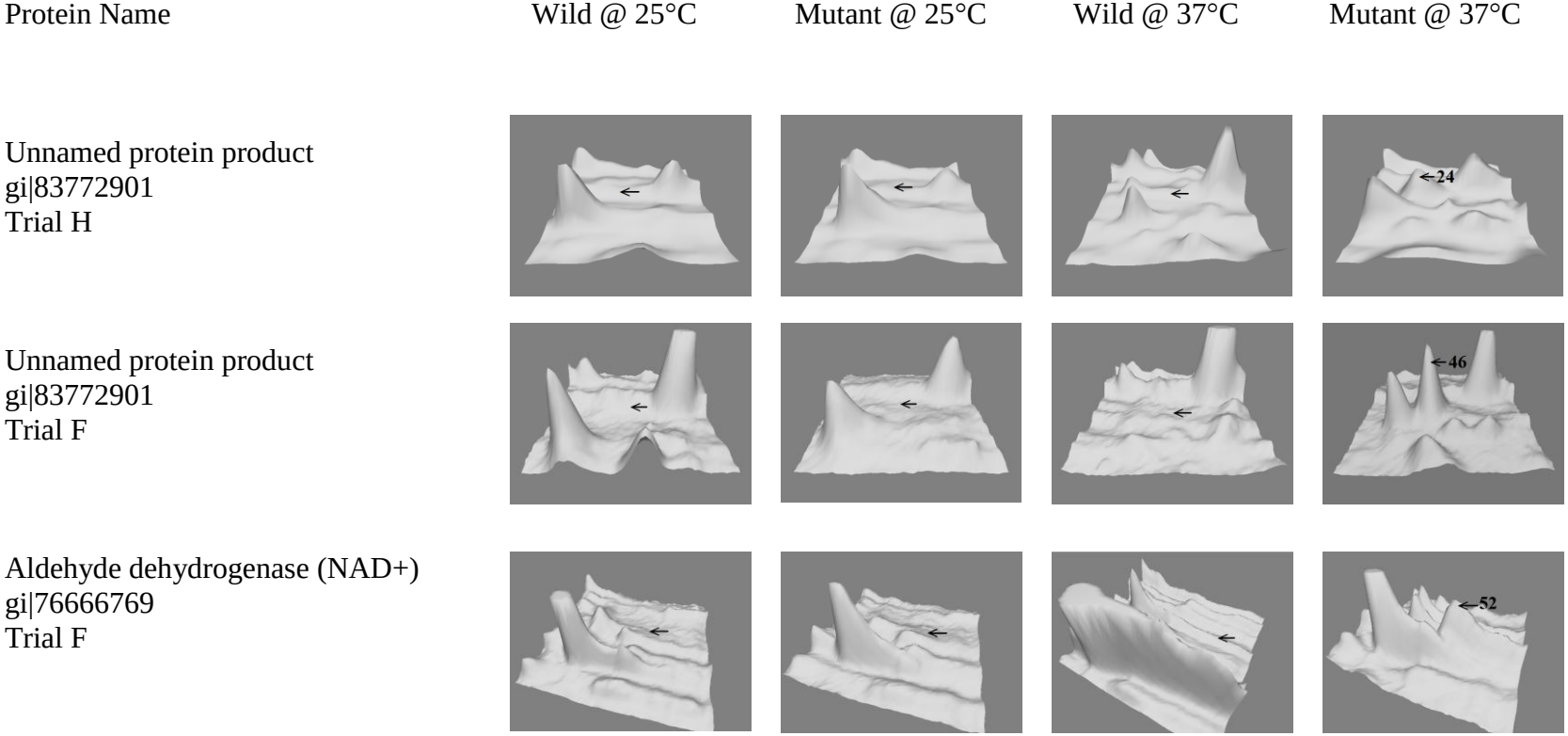


Table 3.1: Table presenting the results obtained from the match set of trials A, B, and C

Match Set Master to Compared	Master Spot Numbers	Compared Spot Numbers	Spots Matched	Unmatched Spots	Match Rate 1 %	Match Rate 2 %
3 to 4	411	500	324	87	64	78
4 to 3	500	411	319	92	77	63
3 to 5	411	599	312	99	52	75
5 to 3	599	411	307	104	74	51
3 to 6	411	540	291	120	53	70
6 to 3	540	411	288	123	70	53
4 to 5	500	599	301	199	50	60
5 to 4	599	500	302	198	60	50
4 to 6	500	540	280	190	51	56
6 to 4	540	500	284	216	56	52
5 to 6	599	540	354	186	65	59
6 to 5	540	599	357	183	59	66

Match Rate 1 Column: list the percentage of matched spots relative to the total number of spots on the gel

Match Rate 2 Column: list the percentage of matched spot on the gel relative to the total number of spots on the master

Table 3.2: Trial F excised and sequenced protein spots

Spot	Gel	Culture type	Spot Description (Relation)
32	F3	Wd 8656 24 Hr 25 C	Landmark (32, 36, 41, 47)
33	F3	Wd 8656 24 Hr 25 C	Down-Regulated (33, 37, 42, 48)
34	F3	Wd 8656 24 Hr 25 C	Landmark (34, 39, 44, 50)
35	F3	Wd 8656 24 Hr 25 C	Down-Regulated (35, 40, 45, 51)
36	F4	Wd MC3 24 Hr 25 C	Landmark (32, 36, 41, 47)
37	F4	Wd MC3 24 Hr 25 C	Down-Regulated (33, 37, 42, 48)
38	F4	Wd MC3 24 Hr 25 C	Up-Regulated (38, 49)
39	F4	Wd MC3 24 Hr 25 C	Landmark (34, 39, 44, 50)
40	F4	Wd MC3 24 Hr 25 C	Down-Regulated (35, 40, 45, 51)
41	F5	Wd 8656 24 Hr 37 C	Landmark (32, 36, 41, 47)
42	F5	Wd 8656 24 Hr 37 C	Up-Regulated (33, 37, 42, 48)
43	F5	Wd 8656 24 Hr 37 C	Up-regualted (solo)
44	F5	Wd 8656 24 Hr 37 C	Landmark (34, 39, 44, 50)
45	F5	Wd 8656 24 Hr 37 C	Up-Regulated (35, 40, 45, 51)
46	F6	Wd MC3 24 Hr 37 C	Up-regualted (solo)
47	F6	Wd MC3 24 Hr 37 C	Landmark (32, 36, 41, 47)
48	F6	Wd MC3 24 Hr 37 C	Up-Regulated (33, 37, 42, 48)
49	F6	Wd MC3 24 Hr 37 C	Up-Regulated (38, 49)
50	F6	Wd MC3 24 Hr 37 C	Landmark (34, 39, 44, 50)
51	F6	Wd MC3 24 Hr 37 C	Up-Regulated (35, 40, 45, 51)
52	F6	Wd MC3 24 Hr 37 C	Up-regualted (solo)
53	F6	Wd MC3 24 Hr 37 C	Up-regualted (solo)

Table 3.3: Trial H excised and sequenced protein spots

Spot	Gel	Culture Type	Spot Description (Relation)
1	H3	Wd 8656 24 Hr 25 C	Down-Regulated (1, 7, 13, 22)
2	H3	Wd 8656 24 Hr 25 C	Landmark (2, 8, 14, 23)
3	H3	Wd 8656 24 Hr 25 C	Down-Regulated (3, 9, 17, 26)
4	H3	Wd 8656 24 Hr 25 C	Up-Regulated (4, 10)
5	H3	Wd 8656 24 Hr 25 C	Down-Regulated (5, 11, 19, 28)
6	H3	Wd 8656 24 Hr 25 C	Landmark (6, 12, 21, 31))
7	H4	Wd MC3 24 Hr 25 C	Down-Regulated (1, 7, 13, 22)
8	H4	Wd MC3 24 Hr 25 C	Landmark (2, 8, 14, 23)
9	H4	Wd MC3 24 Hr 25 C	Down-Regulated (3, 9, 17, 26)
10	H4	Wd MC3 24 Hr 25 C	Up-Regulated (4, 10)
11	H4	Wd MC3 24 Hr 25 C	Down-Regulated (5, 11, 19, 28)
12	H4	Wd MC3 24 Hr 25 C	Landmark (6, 12, 21, 31))
13	H5	Wd 8656 24 Hr 37 C	Up-Regulated (1, 7, 13, 22)
14	H5	Wd 8656 24 Hr 37 C	Landmark (2, 8, 14, 23)
15	H5	Wd 8656 24 Hr 37 C	Up-Regulated (Solo)
16	H5	Wd 8656 24 Hr 37 C	Up-Regulated (16, 25)
17	H5	Wd 8656 24 Hr 37 C	Up-Regulated (3, 9, 17, 26)
18	H5	Wd 8656 24 Hr 37 C	Up-Regulated (18, 27)
19	H5	Wd 8656 24 Hr 37 C	UP-Regulated (5, 11, 19, 28)
20	H5	Wd 8656 24 Hr 37 C	Up-Regulated (20, 29)
21	H5	Wd 8656 24 Hr 37 C	Landmark (6, 12, 21, 31))
22	H6	Wd MC3 24 Hr 37 C	Down-Regulated (1, 7, 13, 22)
23	H6	Wd MC3 24 Hr 37 C	Landmark (2, 8, 14, 23)
24	H6	Wd MC3 24 Hr 37 C	Up-Regulated (Solo)
25	H6	Wd MC3 24 Hr 37 C	Up-Regulated (16, 25)
26	H6	Wd MC3 24 Hr 37 C	Up-Regulated (3, 9, 17, 26)
27	H6	Wd MC3 24 Hr 37 C	Up-Regulated (18, 27)
28	H6	Wd MC3 24 Hr 37 C	UP-Regulated (5, 11, 19, 28)
29	H6	Wd MC3 24 Hr 37 C	Up-Regulated (20, 29)
30	H6	Wd MC3 24 Hr 37 C	Up-Regulated (Solo)
31	H6	Wd MC3 24 Hr 37 C	Landmark (6, 12, 21, 31))

Table 3.4: Identification of primary protein expressed in protein spots of *Wangiella dermatitidis* in trials F and H

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
1	heat shock protein 60, mitochondrial precursor	gi|119182507	XP_001242382	5.56, 62.6	5.6, 61.5	*Coccidioides immitis*	805	13/42	K.FENLGAR.L R.GQLQVAAVK.A R.VVDALNATR.A K.VGGASEVEVEGEK.K K.DRVVDALNATR.A K.EDTIILNGEGSK.D R.NVLIESSYGSPK.I K.GRNVLIESSYGSPK.I K.TIEDELEVTEGMR.F K.GRNVLIESSYGSPK.I R.AAVEEGILPGGGTALLK.A K.EDTIILNGEGSKDAIAQR.C R.TALVDASGVASLLGTTEVAIVEAPEEK.A	22
2	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 59.5	*Aspergillus nidulans*	192	3/9	R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	8
3	alcohol dehydrogenase, zinc-containing, putative	gi|121702865	XP_001269697	6.21, 38.4	6.85, 53.5	*Aspergillus clavatus*	92	2/4	K.AAIVFD.- K.FPAAIDYLDSGK.V	5
4	protein of unknown function DUF861	gi|91773981	YP_566673	6.49, 45.8	6.7, 52	*Methanococcoides burtonii*	67	2/2	R.NALSSIPR.T R.EAARNALSSIPR.T	2
5	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 47	*Alternaria alternata*	262	3/14	R.AVGLHFR.E K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	10

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Species	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
6	Superoxide dismutase [Cu-Zn] (HISOD)	gi|122064579	P83684	6.36, 16.0	6.9, 32.5	*Humicola lutea*	80	2/4	M.VKAVAVLR.G R.HVGDLGNIK.T	11
7	heat shock protein 60, mitochondrial precursor	gi|119182507	XP_001242382	5.56, 62.6	5.6, 61.5	*Coccidioides immitis*	864	15/31	K.EGVITVK.D K.FENLGAR.L K.APGFGDNR.K K.LSGGVAVIK.V R.GQLQVAAVK.A R.VVDALNATR.A K.VGGASEVEVGEK.K K.DRVVDALNATR.A K.EDTIILNGEGSK.D R.NVLIESSYGSPK.I K.TIEDELEVTEGMR.F K.AISLQDKFENLGAR.L R.AAVEEGILPGGGTALLK.A K.EDTIILNGEGSKDAIAQR.C R.TALVDASGVASLLGTTEVAIVEAPEEK.A	28
8	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 59.5	*Aspergillus nidulans*	225	5/13	R.VPFGGVK.Q R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A K.LGPALACGNTVVLKPAEQTPLSILYLAK.F	15
9	alcohol dehydrogenase, zinc-containing, putative	gi|121702865	XP_001269697	6.21, 38.4	6.85, 53.5	*Aspergillus clavatus*	99	2/5	K.AAIVFD.- K.FPAAIDYLDSGKV	9

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
10	protein of unknown function DUF851	gi|91773981	YP_566673	6.49, 45.8	6.7, 52	*Methanococcoides burtonii*	69	2/6	R.NALSSIPR.T R.EAARNALSSIPR.T	2
11	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 47	*Alternaria alternata*	150	2/5	K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	7
12	Superoxide dismutase [Cu-Zn] (HISOD)	gi|122064579	P83684	6.36, 16.0	6.9, 32.5	*Humicola lutea*	80	2/6	R.HVGDLGNIK.T R.TIVVHAGTDDLGR.G	14
13	heat shock protein 60, mitochondrial precursor	gi|119182507	XP_001242382	5.56, 62.6	5.6, 61.5	*Coccidioides immitis*	1188	21/84	K.FENLGAR.L K.APGFGDNR.K K.LSGGVAVIK.V R.GQLQVAAVK.A R.VVDALNATR.A K.APGFGDNRK.S R.VGKEGVITVK.D K.VGGASEVEVGEK.K K.LRGQLQVAAVK.A K.DRVVDALNATR.A K.EDTIILNGEGSK.D R.NVLIESSYGSPK.I K.GRNVLIESSYGSPK.I K.TIEDELEVTEGMR.F K.TIEDELEVTEGMR.F K.VGGASEVEVGEKKDR.V K.AISLQDKFENLGAR.L R.AAVEEGILPGGGTALLK.A K.EDTIILNGEGSKDAIAQR.C K.TIEDELEVTEGMRFDR.G R.TALVDASGVASLLGTTEVAIVEAPEEK.A	28

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
14	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 59.5	*Aspergillus nidulans*	206	4/12	R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A K.LGPALACGNTVVLKPAEQTPLSILYLAK.F	13
15	septin [Neosartorya fischeri NRRL 181]	gi|119479605	XP_001259831	5.04, 43.4	5.9, 57.5	*Neosartorya fischeri*	197	5/8	R.VNVIPVIGK.A R.HSDFLAIR.S R.ELDIELMIKR.L K.ADSLTPAELAESK.K K.SVDGAASGHDSSMNPEDLASQSVR.L	16
17	alcohol dehydrogenase, zinc-containing	gi|70990724	XP_750211	6.21, 38.4	6.85, 53.5	*Aspergillus fumigatus*	121	3/6	K.AAIVFD.- K.SAIKAAIVFD.- K.FPAAIDYLDSGKV	8
19	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 47	*Alternaria alternata*	244	3/11	R.AVGLHFR.E K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	10
20	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.81, 46.5	*Alternaria alternata*	64	1/3	K.VVIVTGASGPTGIGTEAAR.G	7
21	Superoxide dismutase [Cu-Zn] (HISOD)	gi|122064579	P83684	6.36, 16.0	6.9, 32.5	*Humicola lutea*	99	3/5	M.VKAVAVLR.G R.HVGDLGNIK.T K.AVAVLRGDSKI	13

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
22	heat shock protein 60, mitochondrial precursor	gi|119182507	XP_001242382	5.56, 62.6	5.6, 61.5	*Coccidioides immitis*	1112	18/57	K.FENLGAR.L K.APGFGDNR.K R.LLQDVASKT R.GQLQVAAVK.A R.VVDALNATR.A K.APGFGDNRKS K.VGGASEVEVGEK.K K.LRGQLQVAAVK.A K.DRVVDALNATR.A K.EDTIILNGEGSK.D K.VGGASEVEVGEKK.D R.NVLIESSYGSPK.I K.NVAAGCNPMDLR.R K.GRNVLIESSYGSPK.I K.TIEDELEVTEGMR.F R.AAVEEGILPGGGTALLK.A K.EDTIILNGEGSKDAIAQR.C R.TALVDASGVASLLGTTEVAIVEAPEEK.A	28
23	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 59.5	*Aspergillus nidulans*	199	3/13	R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	8
24	unnamed protein product	gi|83772901	BAE83029	5.05, 42.4	5.8, 55	*Aspergillus oryzae*	152	3/6	R.EIDTIGTDGIIK.R R.EIDTIGTDGIIKR.I R.VITLGGDHTITLPLLR.S	7
28	alcohol dehydrogenase, zinc-containing	gi|70990724	XP_750211	6.21, 38.4	6.85, 53.5	*Aspergillus fumigatus*	192	5/21	K.AAIVFD.- K.DPSAQFEK.L R.KDPSAQFEK.L K.FPAAIDYLDTGK.V R.VVADNSELCGECFYCR.R	12

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
28	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 47	*Alternaria alternata*	231	4/12	R.AVGLHFR.E K.SLANEWR.D K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	13
29	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.81, 46.5	*Alternaria alternata*	79	1/3	K.VVIVTGASGPTGIGTEAAR.G	7
31	Superoxide dismutase [Cu-Zn] (HISOD)	gi|122064579	P83684	6.36, 16.0	6.9, 32.5	*Humicola lutea*	141	4/12	K.AVAVLR.G M.VKAVAVLR.G R.HVGDLGNIK.T R.TIVVHAGTDDLGR.G	19
32	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 57.5	*Aspergillus nidulans*	240	5|5	R.VPFGGVK.Q R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A K.LGPALACGNTVVLKPAEQTPLSILYLAK.F	15
33	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	6.1, 58	*Aspergillus nidulans*	126	2/5	K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	6
34	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 42	*Alternaria alternata*	136	2/11	R.AVGLHFR.E K.VVIVTGASGPTGIGTEAAR.G	9
35	alcohol dehydrogenase, zinc-containing	gi|70990724	XP_750211	6.21, 38.4	6.9, 50	*Aspergillus fumigatus*	95	3/4	K.AAIVFD.- K.DPSAQFEK.L R.KDPSAQFEK.L	4

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
36	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 57.5	*Aspergillus nidulans*	238	5/23	R.VPFGGVK.Q R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A K.LGPALACGNTVVLKPAEQTPLSILYLAK.F	15
37	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	6.1, 58	*Aspergillus nidulans*	118	2/3	K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	6
38	hypothetical protein CIMG_06727	gi|90301617	EAS31248	6.37, 41.2	6.55, 49	*Coccidioides immitis*	150	3/5	R.VSVVTGGAR.G K.YVTGADLR.V R.VNCISPGYMLTALTR.K + Oxidation (M)	8
39	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 42	*Alternaria alternata*	214	3/14	R.AVGLHFR.E K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	10
40	alcohol dehydrogenase, zinc-containing	gi|70990724	XP_750211	6.21, 38.4	6.9, 50	*Aspergillus fumigatus*	117	3/7	K.AAIVFD.- K.DPSAQFEK.L R.KDPSAQFEK.L	4
41	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 57.5	*Aspergillus nidulans*	333	5/40	R.VPFGGVK.Q R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A K.LGPALACGNTVVLKPAEQTPLSILYLAK.F	15
42	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	6.1, 58	*Aspergillus nidulans*	145	2/6	K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	6

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
44	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 42	*Alternaria alternata*	245	3/40	R.AVGLHFR.E K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	10
45	alcohol dehydrogenase, zino-containing	gi|70990724	XP_750211	6.21, 38.4	6.9, 50	*Aspergillus fumigatus*	205	5/14	K.AAIVFD.- K.DPSAQFEK.L R.KDPSAQFEK.L R.VVADNSELCGECFYCR.R R.VVADNSELCGECFYCR.R	14
46	unnamed protein product	gi|83772901	BAE63029	5.05, 42.4	5.8, 52	*Aspergillus oryzae*	240	5/13	R.EIDTIGTDGIIK.R R.EIDTIGTDGIIKR.I R.VITLGGDHTITLPLLR.S K.YDIAFIGAPFDTGTSYRPGAR.F	12
47	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	5.9, 57.5	*Aspergillus nidulans*	270	4/27	R.VPFGGVK.Q R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	9
48	hypothetical protein AN1689.2	gi|67522465	XP_659293	5.56, 54.5	6.1, 58	*Aspergillus nidulans*	205	4/13	R.VPFGGVK.Q R.VLSYIESGK.Q K.SPLLVFDDADLEQAAK.W K.VGDPFSDDTFQGPQVTK.A	9
49	hypothetical protein CIMG_06727	gi|90301617	EAS31248	6.37, 41.2	6.55, 49	*Coccidioides immitis*	89	2/5	R.VSVVTGGAR.G K.YVTGADLR.V	4
50	NADP-dependent mannitol dehydrogenase	gi|37780013	AAO91800	5.86, 29.1	6.8, 42	*Alternaria alternata*	199	3/13	R.AVGLHFR.E K.VVIVTGASGPTGIGTEAAR.G K.GKVVIVTGASGPTGIGTEAAR.G	10

Spot	Protein Name	NCBI Accession	Actual Accession	Theo. pI/Mr (kDa)	Expt. pI/Mr (kDa)	Speices	Mascot Score	NP/PD	MS/MS petide sequence	SC (%)
51	alcohol dehydrogenase, zinc-containing	gi|70990724	XP_750211	6.21, 38.4	6.9, 50	*Aspergillus fumigatus*	144	4/11	K.AAIVFD.- K.DPSAQFEK.L R.KDPSAQFEK.L R.VVADNSELCGECFYCR.R	8
52	aldehyde dehydrogenase (NAD+)	gi|76666769	CAA55072	6.23, 53.9	6.95, 57	*Davidiella tassiana*	113	2/3	K.VTLELGGKS K.VAFTGSTWGR.T	3
53	unnamed protein product [Aspergillus oryzae]	gi|83767869	BAE58008	6.36, 25.1	6.9, 28	*Aspergillus oryzae*	125	2/4	K.FPAAIDYLDSGK.V K.SLEAGDEYVALSR.Q	10

Table 3.5: Proteins grouped by common protein spots for trials H and F

Protein	NCBI Accession	Trial H Spot #'s	Trial F Spot #'s	Species
Superoxide dismutase [Cu-Zn] (HISOD)	gi|122064579	6,12,21,31		*Humicola lutea*
hypothetical protein AN1689.2	gi|67522465	2,8,14,23	32 ,36 ,41 ,47 33, 37, 42, 48	*Aspergillus nidulans*
NADP-dependent mannitol dehydrogenase	gi|37780013	5, 11, 19, 28 20, 29	34, 39, 44, 50	*Alternaria alternata*
alcohol dehydrogenase, zinc-containing, putative	gi|121702865	3,9		*Aspergillus clavatus*
alcohol dehydrogenase, zinc-containing	gi|70990724	17,26	35, 40, 45, 51	*Aspergillus fumigatus*
protein of unknown function DUF651	gi|91773981	4, 10		*Methanococcoides burtonii*
hypothetical protein CIMG_06727	gi|90301617		38, 49	*Coccidioides immitis*
heat shock protein 60, mitochondrial precursor	gi|119182507	1, 7, 13, 22		*Coccidioides immitis*
septin [Neosartorya fischeri NRRL 181]	gi|119479605	15		*Neosartorya fischeri*
unnamed protein product	gi|83772901	24	46	*Aspergillus oryzae*
aldehyde dehydrogenase (NAD+)	gi|76666769		52	*Davidiella tassiana*
unnamed protein product [Aspergillus oryzae]	gi|83767869		53	*Aspergillus oryzae*

DISCUSSION

Analysis of temperature on cultures of *Wangiella dermatitidis*.

As shown in Figures 3.1 and 3.2, a morphological shift in cell growth occurred in *Wd* Mc3 when grown at 37°C, the cells became swollen and developed internal septa. This figure demonstrates that the wild type *Wd* 8656 was also affected morphologically by the change in temperature from 25°C to 37°C; cells grown at 37°C also appeared slightly swollen. The effect of temperature change was also disclosed, at a macroscopic level, in color differences between cultures (Figure 3.2). While the liquid colony of *Wd* Mc3 at 37°C was visibly the darkest colony, *Wd* 8656 at 37°C also increased in pigmentation. At 25°C, *Wd* 8656 and *Wd* Mc3 were similar in both morphology (Figure 3.1) and color (Figure 3.2). As expected, we see this same pattern in the protein profiles of *Wd* 8656 and *Wd* Mc3 at 25°C. Likewise, we see more differences between the protein profiles of *Wd* 8656 and *Wd* 8656 at 37°C (Figures 3.14, 3.15), as seen in colony appearance and cellular morphology (Figures 3.1, 3.2). While the greatest number of differences were present comparing either strain by temperature (25°C to 37°C), differences between the protein profiles, cellular morphology, and colony pigmentation were also prevalent comparing *Wd* 8656 and *Wd* Mc3 at 37°C (Figures 3.8, 3.9, 3.14, 3.15). Deciphering these protein differences are key in understanding which proteins are involved in the transformation from the yeast form into the isotropic multicellular form which resembles tissues infected with *W. dermatitidis*.

Temperature is an immense factor in protein production in all organisms. It is not unexpected, then, that many differences found within the protein profiles between the

Wd 8656 and *Wd* Mc3 strains (at 25°C and 37°C) occurred between the temperature variables. Furthermore, it was far more common to see up-regulated protein spots at 37°C than at 25°C. Interestingly, all unique protein spots were found at the higher temperature variable of 37°C (spots: 15, 24, 46, 52, 53).

Yeast growing at 37°C metabolize at a faster rate and reproduction is increased compared to yeast grown at 25°C. Furthermore, colonies grown at 25°C as well as 37°C, if not transferred to fresh nutrient media, also become darker in color over time. Given that the yeasts are in an enclosed space, some of the protein production at the higher temperature could be induced from crowding, reduction in nutrients, or increased waste production.

Prevalence of Dehydrogenases

Three of the putative proteins identified in this study were dehydrogenases. Interestingly, they were all collected from temperature dependent protein spots (Figure 3.14, 3.15). NADP-dependent mannitol dehydrogenase (spots: 5, 11, 19, 28, 20, 29, 34, 44, 50) and alcohol dehydrogenase, zinc-containing (spots: 3, 9, 17, 26, 35, 40, 45, 51) were detected in both trials F and H from the same protein spot locations (Figures 3.8, 3.9, 3.14). Aldehyde dehydrogenase (spot: 52) was also detected from a temperature dependent protein spot but only found in the *Wd*. Mc3 strain (Figures 3.8, 3.15). Since dehydrogenases are important and abundant in metabolism, it makes good sense that these proteins would be detected as temperature dependent. In each of these dehydrogenases, the up-regulated protein spots were at the higher temperature of 37°C.

Dehydrogenases are enzymes that catalyze the removal of hydrogen from a substrate and the transfer of the hydrogen to an acceptor in an oxidation-reduction reaction. Dehydrogenases play important roles in metabolism and alcohol dehydrogenase is an essential enzyme in fermentation (Deacon 2006). At the higher temperature, oxygen depletion is likely a greater factor which could lead to an over expression of alcohol dehydrogenase at 37°C.

Analysis of protein profiles between mutant and wild type strains

While this study was not necessarily looking for protein profile differences between the wild and mutant strains, it is interesting that only one strain dependent protein spot was detected within both temperatures. The hypothetical protein CIMG_06727 (spots: 38, 49) was detected from mutant strain protein spots at comparable intensities in both temperature variables, *Wd* Mc3 at 25°C and *Wd* Mc3 at 37°C (Figures 3.8, 3.13).

The detection of hypothetical protein CIMG_06727 in spot 38 was the only difference noted between the protein profiles of *Wd* 8656 and *Wd* Mc3 at 25°C. At 25°C, cell morphology (Figure 3.1), culture appearance (Figure 3.2), and protein profiles (Figure 3.7 and 3.8) of *Wd* 8656 and *Wd* Mc3 were remarkably similar. In contrast, notable differences in cell morphology (Figure 3.1), culture appearance (Figure 3.2), and protein profiles (Figure 3.7 and 3.8) were found between *Wd* 8656 and *Wd* Mc3 when grown at 37°C. Along with the detection of this hypothetical protein CIMG_06727 (spot: 49) in *Wd* Mc3 at 37°C, there were distinct protein profile differences between *Wd* 8656 and *Wd* Mc3 at 37°C discussed in the following sections.

Heat shock protein spots up-regulated in *Wd* 8656 at 37°C

The heat shock protein 60 (spots: 1, 7, 13, 22,) detected in protein spots from trial H, was greatly up-regulated in *Wd* 8656 at 37°C (Figure 3.9, 3.14). This protein was so overly expressed that in trial F (Figure 3.8: A5) differentiation of this spot from other nearby spots was not possible. Recall that trial F was processed with 400 µg of protein while trial H was processed with 250 µg. Also, in trial A loaded at 250 µg, the heat shock protein spot is visible (Figure 3.6: A5).

It is not unusual to find an over-expression of heat shock protein at the higher temperature compared to the lower temperature but it is worth noting that over-expression was only seen in the wild type, *Wd* 8656, and not in the mutant, *Wd* Mc3. The expression of heat shock proteins (HSP) are increased when the cells are exposed to elevated temperature or other stress. The function of heat shock protein 60 (HSP60) is to transport and refold proteins from the cytoplasm into the mitochondrial matrix; it is a mitochondrial chaperonin (Schlesinger 1990). Heat shock proteins are known immunogens and are involved in a variety of different types of infections and appear to play a role in morphogenesis (Swoboda, et al. 1995). One explanation for the abundance of heat shock protein production in *Wd* 8656 but not in *Wd* Mc3 is that the mutant has some other method, protein expression or cellular morphology, which is utilized to tolerate the higher temperature. Culture *Wd* 8656 is responding to the increase in temperature as expected. The mutation that allows the *Wd* Mc3 strain to switch to the isotropic multicellular morphology at 37°C may buffer it from temperature shock thus rendering the up-regulation of the heat shock protein unnecessary.

Septin protein spot up-regulated only in *Wd* 8656 at 37°C

Septins were named for their role in septum formation; they were discovered in budding yeast of *Saccharomyces cerevisiae* (Douglas et al. 2005). Septins are conserved proteins involved in the cell cycle and essential in cytokinesis. *Saccharomyces cerevisiae* has been the focus of much of the septin research (Warenda and Konopka 2002). Over the last decade research has linked septins to morphogenesis of multimorphic opportunistic pathogenic fungi. (González et al. 2006). The focus of this research has been conducted with *Candida albicans*. The ability to switch between budding and filamentous forms has been shown to be an important virulence determinant for the pathogenicity of *C. albicans* (Warenda and Konopka 2002).

A septin protein was detected in *Wd* 8656 at 37°C but not in any other variable (Figure 3.14). Since this protein was undetected in *Wd* 8656 at 25°C as well as being undetected in the mutant at either temperature, it is logical to consider that this protein is connected to virulence for pathogenicity in morphogenesis. The *Wd* Mc3 strain has a mutation that allows it to switch to the isotropic morphology at 37°C thus mimicking the growth of infected tissue without a host and without the aid of this particular septin protein.

Analysis of time on cultures of *Wangiella dermatitidis*.

While this research sought to capture changes in the protein profiles at the onset of the morphological shift induced by temperature, the question of when this onset occurs may require additional study. Furthermore, the length of time in which these up-regulated proteins, stimulated by this temperature shift, remain active in the organism is

also in question. Recent studies have demonstrated that some temperature induced protein expression may occur immediately, persist for a mere 12 hours and then gradually decrease (Liu and Szaniszlo 2007). Likewise, the question of which protein shifts occur beyond the 24 hour time period, investigated in the current study, is also worth further investigation. Preliminary results from samples collected after a 48 hour growth period showed several proteins spots that were present in all variables except *Wd* Mc3 at 37°C (Figure 1.3). Interestingly, in this current study, proteins collected at 24 hours, lacked examples of protein spots in all but *Wd* Mc3 at 37°C. It is likely that the difference in protein profiles between the variables shifts over time and is that protein shift is favored at higher temperatures (37°C). Certainly, the effect of temperature and time must be considered when deciphering the protein profiles of the given variables.

Conclusion

This research used two-dimensional gel electrophoresis to created protein profiles of *Wd* 8656 and *Wd* Mc3 at 25°C and 37°C, in which specific protein spots were analyzed through mass spectrometry and peptide sequences compared against other known fungal proteins thus laying the ground work for the investigation of key proteins involved in morphogenesis of *Wangiella dermatitidis*. The up-regulation of a heat shock protein in *Wd* 8656 at 37°C, the detection of septin in only the *Wd* 8656 at 37°C variable, and the up-regulation of dehydrogenases at 37°C are substantial findings that appear to be associate with the morphological difference within the variable of this study.

Future studies should seek to replicate and expand upon these findings. Possibilities include utilizing different isoelectric ranges, varying protein concentrations,

using a median temperature between 25°C and 37°C as well as an extreme temperature above 37°C, and collecting cells at 12, 24, and 48 hours. The results generated from this study present a foundation for future research in the investigation of protein function involved in the morphological phase changes related to virulence of this potentially pathogenic fungus.

REFERENCES

Beranova-Giorgianni, S. (2003) Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: strengths and limitations. *Trends in Analytical Chemistry* 22, 273-281.

Brown, D.E. (2004) Proteomic profile of *Wangiella dermatitidis*. [MS thesis]. Youngstown State University, OH. 81 p

Brush, L., Money, N.P. (1999) Invasive hyphal growth in *Wangiella dermatitidis* is induced by stab inoculation and shows dependence upon melanin biosynthesis. *Fungal Genetics and Biology* 28, 190-200.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Journal of Biological Chemistry* 72, 248-254.

Chandler, J.M., Treece, E.R., Trenary, H.R., Brenneman, J.L., Flickner, T.J., Frommelt, J.L., Oo, Z.M., Patterson, M.M., Rundle, W.T., Valle, O.V., Kim, T.D., Walker, G.R., Cooper, C.R. (2008) Protein profiling of the dimorphic, pathogenic fungus, *Penicillium marneffei*. *Proteome Science* 6, 17.

Cooper, C.R., Szaniszlo, P.J. (1993) Evidence for two cell division cycle (CDC) genes that govern yeast bud emergence in the pathogenic fungus *Wangiella dermatitidis*. *Infection and Immunity* 61, 2069-2081.

Deacon, J. (2006) Fungal Biology, 4th edition. Blackwell Publishing, Main

De Hoog, G.S.; Matos, T., Sudhadham, M., Luijsterburg, K.F., and Haase, G. (2005) Intestinal prevalence of the neurotropic black yeast *Exophiala (Wangiella) dermatitidis* in healthy and impaired individuals. *Mycoses* 48, 142-145.

Diemert, D., Kunimoto, D., Sand, C., Rennie, R. (2001) Sputum isolation of *Wangiella dermatitidis* in patients with cystic fibrosis. *Scandinavian Journal of Infectious Diseases* 33, 777-779.

Douglas, L.M., Alvarez, F.J., McCreary, C., and Konopka, J.B. (2005) Septin Funcion in Yeast Model Systems and Pathogenic Fungi. *Eukaryotic Cell*. Sept., 15003-1512

Geis, P.A., Jacobs, C.W. (1985) Polymorphism of *Wangiella dermatitidis*, p. 205-233. In P. J. Szaniszlo (ed.), Fungal dimorphism: with emphasis on fungi pathogenic for humans. Plenum Press, New York.

Graves, P.R., Haystead, T.A.J. (2002) Molecular biologist's guide to proteomics. *Microbiology and Molecular Biology Reviews* 66, 39-63.

Gonzàlez-Novo, A., Labrador, L., Jimènez, A., Sànchez-Pèrez, M., and Jimènez, J. (2006) Role of the Septin Cdc10 in the Virulence of *Candida albicans. Microbiol. Immunol.* 50, 499-511.

Harder, A., Wildgruber, R., Nawrocki, A., Fey, S.J., Larsen, P.M., Gorg, A. (1999) Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis* 20, 826-829.

Jacobs, C.W., Szaniszlo, P.J. (1985) Altered development in a temperature-sensitive morphological mutant of *Wangiella dermatitidis*. *Mycologia* 77, 142-148.

Liu, H., and Szaniszlo, P.J. (2007) Transcription and expression analyses of *Wd*CHS5, which encodes a class V chitin synthase with a myosin motor-like domain in *Wangiella (Exophiala) dermatitidis. FEMS Microbiology Letters* 276, 99-105.

Liu, H., Kauffman, S., Becker, J.M., and Szaniszlo, P.J. (2004) *Wangiella* (*Exophiala*) *dermatitidis Wd*Chs5p, a class V chitin synthase, is essential for sustained cell growth at temperature of infection. *Eukaryotic Cell.* 3, 40-51.

McGinnis, MR. (1983) Chromoblastomycosis and phaeohyphomycosis: New concepts, diagnosis, and mycology. *Journal of the American Academy of Dermatology, St. Louis* 8, 1-16.

Peng, M., Cooper, C.R., Szaniszlo, P.J. (1995) Genetic transformation of the pathogenic fungus *Wangiella dermatitidis. Applied Microbiology Biotechnology. 44: 444-450*

Perkins, D.N., Pappin, D.J.C., Creasy, and D.M., Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20, 3551-3567.

Roberts, R.L., and Szaniszlo, P.J. (1978) Temperature-Sensitive Multicellular Mutants of *Wangiella dermatitidis*. *Journal of Bacteriology*. 135, 622-632.

Schell, W.A. (2003) Dematiaceous Hyphomycetes. In: Howard DH, ed. Fungi Pathogenic for Humans and Animals: Second Edition, Revised and Expanded, 565-636. Marcel Dekker, Inc., New York.

Schlesinger, M.J. (1990) Heat Shock Proteins. *The Journal of Biological Chemistry*. 21, 12111-12114.

Swoboda, R.K., Bertram, G., Budge, S., Gooday, G.W., Gow, N.A.R., and Brown, A.J.P. (1995) Structure and Regulation of the HSP90 Gene from the Pathogenic Fungus *Candida albicans*. *Infection and Immunity.* Nov., 4506-4512.

Szaniszlo, PJ. (2002) Molecular genetic studies of the model dematiaceous pathogen *Wangiella dermatitidis*. *Int. J. Med. Microbiol*. 292, 391-390.

Szaniszlo, PJ. (2006) Virulence factors in black molds with emphasis on melanin, chitin, and *Wangiella* as a molecularly tractable model. In J. Heitman et al (eds.), Molecular Principles of Fungal Pathogenesis. ASM Press, Washington, DC.

Ye, X., Feng, B., and Szaniszlo, P.J. (1999). A color-selectable and site-specific integrative transformation system for gene expression studies in the dematiaceous fungus *Wangiella (Exophiala) dermatitidis*. *Current Genetics* 36, 241-247.

Twyman, R.M. (2004) Principles of Proteomics. Garland Science/BIOS Scientific Publishers, New York.