Proteomic Profile of *Wangiella dermatitidis*

By

Donald E. Brown

Submitted in Partial Fulfillment of the Requirements

For the Degree of

Master of Sciences

In the

Biological Sciences

Program

Youngstown State University

December, 2004

Proteomic Profile of *Wangiella dermatitidis*

Donald E. Brown

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Donald Brown 12/3/04
Donald Brown, Student Date

Approvals:

12/3/04
Dr. Chester R. Cooper Jr., Thesis Advisor Date

Thomas D Kim 12/3/04
Dr. Thomas D. Kim, Committee member Date

Gary R. Walker 12/3/04
Dr. Gary R. Walker, Committee member Date

12/3/04
Peter J. Kasvinsky, Dean of Graduate Studies Date

Abstract

Wangiella dermatitidis is a pathogenic fungus capable of expressing three distinct modes of cellular development. These forms include yeast, multicellular, and mould phases. In particular, the latter two morphologies are induced from the yeast phase by specific environmental conditions. Temperature-sensitive mutants, designated Mc strains, have also been derived that grow as yeasts at 25°C, but develop in the multicellular phenotype when incubated at 37°C. The latter strongly resembles that tissue form of certain pathogenic fungi phylogenetically related to *W. dermatitidis*. These observations form the basis of the following hypothesis: the conversion from the yeast-like budding phase to the multicellular growth pattern is associated with the expression of specific proteins. Therefore, the present study seeks to establish a proteome map of *W. dermatitidis* for subsequent studies targeting virulence-associated proteins.

Yeast-phase cultures of *W. dermatitidis* were incubated for 24 hours at 25°C and 37°C prior to homogenization. Protein components of the homogenate were resolved by two-dimensional gel electrophoresis, then analyzed by fluorescence imaging using Sypro Ruby Stain. A proteomic profile was created for the wild-type strain 8656 at both temperatures as well as for the mutant strain Mc3. Distinct protein spots separated in the profiles were excised and are being further identified by mass spectrometry. The collective results of the present study demonstrate the potential of standard proteomic techniques for dissecting the molecular mechanisms of virulence in *W. dermatitidis*.

ACKNOWLEDGEMENTS

Dr. Chester R. Cooper Jr. – I would like to express my gratitude to you for taking the chance to allow me to work in your laboratory. Your encouragement and confidence in me pushed me to succeed. It has been an honor and a privilege to work with such an accomplished advisor.

Dr. Walker – Thank you for spending so much time as a committee member. Without your help and advice, I could not have accomplished what I did with my research.

Dr. Kim – Your help was invaluable to my success. Thank you for providing so much knowledge in the field of proteomics and for the hours you dedicated to me as a committee member.

Ron Brown – Thank you for encouraging me and for always having faith in my success. You always kept me going when I thought I couldn't make it.

Mom and Dad – Even when I doubted myself, you never thought I would do anything but succeed. Thank you for supporting me and the decisions I have made in my life. Without your love and encouragement, I would never have made it this far.

Table of Contents

ABSTRACT……………………………………………………..iii

ACKNOWLEDGEMENTS……………………………………..iv

TABLE OF CONTENTS………………………………………..v

LIST OF FIGURES……………………………………………..vi

LIST OF TABLES………………………………………………vii

CHAPTERS

I. Introduction……………………………………………...01

II. Methods and Materials………………………………..09

III. Results…………………………………………………...22

IV. Discussion……………………………………………….75

V. References……………………………………………….80

LIST OF FIGURES

Figure		Page
1.	Protein Standard Curve	28
2.	Raw image of wild-type strain 8656 at 25°C	30
3.	Filtered image of wild-type strain 8656 at 25°C	32
4.	Filtered image of wild-type strain 8656 at 37°C	34
5.	Filtered image of wild-type strain 8656 at 25°C	36
6.	Filtered composite image of wild-type strain 8656 at 25°C	42
7.	Filtered composite image of wild-type strain 8656 at 37°C	44
8.	Filtered composite image of mutant strain Mc3 at 25°C	46
9.	Filtered composite image of mutant strain Mc3 at 37°C	48
10.	Filtered composite image of wild-type strain 8656 at 25°C	50
11.	Filtered composite image of mutant strain Mc3 at 25°C	52
12.	Filtered composite image of wild-type strain 8656 at 37°C	54
13.	Filtered composite image of mutant strain Mc3 at 37°C	56
14.	Filtered composite image of wild-type strain 8656 at 25°C	58
15.	Filtered composite image of mutant strain Mc3 at 37°C	60
16.	Filtered composite image of wild-type strain 8656 at 37°C	62
17.	Filtered composite image of mutant strain Mc3 at 25°C	64
18.	Filtered composite image of wild-type strain 8656 at 25°C	68
19.	Filtered composite image of wild-type strain 8656 at 37°C	70
20.	Filtered composite image of mutant strain Mc3 at 25°C	72
21.	Filtered composite image of mutant strain Mc3 at 37°C	74

LIST OF TABLES

Table		Page
1.	Matchsets	21
2.	Bradford Assay standard concentrations and absorbances	24
3.	Fungal Protein Concentrations	26
4.	Higher order matchsets	39

Chapter I

Introduction

Cellular Development of *Wangiella dermatitidis*

Wangiella dermatitidis is a darkly pigmented fungus that exhibits yeast-like budding. It also displays other polymorphic patterns of vegetative growth. The dark color is due to melanin depositions in the cell wall of this pathogenic fungus (Szaniszlo, 2002).

There are three well-characterized morphologies that constitute the polymorphic nature of *W. dermatitidis*. The first is composed of moniliform or true hyphae. The second form exists as unicellular budding yeasts. The third is a multicellular form, in which cells are thick-walled and enlarged, with internal septa (Geis and Jacobs, 1985). The latter resembles the sclerotic bodies of chromoblastomycosis and similar forms associated with phaeohyphomycosis (Ye *et al*, 1999).

The hyphal growth morphology displays apical extension and budding growth that yields blastoconidia. Hyphae possess thin-layered walls and fairly invariable diameter. Septa originate in the inner layers of the cell wall. Lateral hyphal buds also derive from the inner layers of the cell wall by blastic growth. Conidia come from two places: the apices of undifferentiated conidiophores on hyphal branches and directly from hyphal walls (Geis and Jacobs, 1985).

The yeast morphology begins with cell-wall synthesis in a directional, site-specific fashion. This is followed by isotropic growth causing an increase in cell volume. Cells are ovoid in shape and bud at the poles. Both yeast cells and buds contain a single nucleus. Bud cells separate from the mother cells following nuclear division. The population is then composed of unicellular yeasts (Geis and Jacobs, 1985).

Yeast cells have thin cell walls of which the outer surface is a thin fibrillar capsule. Glycogen deposits are located interior to the plasma membrane. The cytoplasm contains ribosomes and mitochondria, as well as other vesicles that may or may not be paired with a Golgi apparatus. The nucleus and nucleolus are fairly conspicuous, as are sizeable, electron-dense bodies often seen in the cytoplasm (Geis and Jacobs, 1985).

Only one bud is formed each cell cycle during yeast cell reproduction. As budding proceeds, the cell wall is contiguous between the mother cell and the bud. Once the bud has reached approximately two thirds the size of the mother cell, the nucleus travels to the region between the mother cell and the bud. Mitosis follows, after which the daughter nuclei migrate to the respective bud and mother cell. A septum forms across the neck between the mother and daughter cells, and the two then disassociate (Geis and Jacobs, 1985).

The wild type of *W. dermatitidis*, designated strain 8656, grows as a budding yeast at both the permissive temperature of 25°C and the restrictive temperature of 37°C (Cooper and Szaniszlo, 1993). The process of bud formation is identical whether it originates in a yeast cell or a hyphal element (Grove, 1973). Blastoconidia of hyphae have an identical structure to yeast cells. Budding yeasts result when any of these cell types are inoculated into fresh media (Geis and Jacobs, 1985).

The multicellular form occurs as a result of infection in humans (McGinnis, 1983). The wild type can be induced to grow multicellularly under acidic conditions and long-term growth on solid medium (Szaniszlo *et al*, 1976, Roberts and Szaniszlo, 1978).

This is consistent with studies showing that stresses that limit growth transform the cells into a transversely septated form (Grove, 1973).

Methods have been developed to generate and analyze mutants of *W. dermatitidis*. By taking advantage of spheroplast fusion, certain temperature-sensitive mutants of *W. dermatitidis* were shown to be deficient in cell division processes. These temperature-sensitive mutants of *W. dermatitidis* display yeast-like budding at 25°C similar to the wild type, but convert to multicellular growth at 37°C. Analysis of a mutant strain designated Mc3 showed that it possesses a defective cell division cycle gene. Mutant strain Mc3 is able to complete cytokinesis and finish the mitotic cell cycle using alternative pathways, leading to septated, multinucleated cells (Cooper and Szaniszlo, 1993).

The multicellular morphology has two separate stages of growth. In the first stage, cell walls grow isotropically without budding or displaying apical growth. The cell walls thicken in comparison to yeast-phase cells. The cell increases in size and develops multiple nuclei in acid-induced cells of the wild-type and in temperature-sensitive mutant strain Mc3 cells grown at 37°C. In stage II, growth continues and cell walls become increasingly thick and hyper-pigmented. Transverse septa develop into two types. Simple septa with septal pores and pore plugs form, as well as complete septa composed of several layers. Complete septa display no cytoplasmic continuity between the cells they separate and appear to derive from simple septa that mature. In stage II, the mutant strain Mc3 grown at 37°C differs from the acid-induced wild-type cells only in that they contain smaller lipid deposits and almost no glycogen (Geis and Jacobs, 1985).

Several methods exist for the induction of stage I of the multicellular morphology. The wild type can be induced by incubation on solid medium for 2-4 weeks. It can also be induced in a few days by growing it in acidic medium. The mutant strain Mc3 can be induced to stage I by growing it at 37°C. This last method is rapid, occurring after one cell cycle (Geis and Jacobs, 1985).

The wild type develops thicker cell walls after 4-7 days of incubation on solid medium or 48 hours after inoculation in acidic medium. Glycogen storage areas enlarge and sizeable lipid deposits appear in the cytoplasm. The cells also contain refractile inclusions, as shown by phase-contrast microscopy. Cell walls become extremely thick after 3-4 weeks on solid medium and 3-3 days in acidic broth. Several layers are apparent through electron microscopy. Aged cells differ from acid-grown cells by a lack of glycogen storage areas. In both types, lipid bodies inhabit much of the cytoplasm (Geis and Jacobs, 1985).

Cells of the mutant strain Mc3 display the minor increase in cell wall thickness characteristic of early stage I after approximately 6-12 hours of growth at 37°C. The cytoplasm contains the same organelles that are present in cells of the wild type that have been induced to stage I. Lipid bodies are smaller in comparison to those of the induced forms of the wild-type. Glycogen storage areas do not appear to be present. Within 24-72 hours, cells of the mutant strain Mc3 have developed the very thick, layered cell walls that were seen in late stage I cells of the wild type. Throughout stage I, mutant strain Mc3 cells remain consistent in having smaller lipid bodies and no glycogen storage areas (Geis and Jacobs, 1985).

Cells of the wild-type strain 8656 and mutant strain Mc3 strain enter stage II if the conditions that led to stage I are maintained. Stage II cells develop transverse septa. It may be blocked in mutant strain Mc3 cells when they are inoculated at 10^7 cells/mL or higher. Stage II development is minimal in wild type cells under long-term incubation conditions. Stage II development may therefore depend on the availability of certain nutrients (Geis and Jacobs, 1985).

Stage I cells of the wild type grown on solid medium convert back to the yeast growth pattern when inoculated into fresh medium. Likewise, stage I cells of the mutant strain Mc3 created by growth at 37°C revert to the yeast morphology when they are exposed to a 25°C environment (Geis and Jacobs, 1985, Roberts and Szaniszlo, 1978).

The generation of hyphae is not so simple. Hyphae cannot be derived from the yeast morphology. They develop when stage I cells grown on solid medium for 3 weeks are transferred to fresh medium. Hyphae are also generated when stage II cells are transferred to fresh medium. There is an increase in both ribosomes and mitochondria preceding the growth of hyphae. Moniliform transition cells develop. These cells have thin walls generated by the inner layer of the thick wall of stage I or stage II cells. They have simple septa with pores, Woronin bodies, and pore plugs. True hyphae grow at the end of the transition cells. They are uniform in diameter and the septal region has no constrictions (Geis and Jacobs, 1985).

Analysis of Morphogenesis

The simplicity of growth pattern conversion in *W. dermatitidis* provides a useful model for the study of this and other pathogenic fungi. The multicellular growth pattern greatly resembles the sclerotic body typical of chromoblastomycosis (Geis and Jacobs, 1985). Analysis of the multicellular growth pattern and the proteins associated with it might therefore lead to a better understanding of the pathogenicity of *W. dermatitidis*.

Proteomic analysis is a method used to study proteins with regard to the identification and quantification of specific proteins. Literally thousands of proteins can be separated and analyzed in this fashion. The three main steps in proteomic analysis include separation of proteins, isolation of the protein of interest, and sequencing. Proteomics is extremely useful in comparison studies in which normal subjects may be compared to test subjects (Beranova-Giorgianni, 2003).

Proteomics differs from protein chemistry in a few ways. For one, conglomerate mixtures of proteins are studied, as opposed to the individual proteins that are the subject of protein chemists. Partial sequences are generally enough to compare in a database, rather than complete sequence analysis. The focus of protein chemistry is structural biology, and the function of a single entity. By comparison, proteomics looks instead at the interaction of multiple proteins in a system (Liebler, 2002).

Proteomics follows a very general protocol. First cells must be lysed. Proteins must then be solubilized. Following solubilization is the separation of proteins by two-dimensional (2D) gel electrophoresis. Digital images of 2D gels are acquired in order to analyze them with the help of computer software. The features of particular proteins may be revealed by mass spectrometry. These characteristics are then used to search databases in an effort to identify the protein and its function (Beranova-Giorgianni, 2003).

Two-dimensional gel electrophoresis is very effective in resolving complex protein mixtures. The method takes advantage of two distinct protein qualities. In the first dimension, proteins are separated by their charge in a process called isoelectric focusing. Separation in the second dimension is done by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The second dimension separation through a polyacrylamide gel relies on the molecular weight of proteins (Beranova-Giorgianni, 2003).

The development of commercial immobilized pH gradient gels greatly enhanced success and reproducibility in isoelectric focusing. As a result, 2D gels became much easier to recreate. Once 2D gel electrophoresis has been carried out, proteins are delineated by stains such as Coomassie Blue stain or silver stain. 2D gels are digitized so that protein spot patterns can be characterized using computer software. Multiple protein maps may be analyzed simultaneously in order to compare consistencies and differences between samples (Beranova-Giorgianni, 2003).

Proteins isolated by 2D gel electrophoresis can be analyzed by mass spectrometry to obtain their identification (Beranova-Giorgianni, 2003). Proteins are digested with proteolytic enzymes such as trypsin because mass spectrometers are unable to analyze complete proteins (Liebler, 2002). Peptide sequences are generated by MS analysis (Beranova-Giorgianni, 2003). A peptide sequence 6 amino acids in length is enough to be unique in an organism's proteome (Liebler, 2002). The peptide mass fingerprint is then used to search databases (Beranova-Giorgianni, 2003).

Proteomic methods will be used to compare the proteins of the temperature-sensitive mutant strain Mc3 and the wild-type strain 8656. A proteomic profile will be constructed for the wild type and the mutant strain Mc3. The profiles will be used to help test the following hypothesis: *the conversion from yeast-like budding to the multicellular growth pattern is associated with the expression of specific proteins.* The proteomic profiles will be used to identify proteins that dictate multicellular growth. These proteins can then be isolated and sequenced.

Goals of Proposed Study

First, the proteomic profile of the wild-type strain 8656 grown at both 25°C and 37°C will be created. This will set up a control because the wild type grows as a budding yeast at both temperatures. Any unique proteins revealed in the proteome are clearly not involved in the multicellular morphology. Next, the proteomic profile of the mutant strain Mc3 will be constructed. The permissive temperature of 25°C and the restrictive temperature of 37°C will be compared. The restrictive temperature is of particular interest in identifying proteins responsible for multicellular growth. Any unique proteins of the mutant strain Mc3 at 37°C not matching in the profile of the wild type may be a candidate associated with multicellular development. Proteins over-expressed at the restrictive temperature may be of interest as well. Proteins under-expressed in the wild type or the permissive temperature of the mutant strain may also reveal some information about the multicellular growth process. Finally, such proteins will be excised and sequenced by mass spectrometry. Ultimately, proteins involved in multicellular growth may reveal agents involved in the infection process of *W. dermatitidis* and related fungi.

Chapter II

Methods and Materials

Fungal Strains and Media:

Cultures of *Wangiella dermatitidis* strains 8656 and Mc3 were maintained on YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 5% agar) slants and in YPD broth (1% yeast extract, 2% peptone, and 2% glucose) (Szaniszlo *et al*, 1999). Broth cultures were incubated at 25°C under shaking conditions and transferred every 48 hours. The inoculum for all experiments was derived from cultures inoculated at an initial concentration of 10^6 cells/mL and grown for 24 hours (Roberts and Szaniszlo, 1978). Cell numbers were determined using a hemocytometer. Experimental cultures were grown in YPD broth and incubated at 25°C and 37°C with shaking at 130 rpm for 24 hours. The YPD medium was pre-warmed to the proper temperature before inoculation. Individual cell lines for each condition were grown in triplicate.

Hemocytometer Count:

Cells to be used as inoculum were diluted 1:100 and 1:1000 in sterile water. For each dilution, 15 microliters was inserted under the cover slip of the hemocytometer slide cover on both the top and bottom grid sections. The top grid was then focused at a magnification of 400 X using phase contrast.

Each grid section was composed of a series of 4 individual grids separated by markings. Each of these individual grids contained 16 squares, from which cell numbers were counted. Once the cell numbers were counted for each of the 4 grids, they were averaged by adding them up and dividing by 4. This process was repeated for the bottom grid. The averages for the top grid section and the bottom grid section were then averaged together. This number was then multiplied by 10^4 (the dilution factor for the hemocytometer slide) and the dilution factor of the original cell dilution (10^3 for the 1:1000 dilution or 10^2 for the 1:100 dilution). The resulting number described the number of cells per mL in the 24 hour cell culture and was used to determine what volume of the original culture was to be used to inoculate another 24 hour culture at 10^6 cells per mL.

Protein Isolation Protocol:

In general, cell lysis was performed previously described methods (Harder *et al*, 1999). Fungal cells were disrupted by sonication using an Artek Sonic Dismembrator, Model 300 from Dynatech Laboratories. Proteins were isolated from the cellular debris using TRI Reagent (Molecular Research Center). Protein concentration was determined by performing a Bradford Protein Assay (Bradford, 1976). A detailed procedure is provided below.

A 25 mL suspension of yeast cells was aseptically transferred to a sterile, screw-capped, pre-chilled 50 mL conical tube (Fisherbrand polypropylene [Cat. No. 05-539-6] or similar tube). The tube(s) were placed in the yellow tube holder in the cup rotor (Model GH 3.7) of the Beckman GPR centrifuge. The cells were collected by operating the centrifuge at 3000 x g for 5 minutes at 4°C. The supernatant was discarded into disinfectant (10% [v/v] bleach). Care was taken to avoid disturbing the cell pellet at the bottom of the tube.

The cells were washed by aseptically adding 1 mL of pre-chilled, sterile TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.0) to the tube and suspending the cell pellet using a vortex set at the highest operating speed. 250 μL aliquots of the cell suspension were removed and placed in pre-chilled, sterile 1.5 mL microcentrifuge tubes. The cells were collected into a pellet operating a microcentrifuge at 7500 x g for 1 minute at 4°C. The supernatant was removed using a micropipette and discarded into disinfectant, again taking care not to disturb the cell pellet. At this point the cell pellets were able to be stored for at least a week by placing the microcentrifuge tubes at -20°C. Before continuing, frozen cell pellets were thawed on ice.

500 μL of TRI Reagent (Molecular Research Center, Cat. No. TR 118; or Sigma, Cat. No. T-9424) were added to the microcentrifuge tube and the cell pellet was suspended using a vortex set at the highest operating speed. The cell suspension was placed on ice until performing the next step in the protocol.

The microprobe tip of the sonicator (Artek Sonic Dismembrator, Model 300; Dynatech Laboratories) was placed midway into the cell suspension so that there was no contact with the sides of the microcentrifuge tube. The tube was supported in a small ice bath while the microprobe tip was immersed in the cell suspension. The sonicator was operated at 57 watts for 30 seconds. The microcentrifuge tube was removed and placed on ice for at least 1 minute. Before using the sonicator, it was necessary to clean and disinfect the microprobe with 70% [v/v] ethanol. Similarly, the microprobe had to be cleaned and disinfected between multiple samples of different types and also when finished. The sonication step was repeated two more times for each sample being processed. The samples were place at room temperature for 5 minutes.

100 μL of chloroform were added to the sonicated cell suspension. The contents of the microcentrifuge tube were mixed using a vortex set at the highest operating speed. The samples were placed at room temperature for 15 minutes. The cell suspension/chloroform mixture was separated by centrifugation at 12,000 x g for 15 minutes at 4°C. This centrifugation separated the mixture into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. In this procedure, DNA and proteins are found in the interphase and organic phase.

The upper aqueous phase was removed using a micropipette, taking care not to disturb the interphase and lower organic phase. The aqueous phase was discarded in the organic waste container. The microcentrifuge tubes containing the phenol phase and interphase can be stored overnight at 4°C.

150 μL of 100% ethanol were added to the microcentrifuge tube, and the interphase and organic phase were mixed by inverting the tube several times by hand. The mixed sample was placed at room temperature for 2-3 minutes. The DNA was then sedimented by centrifugation at 2000 x g for 5 minutes at 4°C. The supernatant was carefully removed using a micropipette and placed in a new microcentrifuge tube. The microcentrifuge tube containing the DNA pellet was discarded in the plastics/organic waste container.

750 μL of isopropanol was added to the collected supernatant and mixed by inverting the tube several times by hand. The mixture was allowed to stand at room temperature for at least 10 minutes. The protein was collected by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was carefully removed using a

micropipette and discarded into the organic waste container, being careful not to disturb the protein pellet.

1 mL of protein wash buffer (0.3 M guanidine hydrochloride in 95% ethanol) was pipetted into the microcentrifuge tube containing the protein pellet. The protein pellet was suspended in the protein wash buffer using a vortex set at the highest operating speed. The sample was allowed to remain at room temperature for 20 minutes. The protein was collected as a pellet by centrifugation of the microcentrifuge tube(s) at 7,500 x g for 5 minutes at 4°C. The supernatant was carefully removed using a micropipette and discarded into the organic waste container. The protein pellet was subjected to two additional washes in this fashion.

Once the supernatant was removed after the final wash, protein pellets were combined in one microcentrifuge tube if multiple aliquots were processed. 1 mL of 100% ethanol was added to the protein pellet which was then shaken for 15 seconds using a vortex set at the highest operating speed. Subsequently, the sample was incubated at room temperature for 20 minutes. The protein from this suspension was collected in a pellet by centrifugation at 7,500 x g for 5 minutes at 4°C. The ethanol supernatant was carefully removed without disturbing the protein pellet and discarded into the organic waste container. The protein pellet was allowed to dry for 1 hour at room temperature. 1 mL of active rehydration buffer (8-9.8 M Urea, 1-4% CHAPS, 15-100 mM dithiothreitol, 0-0.2% (w/v) Biolytes, 0.001% bromophenol blue) was transferred to the microcentrifuge tube. A sonicator was used to disrupt and dissolve the pellet.

Modified Bradford Assay:

A modified Bradford Assay was performed to determine the concentration of the protein extracted from the fungal cells (Bradford, 1976). Standards for the assay were prepared using bovine serum albumin (BSA) in microgram amounts of 10, 15, 20, 25, 30, 35, and 40. The standards were prepared by adding the appropriate amount of BSA, 10 microliters of 2-dimensional electrophoresis (2DE) Buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 25 mM spermine base, 50 mM dithiothreitol), 10 microliters of 0.1 M hydrochloric acid, 80 microliters of distilled/deionized water, and 4 mL of Bradford Dye (50 mL 95% ethanol, 100 mL 85% (w/v) phosphoric acid, 100 mg Coomassie Brilliant Blue G-250, dilute to 1 L) (Bradford, 1976). A blank was prepared by mixing 10 microliters of 0.1 M hydrochloric acid, 10 microliters of 2DE buffer, 80 microliters of distilled/deionized water, and 4 mL of Bradford Dye. The unknown samples were prepared by adding 10 microliters of the fungal protein solution in active rehydration buffer, 10 microliters of 2DE buffer, 10 microliters of 0.1 M hydrochloric acid, 80 microliters of distilled/deionized water, and 4 mL of Bradford Dye.

In all cases, the hydrochloric acid, protein, and 2DE buffer were added before the other reagents. Prior to mixing the standards and unknowns, the red lamp in the spectrophotometer (Hewlett-Packard 8453 UV-Visible System) was turned on and warmed up for at least 15 minutes. The wavelength was set to 595 nanometers. Once the spectrophotometer was blanked, the absorbances of the standards and unknowns were recorded using the UV-Visible Chemstation Software Rev. A.06.04 [48].

The values of the absorbances and protein amounts of the standards were plotted on a linear graph. The protein amounts of the unknowns were extrapolated from the equation derived from the standards using the recorded absorbances. The concentrations of the unknowns were deduced by dividing by 10 microliters, for the amount originally added.

Protein Separation by 2-Dimensional Gel Electrophoresis:

To summarize, proteins were separated in the first dimension by isoelectric focusing on a Bio-Rad Protean IEF System (Bio-Rad Cat. No. 165-4000). Equal amounts of protein were loaded and actively rehydrated on 7 cm and 17 cm immobilized pH gradient strips with a pH range of 3-10 as well as on both 7 cm and 17 cm immobilized pH gradient strips with a pH range of 4-7. Separation in the second dimension was accomplished by electrophoresis on a 17 cm 10% polyacrylamide gel with a Bio-Rad Protean II XL Electrophoresis Cell. Gels were stained with Sypro Ruby Red fluorescent stain. Gels were analyzed and compared using the Bio-Rad PD Quest 2-D Image Analysis Software. Details of each of these procedures are given below.

Isoelectric Focusing:

100 micrograms of protein were loaded into the appropriate volume of active rehydration buffer in the focusing tray. A total volume of 125 microliters was added for the 7 cm immobilized pH gradient (IPG) strip and 300 microliters for the 17 cm IPG strip. Care was taken to avoid introducing bubbles in the channel. The plastic backing of the IPG strips was removed using forceps and placed gel side down on the sample. The acidic (marked with a "+") end of the strip was placed at the anode (red/+) of the IEF

cell. The strips were then over-lain with 2-3 mL of mineral oil. The focusing tray was placed in the IEF cell.

Conditions for active rehydration were as follows: 50 V, 20°C, 12-16 hours. Focusing conditions were dependent on strip size, but did not vary with IPG strip pH ranges of 3-10 or 4-7. The ending voltage was 4000 V for the 7 cm IPG strips and 6000 V for the 17 cm IPG strips. The volt-hours were 20,000 for the 7 cm IPG strips and 60,000 for the 17 cm IPG strips. In all cases the temperature was kept at 20°C. Once isoelectric focusing was complete, the IPG strips were kept at a holding voltage of 500 V until the focusing tray was removed from the IEF cell.

SDS-Polyacrylamide Gel Electrophoresis:

The IPG strips were equilibrated in a buffer containing SDS. The mineral oil was removed from the strips by placing them gel side up on a piece of dry filter paper and blotting them with a second piece of wet filter paper. Equilibration buffer I (6 M urea, 0.375 M Tris, pH 8.8, 2% sodium dodecyl sulfide (SDS), 20% glycerol, 2% (w/v) dithiothreitol) was added to an equilibration/rehydration tray, using one channel per IPG strip. 6 mL of the buffer were added for the 17 cm IPG strip and 2.5 mL were added for the 7 cm IPG strip. The IPG strips were then placed gel side down in the channels with Equilibration buffer I. The tray was placed on an orbital shaker and shaken gently for 10 minutes.

At the end of the 10 minute shaking period, the used equilibration buffer was discarded. The proper volume of Equilibration buffer II (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) was added to the channels of the equilibration/rehydration tray and the IPG strips were placed gel side down in the buffer. The tray was returned to the orbital shaker and shaken gently for 10 minutes. At the end of the 10-minute shaking period, Equilibration buffer II was discarded.

The IPG strips were removed from the rehydration/equilibration tray and dipped briefly into a graduated cylinder containing 1X Tris-glycine-SDS (TGS) running buffer (25 mM Tris Base, 250 mM glycine, 0.1% SDS). The IPG strips were placed above the IPG well on the back plate of a 10% polyacrylamide gel with the gel side facing out. Overlay agarose solution (0.5 % (w/v) low melting agarose, 1X TGS, trace bromophenol blue) was placed into the IPG well of the gel using a disposable plastic transfer pipette. The IPG strip was carefully pushed into the well with forceps, being careful not to introduce air bubbles underneath the strip. Care was taken to ensure that the forceps were pushing the plastic backing of the strip and not the gel matrix. The polyacrylamide gel was stood up straight and the agarose allowed to solidify for 5 minutes.

The gels were placed in the Bio-Rad Protean II XL Electrophoresis Cell and 1X TGS running buffer was added to the inner and outer reservoirs. The gels were run with constant current, depending on the size of the gels. For 7 cm gels, the power source was set at 16 mA. For 17 cm gels, the power source was set at 24 mA. The progress of the gel run was monitored by observing the migration of the bromophenol blue present in the overlay agarose solution.

The gels were removed from the glass plates encasing them. The IPG strips were discarded and the positive side of the gel was marked by slicing off a corner of the gel. Gels were placed in containers and an adequate amount of fixing solution was added to cover them. The containers were placed on an orbital shaker and shaken gently for 1 hour.

The fixing solution was discarded in the appropriate waste container at the end of the shaking period. A sufficient quantitiy of 1X Sypro Ruby red stain (excitation peaks at 280 nm and 450 nm, emission maxima at 610 nm) was added to the gels to cover them and the containers were placed back on the orbital shaker. The gels were shaken gently overnight. At the end of the shaking period, the stain was placed in the appropriate waste container.

Destain solution was added to the gels. The containers were placed on the orbital shaker and shaken gently for 2 hours. At the end of the shaking period, the destain solution was removed and placed in the appropriate waste container. Distilled/deionized water was added to the gels. They were allowed to rinse for at least 15 minutes before viewing. The gels were also stored in distilled/deionized water.

Imaging and Matching:

Digital images of the gels were acquired using the Bio-Rad Gel Chemidoc™ XRS Gel Documentation System. The gels were visualized using ultraviolet transillumination. The digital images were saved to a computer for analysis. Gels were analyzed using the Bio-Rad PD Quest 2-D Image Analysis Software.

Once the raw gel images were saved, they were cropped to an area just containing the protein spots. The same crop settings were used for multiple gels. A major common spot was used to line up the crop area on all gels processed. The cropped gels were then used to create a matchset.

Matchsets were created for each growth temperature for the wild type strain 8656 and for each growth temperature for the mutant strain Mc3. Each matchset consisted of 3 cropped gels derived from proteins extracted from individual cell lines. The Bio-Rad PD Quest 2-D Image Analysis Software was used to match spots through an automated detection and matching process. Some manual matching was necessary due to oversights and mismatches in the automated process.

When the matchset was created, a master gel was selected from one of the 3 cropped gels in the matchset. The master was representative of the 3 gels in the matchset. The masters were filtered for speckling and streaking. Higher-order matchsets were created from these masters in order to compare different conditions. Each condition was compared in turn to the others, for a total of 10 matchsets. Table 1 lists the components of the primary and the higher order matchsets. 4 of the matchsets were primary. 6 of the matchsets were higher order.

Once the matchsets were created and the spots were matched, differences were analyzed between different conditions. The software allowed different overlays to be added to the gel images in the matchsets. There were overlays available to mark matched spots, mismatched spots, landmark spots, and manual matches. Spots that were analyzed were annotated with brief text descriptions that could be accessed through the software.

Matchsets

Primary Matchsets	**Higher order Matchsets**
	WD 8656 25° and WD Mc3 25°
WD 8656 25°	**WD 8656 25° and WD Mc3 37°**
WD 8656 37°	**WD Mc3 25° and WD Mc3 37°**
WD Mc3 25°	**WD Mc3 25° and WD 8656 37°**
WD Mc3 37°	**WD 8656 37° and WD Mc3 37°**
	WD 8656 25° and WD 8656 37°

Chapter III

Results

Preliminary results reveal that the wild type expresses different proteins at 25°C and 37°C. Each spot on the gel represents a specific protein separated both by its isoelectric point and by its size. Expression levels of specific proteins differed between the permissive and restrictive temperatures, as shown by differences in spot size and intensity.

Table 2 shows typical absorbance readings from BSA standards in a Modified Bradford Assay. Table 3 displays typical absorbance readings for fungal protein samples along with concentrations derived from the standard curve. Figure 1 depicts a standard curve of absorbance versus protein concentration in micrograms for a Bradford Assay.

Figure 2 is a raw unfiltered digital image of a 17 cm 2DE gel of the wild-type strain 8656 grown at 25°C. Isoelectric focusing was done with IPG strips with a pH range of 3-10. Some vertical and horizontal streaking can be seen in the gel. Some speckling is also visible. The large vertical streak on the right side marks where the immobilized pH gradient ended on the strip from the isoelectric focusing. It is composed of excess sample that was not separated by isoelectric point.

Figure 3 is a filtered image of the17 cm 2DE gel of the wild-type strain 8656 grown at 25°C in Figure 1. Each white spot represents a different protein. The red circles indicate proteins unique to the 25°C sample as compared to the 37°C sample in Figure 4. Preliminary automated analysis reveals 242 proteins unique to cultures incubated at 25°C for 24 hours.

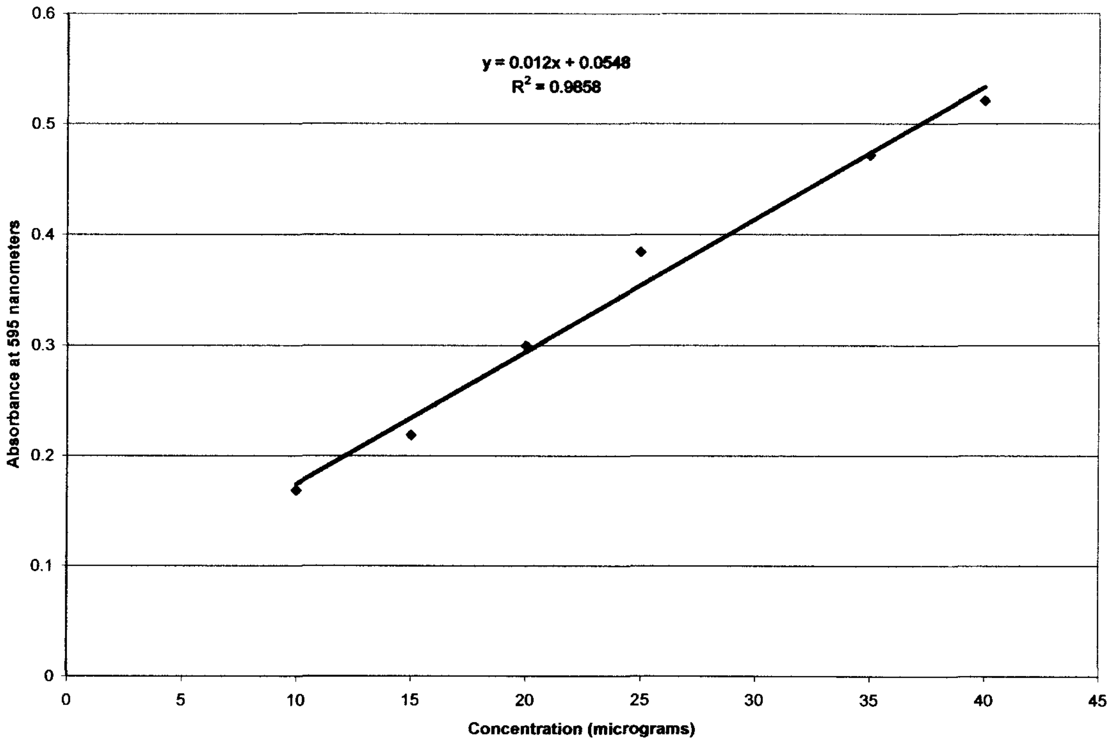
Bradford Assay Standard Concentrations and Absorbances

Standard Concentration (micrograms)	Absorbance
10	0.16879
15	0.2189
20	0.29979
25	0.38423
35	0.47153
40	0.52081

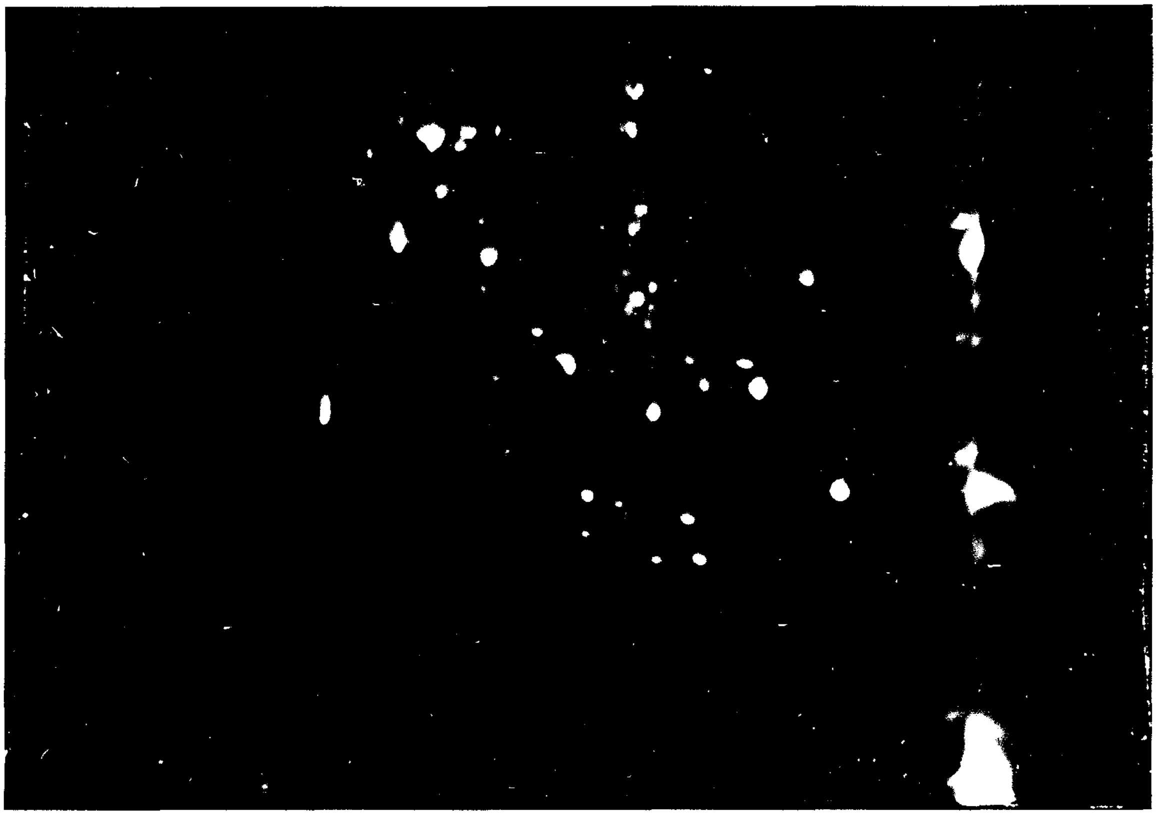
Fungal Protein Concentrations

Unknown Sample	Absorbance	Concentration (micrograms/microliter)
mutant strain Mc3 H 25	0.231	1.47
mutant strain Mc3 H 37	0.26111	1.72
mutant strain Mc3 I 25	0.21039	1.30
mutant strain Mc3 I 37	0.25206	1.64
mutant strain Mc3 J 25	0.30299	2.07
mutant strain Mc3 J 37	0.32487	2.25

Protein Standard Curve



Raw Image of Wild-type Strain 8656 at 25°C



Filtered Image of Wild-type Strain 8656 at 25°C

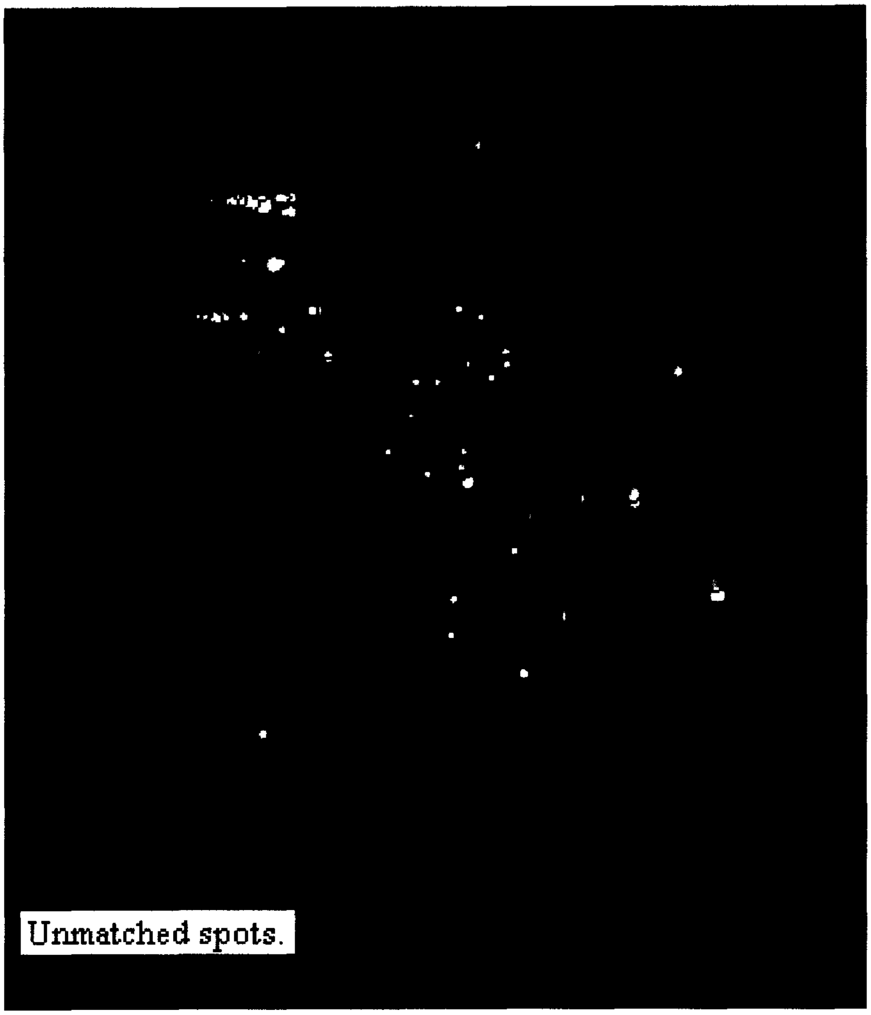

Unmatched spots.

Figure 4: Filtered Image of Wild-type Strain 8656 37°C

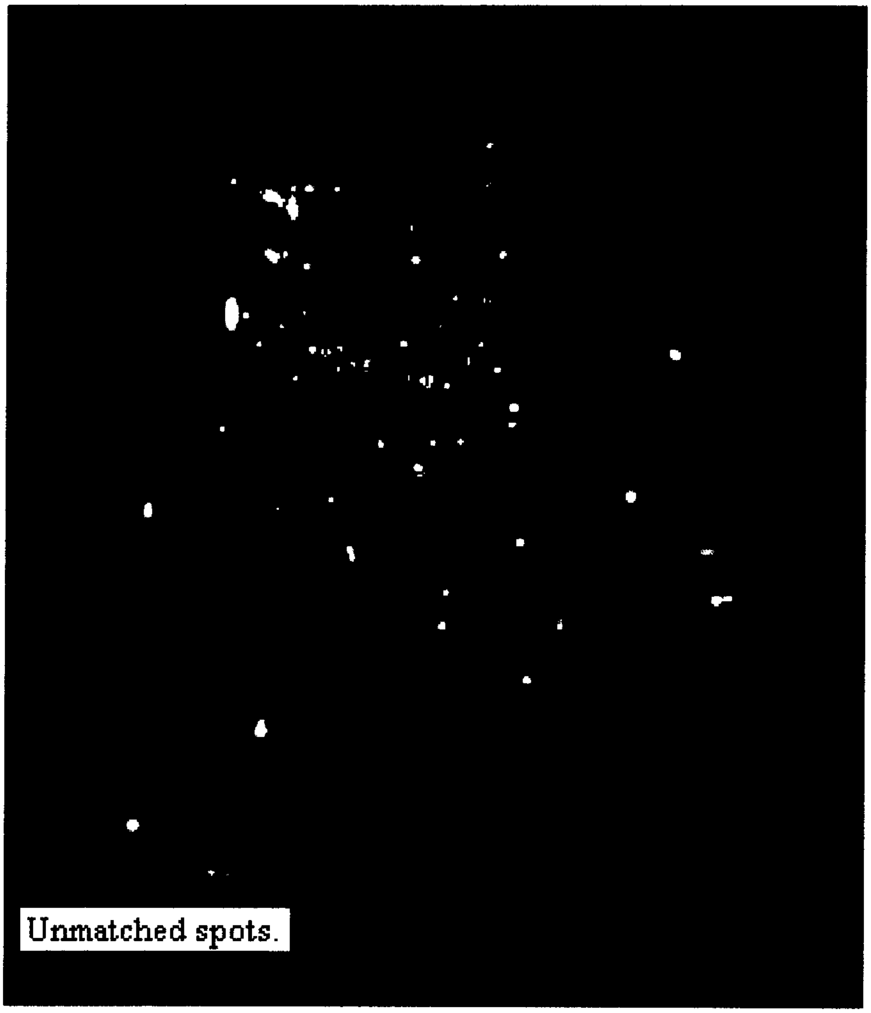

Unmatched spots.

Figure 5: Digital Overlay of the 2DE Gels of the Wild-type strain 8656 Grown at 25°C and 37°C

Figure 4 is a filtered 17 cm 2DE gel of the wild-type strain 8656 grown at 37°C, under the same conditions as those used for the 25°C sample. Both Figures 3 and 4 represent samples separated in the first dimension using 17 cm IPG strips with a pH range of 3-10. There are 112 unique proteins detected by automated matching in the 37°C sample. There were 106 proteins that were automatically matched in both growth temperatures.

Figure 5 is a view of both gels overlaid one on top of the other. Proteins from the 25°C sample are shown in green, whereas proteins from the 37°C sample are shown in red. The work done to obtain the results for Figures 3, 4, and 5 was not replicated.

A second series of proteomic profiles was created using a pH range of 4-7. Subsequent separation in the second dimension was performed as for previously described experiments (see above). For figures 6 through 17, isoelectric focusing was performed with IPG strips manufactured to a pH range of 4-7. Each dark spot represents a different protein. The red circles indicate proteins unique to that condition in the matchset.

Table 4 summarizes the 6 higher-order matchsets that were created and analyzed from this protein profiling series. It displays the matched proteins for each matchset, as well as the proteins unique to each strain and temperature of each of the matchsets. The wild-type strain 8656 at 25°C, the wild-type strain 8656 at 37°C, the mutant strain Mc3 at 25°C, and the mutant strain Mc3 at 37°C were the conditions used to create the matchsets.

Higher Order Matchsets

Matchset	Matched Proteins	Unique Proteins to wild-type strain 8656 at 25°C	Unique Proteins to wild-type strain 8656 at 37°C	Unique Proteins to mutant strain Mc3 at 25°C	Unique Proteins to mutant strain Mc3 at 37°C
8656 25°C to 8656 37°C	335	118	126	N/A	N/A
Mc3 25°C to Mc3 37°C	321	N/A	N/A	111	158
8656 25°C to Mc3 37°C	276	177	N/A	N/A	203
Mc3 25°C to 8656 37°C	215	N/A	246	217	N/A
8656 25°C to Mc3 25°C	219	234	N/A	213	N/A
Mc3 37°C to 8656 37°C	271	N/A	190	N/A	208

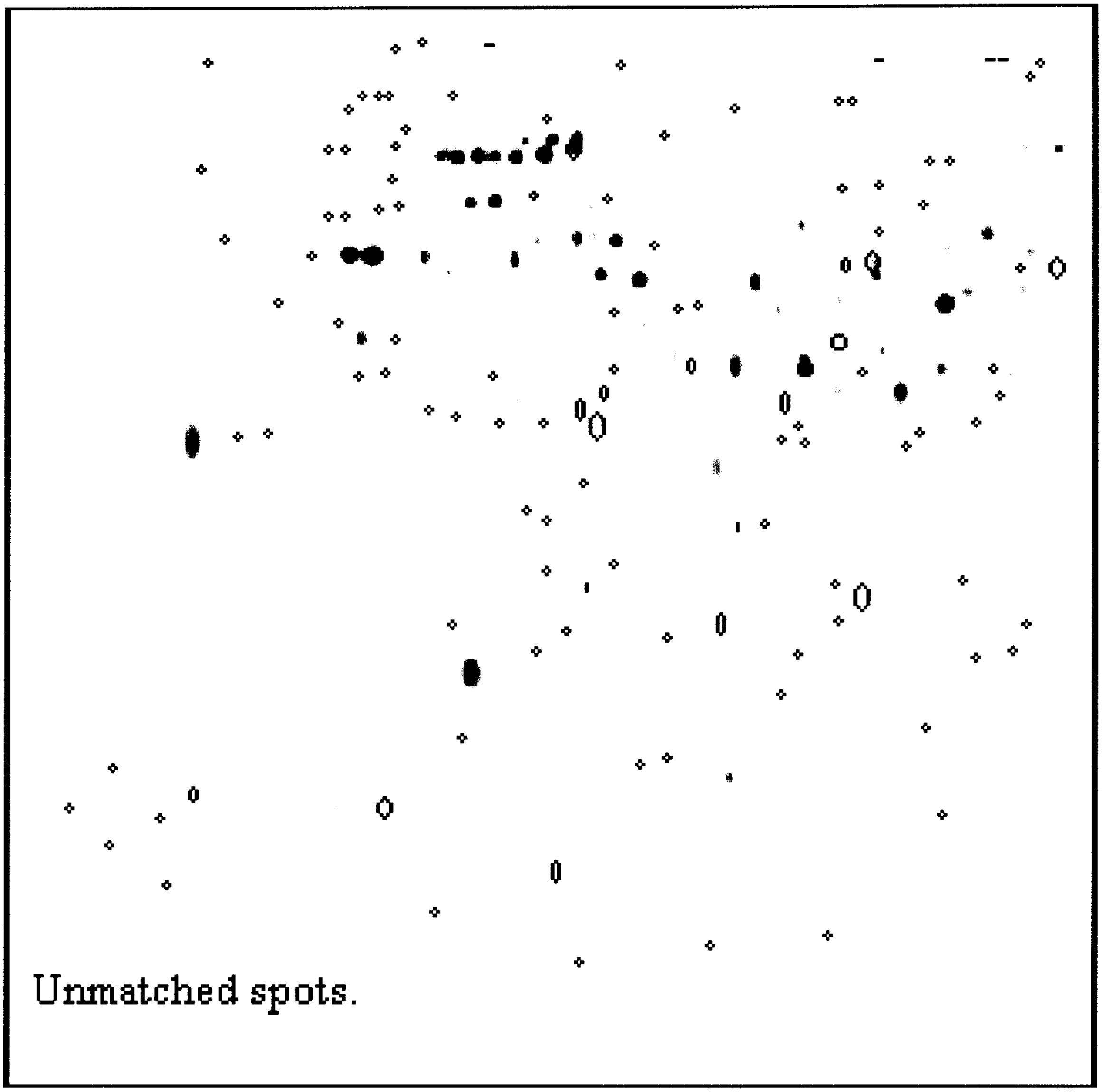
Figure 6 is a composite of three 17 cm 2DE gels of wild-type strain 8656 grown at 25°C. 118 unique proteins are present in the 25°C sample. Figure 7 is a composite of three 17 cm 2DE gels of wild-type strain 8656 grown at 37°C. There are 126 unique proteins to the 37°C sample. There are 335 proteins that are matched in both growth temperatures.

Figure 8 is a composite of three 17 cm 2DE gels of the mutant strain Mc3 grown at 25°C. 111 unique proteins are present in the 25°C sample. Figure 9 is a composite of three 17 cm 2DE gels of the mutant strain Mc3 grown at 37°C. There are 158 unique proteins to the 37°C sample. There are 321 proteins that are matched in both growth temperatures.

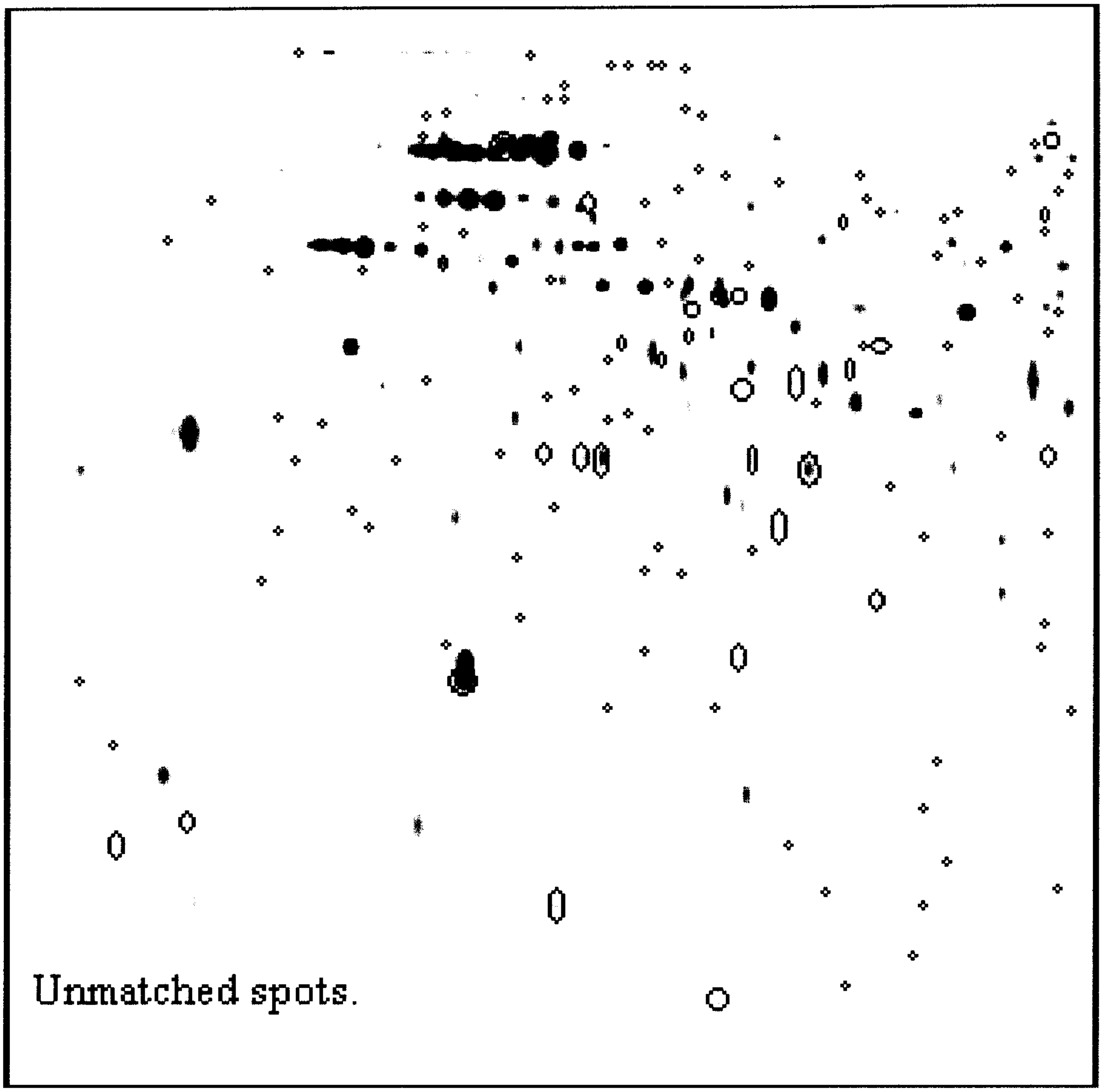
Figure 10 is a composite of three 17 cm 2DE gels of wild-type strain 8656 grown at 25°C. 234 unique proteins are present in the wild-type sample. Figure 11 is a composite of three 17 cm 2DE gels of the mutant strain Mc3 grown at 25°C. There are 213 unique proteins to the mutant sample. There are 219 proteins that are matched in both samples.

Figure 12 is a composite of three 17 cm 2DE gels of wild-type strain 8656 grown at 37°C. 190 unique proteins are present in the wild-type sample. Figure 13 is a composite of three 17 cm 2DE gels of the mutant strain Mc3 grown at 37°C. There are 208 unique proteins to the mutant sample. There are 271 proteins that are matched in both samples.

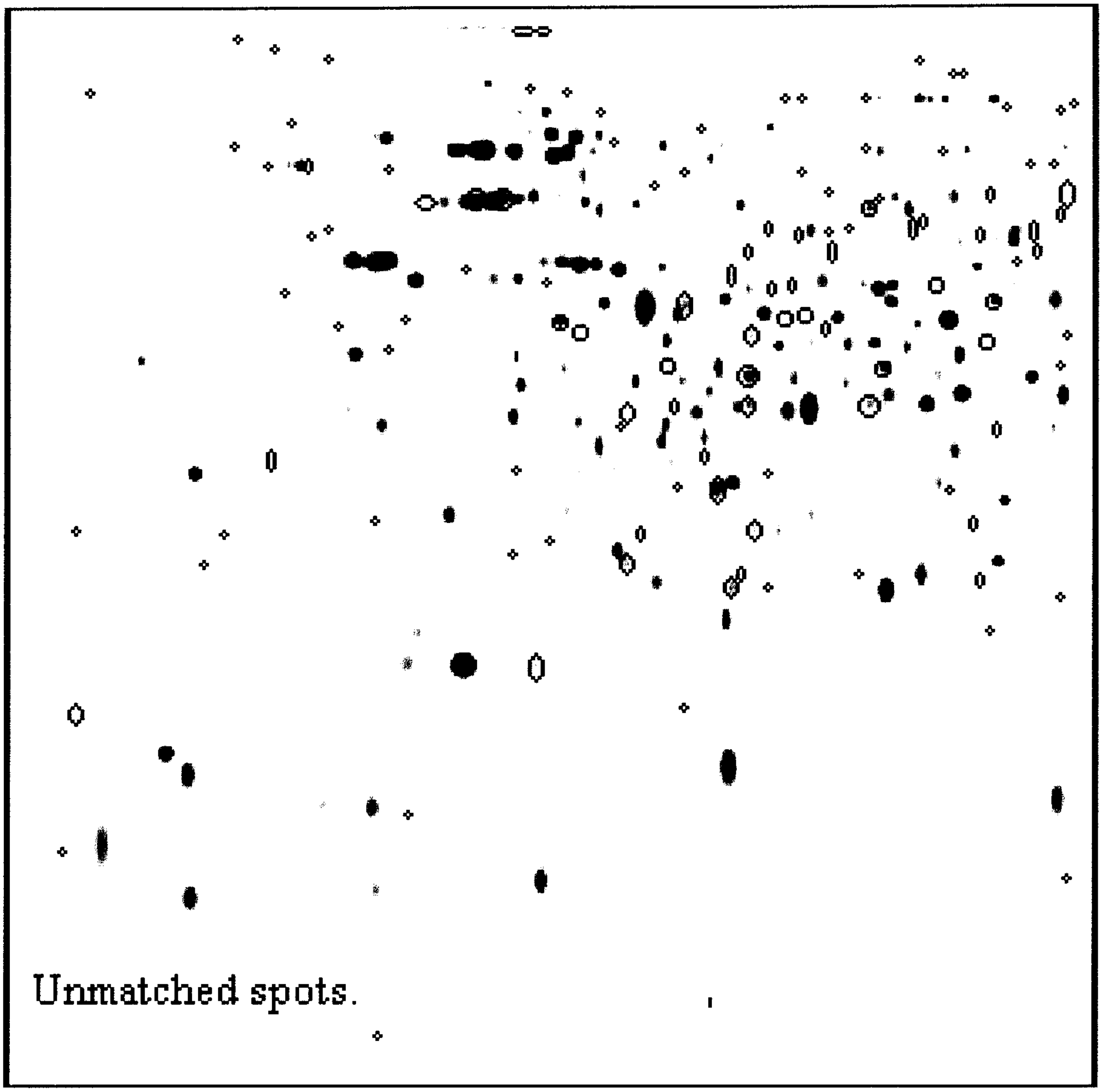
Filtered Composite Image of Wild-type Strain 8656 at 25°C


Unmatched spots.

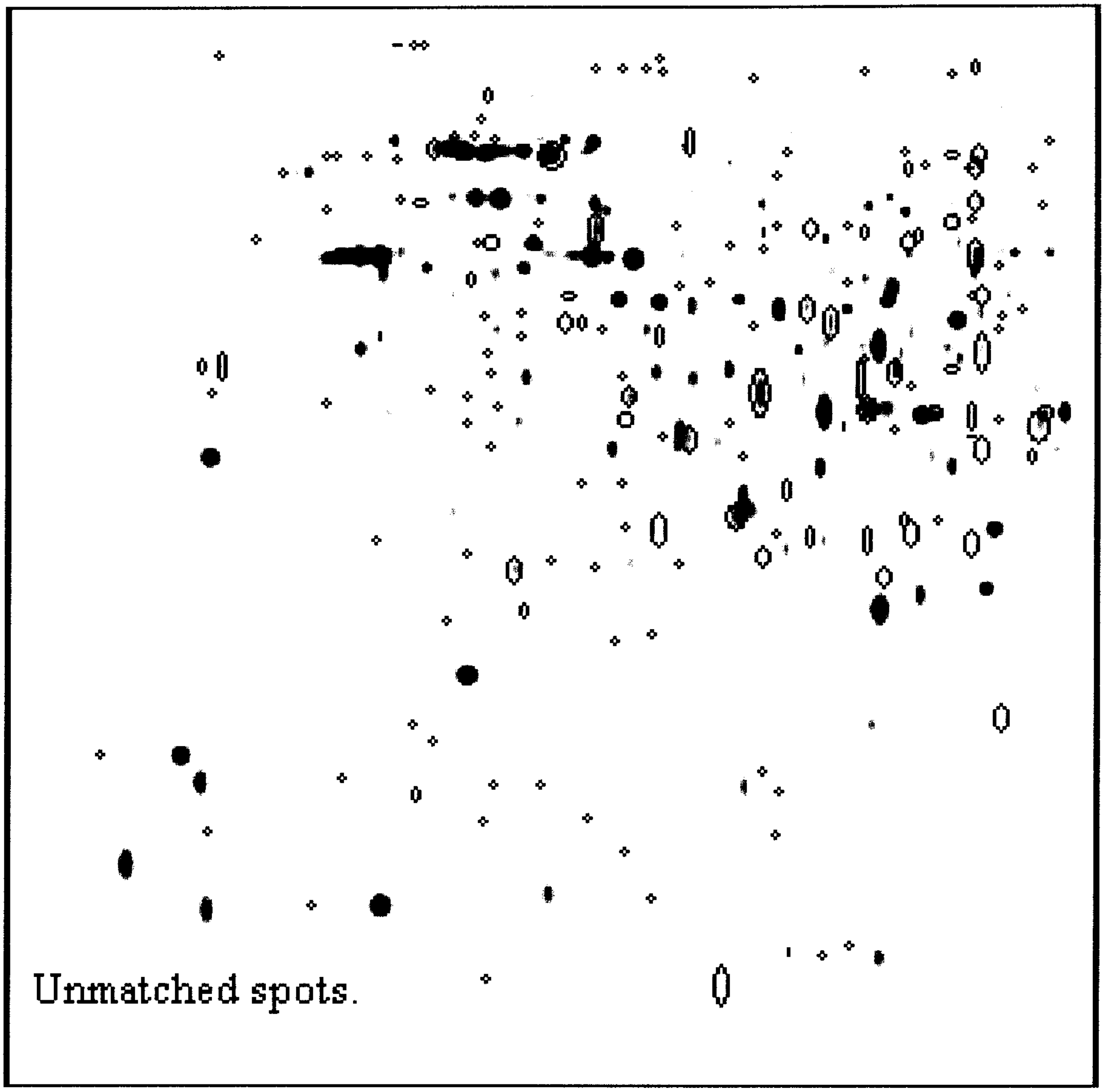
Filtered Composite Image of Wild-type Strain 8656 at 37°C


Unmatched spots.

Filtered Composite Image of Mutant Strain Mc3 at 25°C

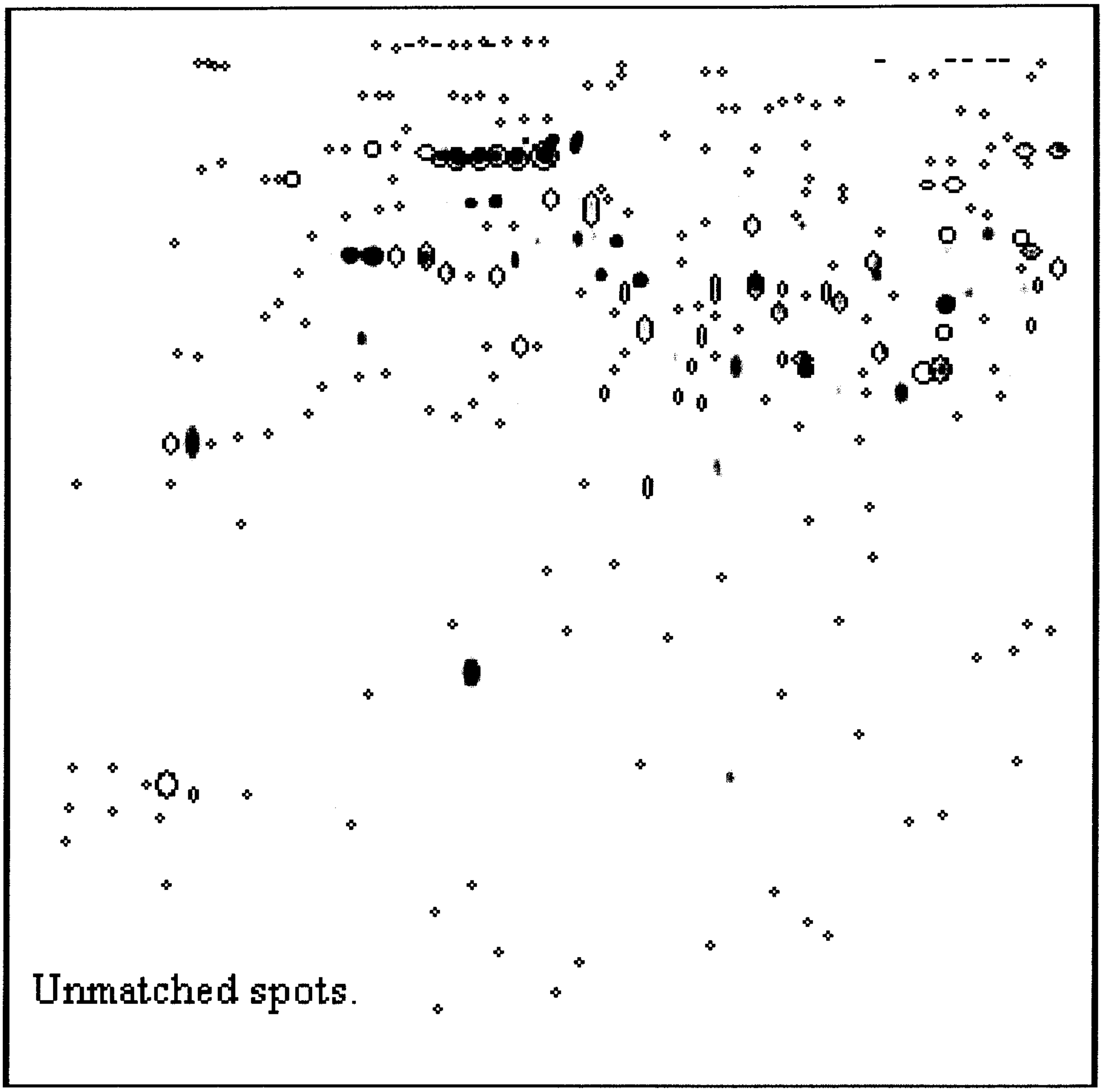

Unmatched spots.

Filtered Composite Image of Mutant Strain Mc3 at 37°C

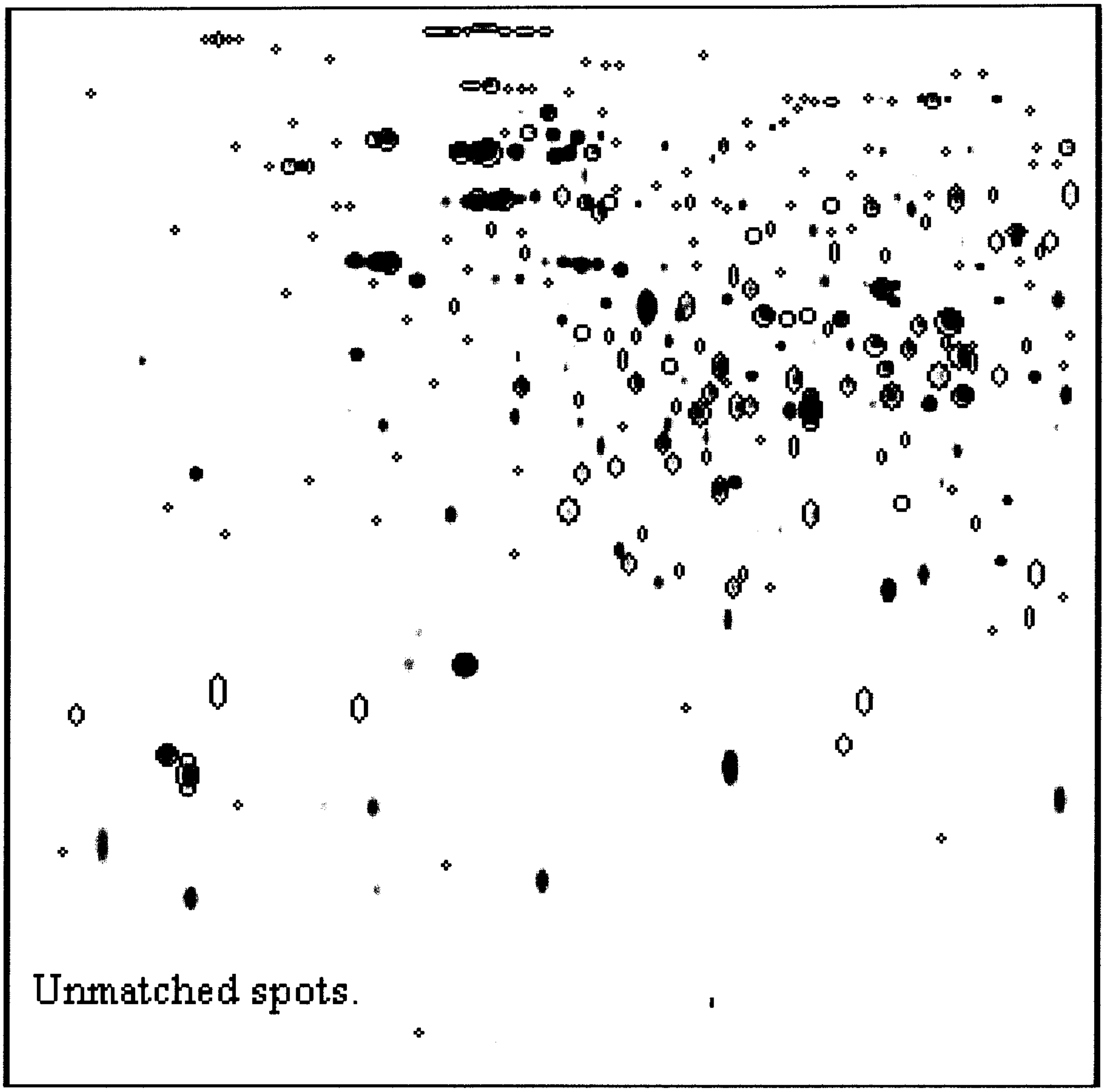


Unmatched spots.

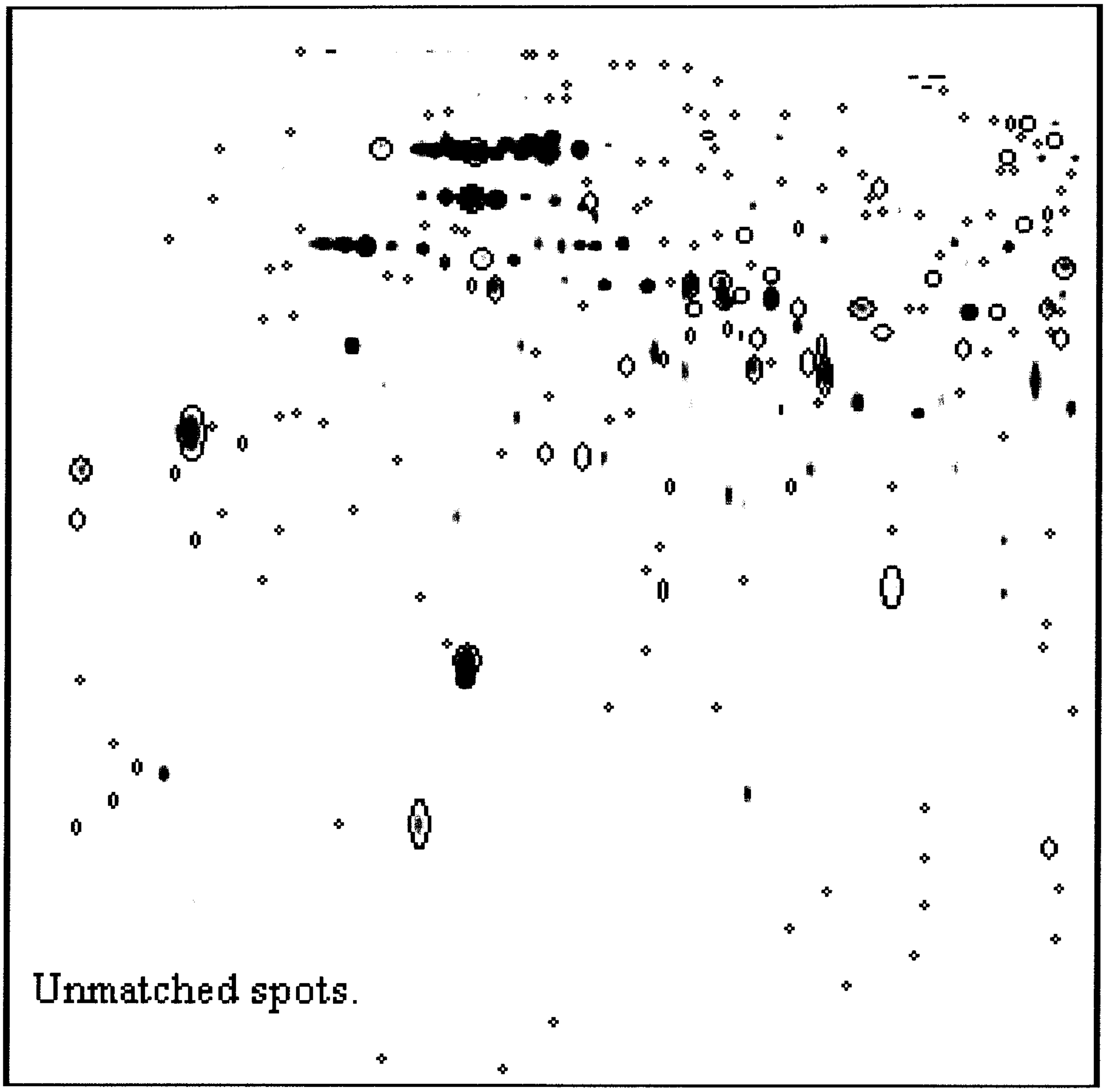
Filtered Composite Image of Wild-type Strain 8656 at 25°C


Unmatched spots.

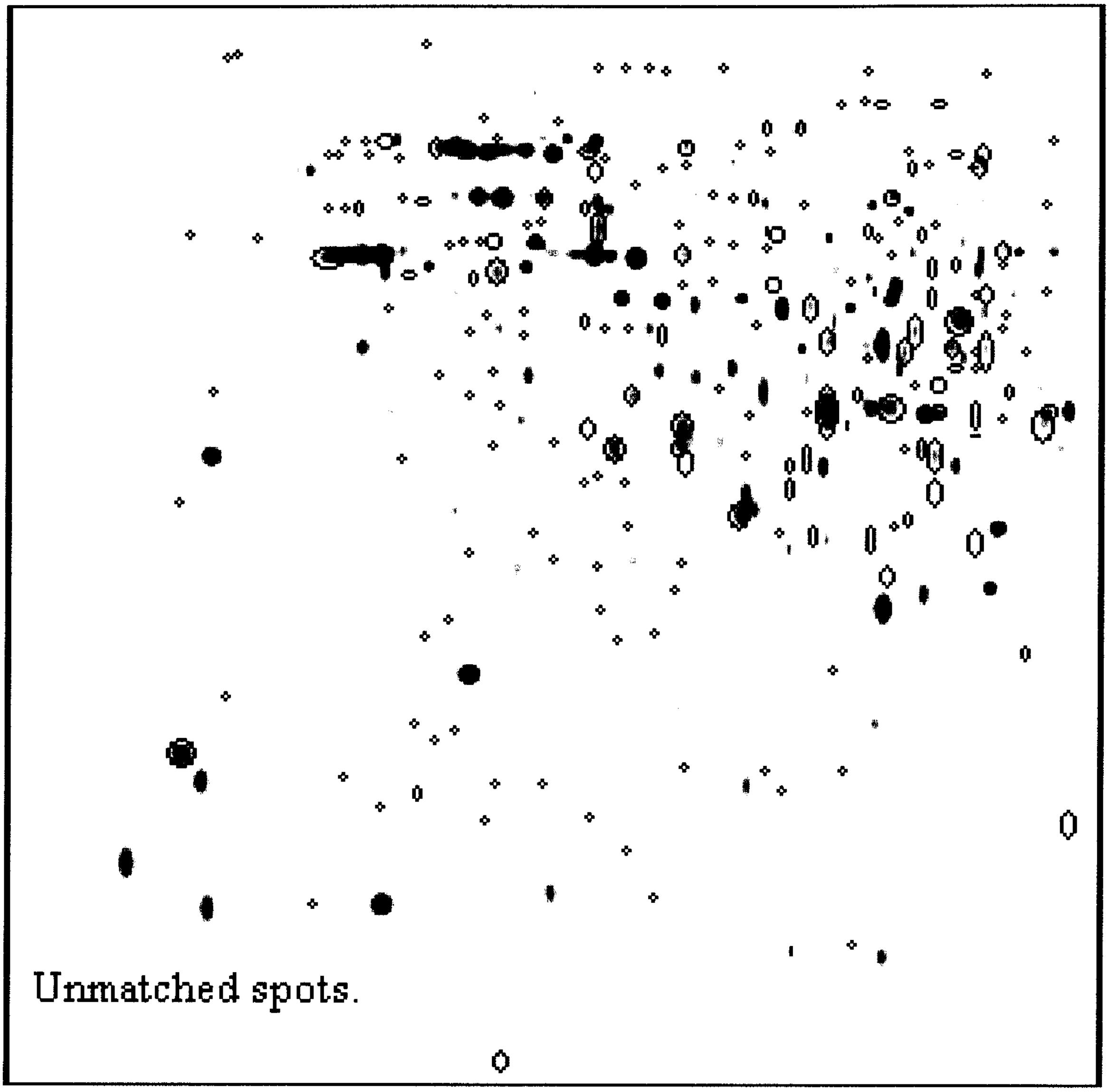
Filtered Composite Image of Mutant Strain Mc3 at 25°C


Unmatched spots.

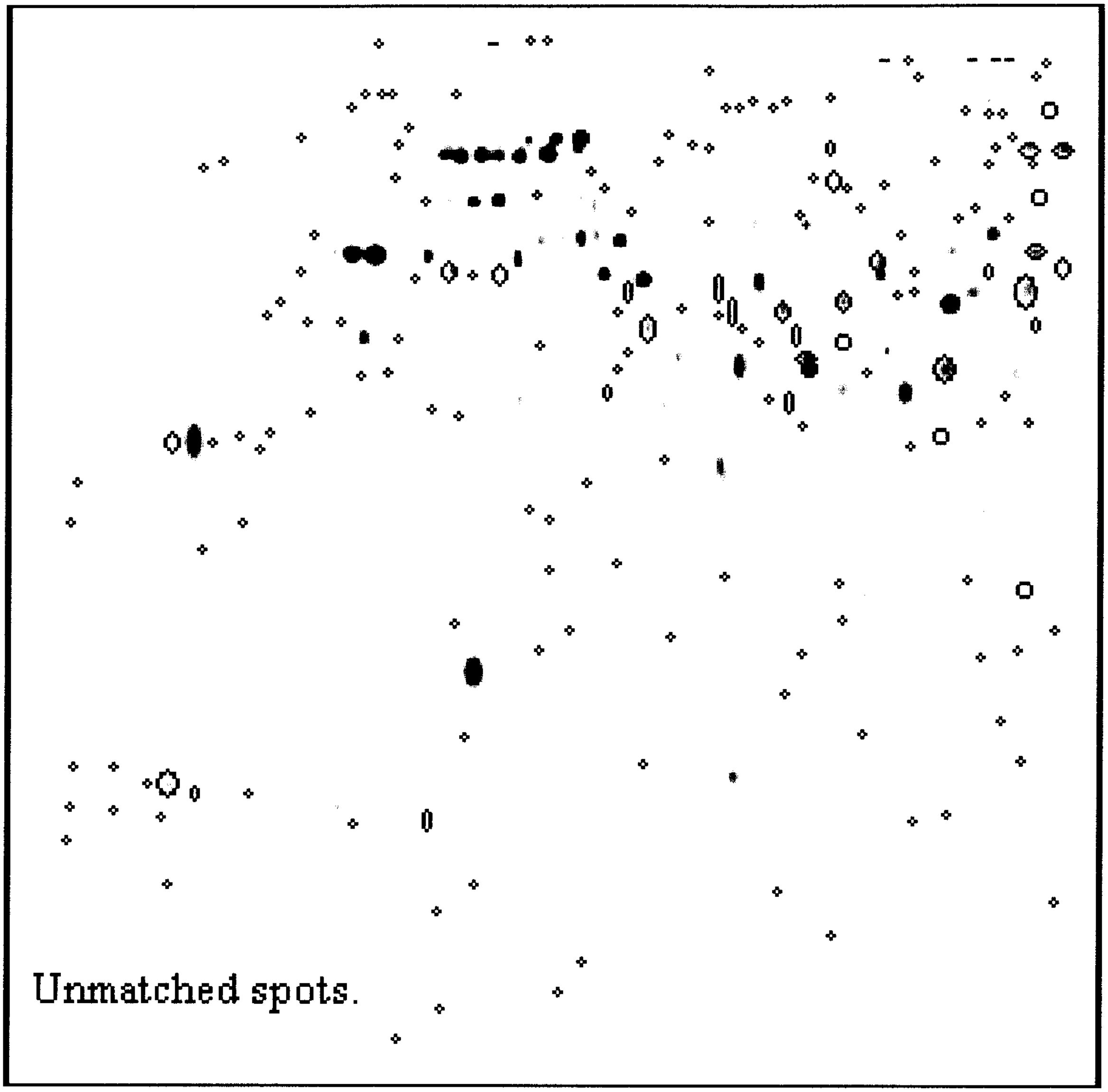
Filtered Composite Image of Wild-type Strain 8656 at 37°C


Unmatched spots.

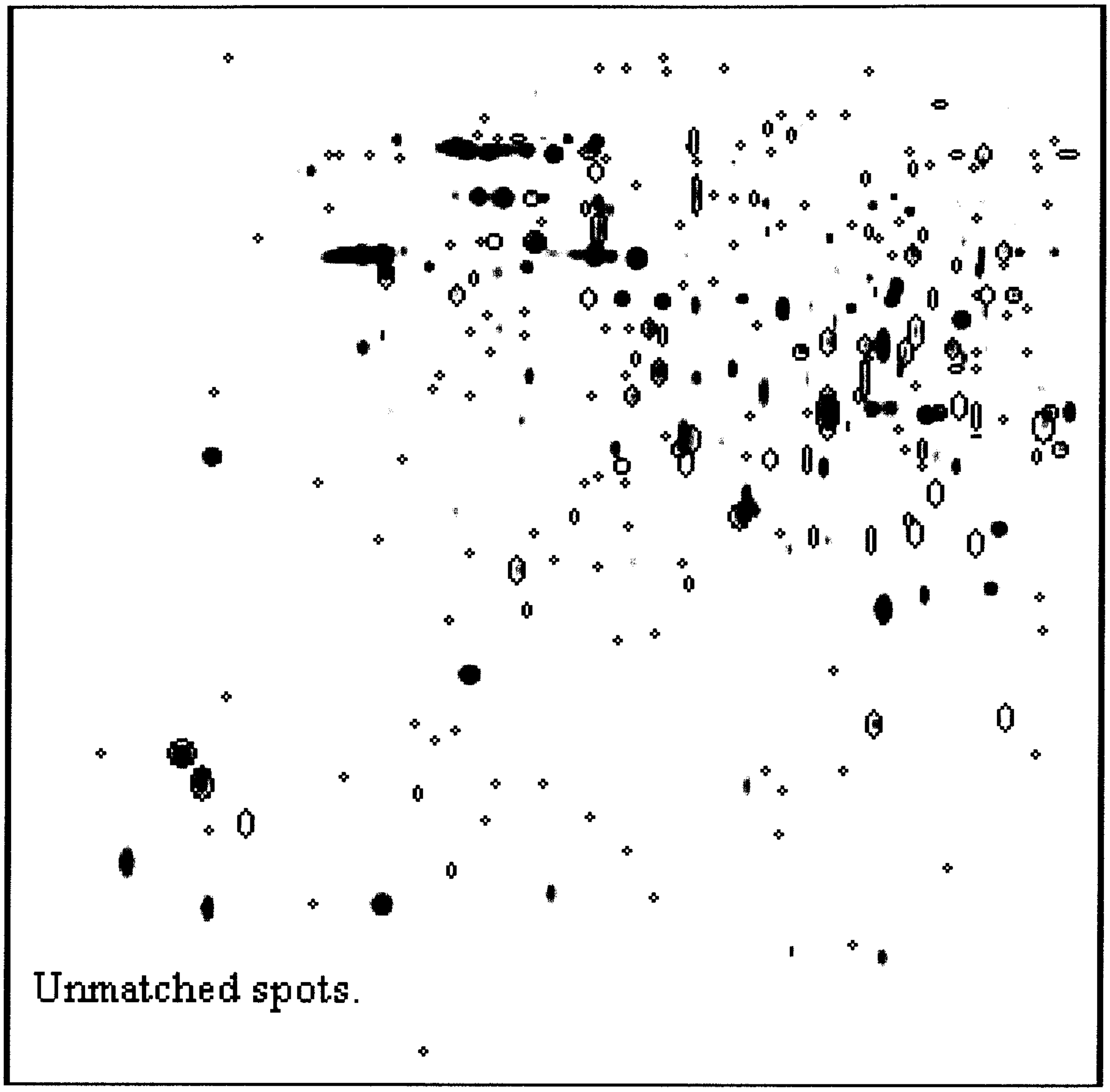
Filtered Composite Image of Mutant Strain Mc3 at 37°C



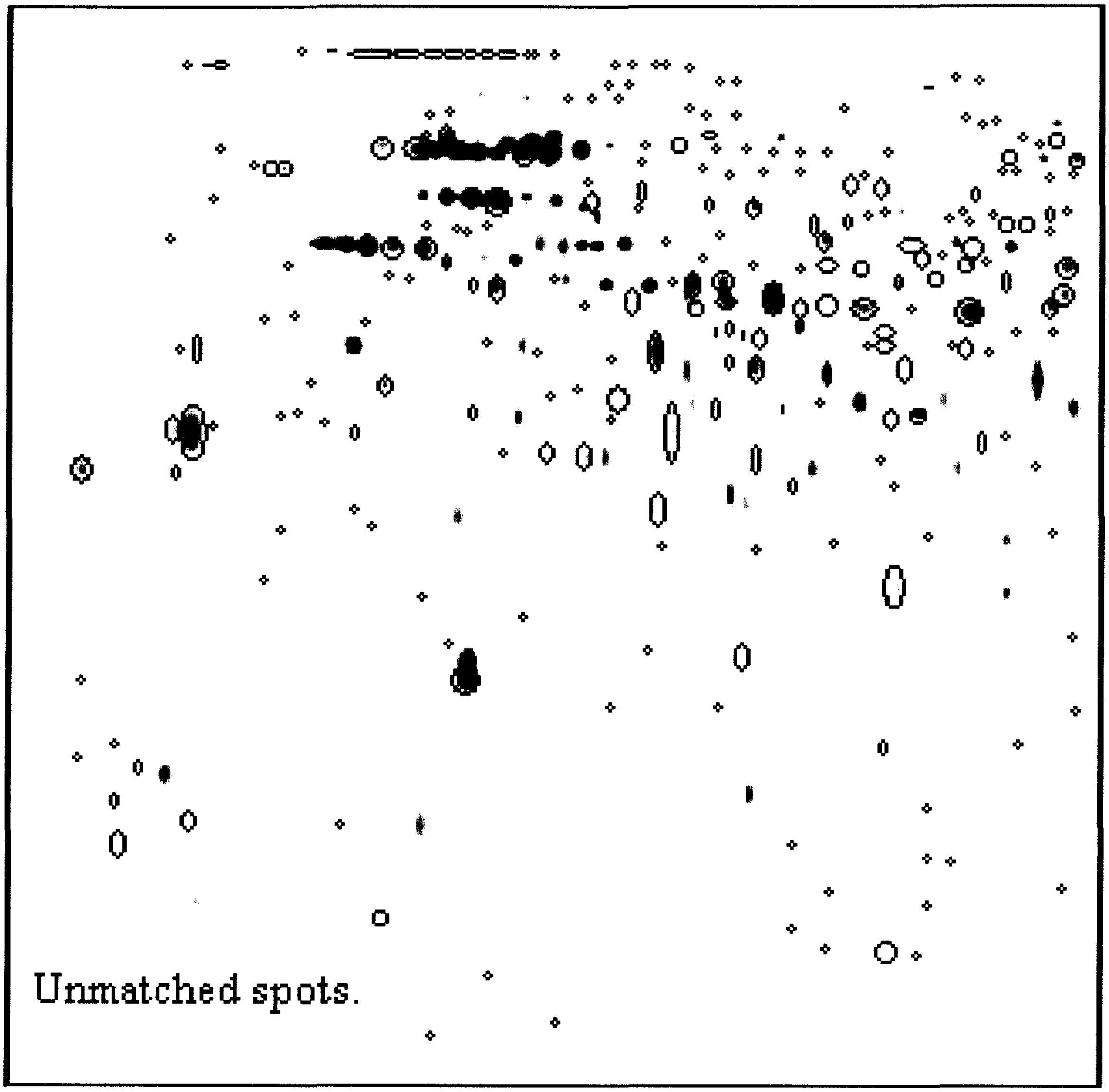
Filtered Composite Image of Wild-type Strain 8656 at 25°C


Unmatched spots.

Filtered Composite Image of Mutant Strain Mc3 at 37°C


Unmatched spots.

Filtered Composite Image of Wild-type Strain 8656 at 37°C


Unmatched spots.

Filtered Composite Image of Mutant Strain Mc3 at 25°C

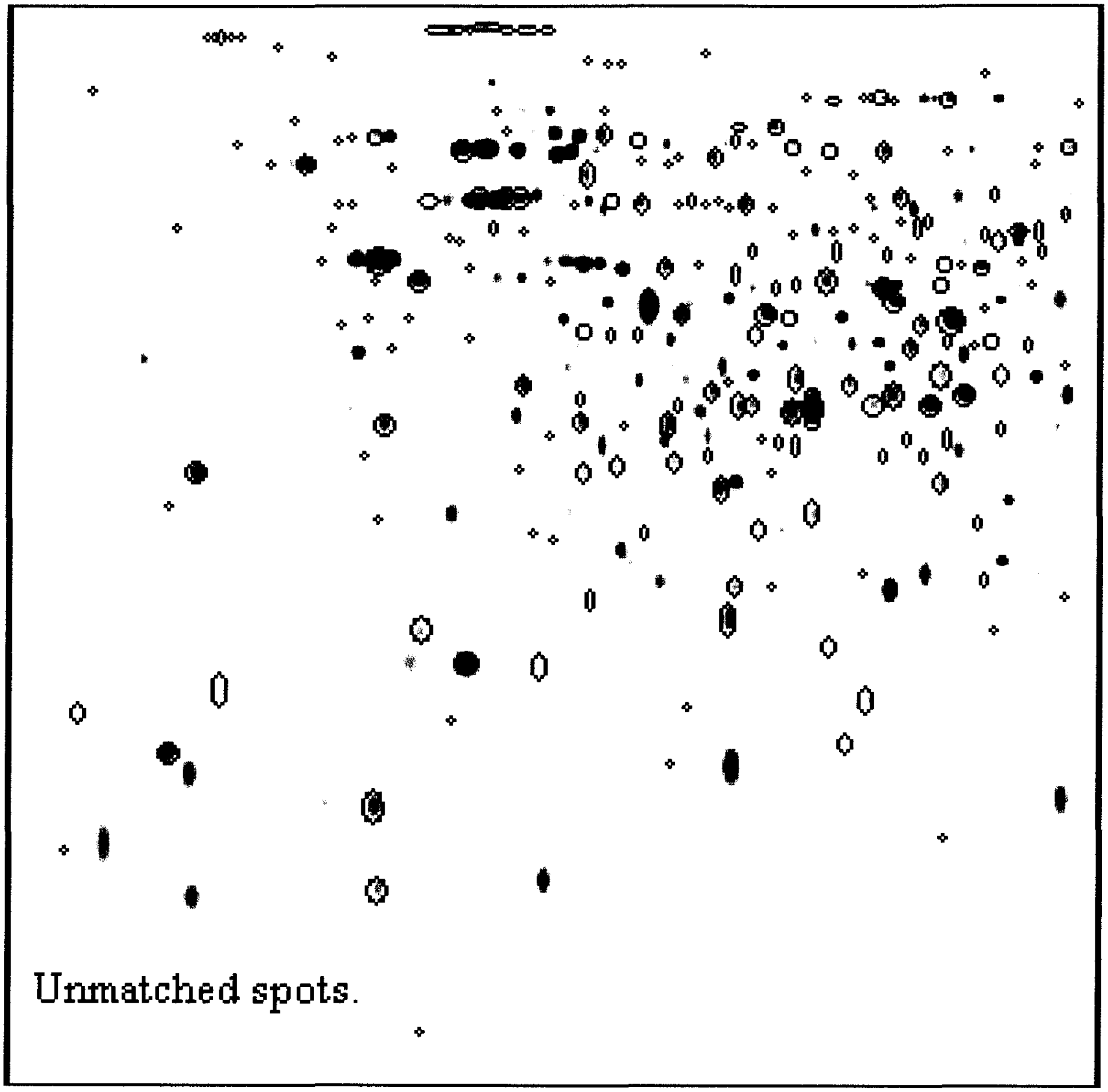

Unmatched spots.

Figure 14 is a composite of three 17 cm 2DE gels of wild-type strain 8656 grown at 25°C. 177 unique proteins are present in the wild-type sample. Figure 15 is a composite of three 17 cm 2DE gels of the mutant strain Mc3 grown at 37°C. There are 203 unique proteins to the mutant sample. There are 276 proteins that are matched in both samples.

Figure 16 is a composite of three 17 cm 2DE gels of wild-type strain 8656 grown at 37°C. 246 unique proteins are present in the wild-type sample. Figure 17 is a composite of three 17 cm 2DE gels of the mutant strain Mc3 grown at 25°C. There are 217 unique proteins to the mutant sample. There are 215 proteins that are matched in both samples.

There were 6 proteins chosen for further analysis. They were chosen for a variety of reasons, including over-expression or under-expression. Spots were also chosen if they were seen to be missing in a component of a matchset. Spots I, II, A, B, C, and D are visible in Figures 18-21.

Spot I is present in the wild-type strain 8656 at 25°C. It is also present in the wild-type strain 8656 at 37°C. Spot I appears to be under-expressed in the mutant strain Mc3 at 25°C. It is over-expressed in the mutant strain Mc3 at 37°C. Spot I was sent for sequencing. Preliminary data was not sufficient to establish the identity of the protein.

Spot II is present in the wild-type strain 8656 at 25°C. It is also present in the wild-type strain 8656 at 37°C. Spot II is expressed in the mutant strain Mc3 at 37°C. It is over-expressed in the mutant strain Mc3 at 25°C.

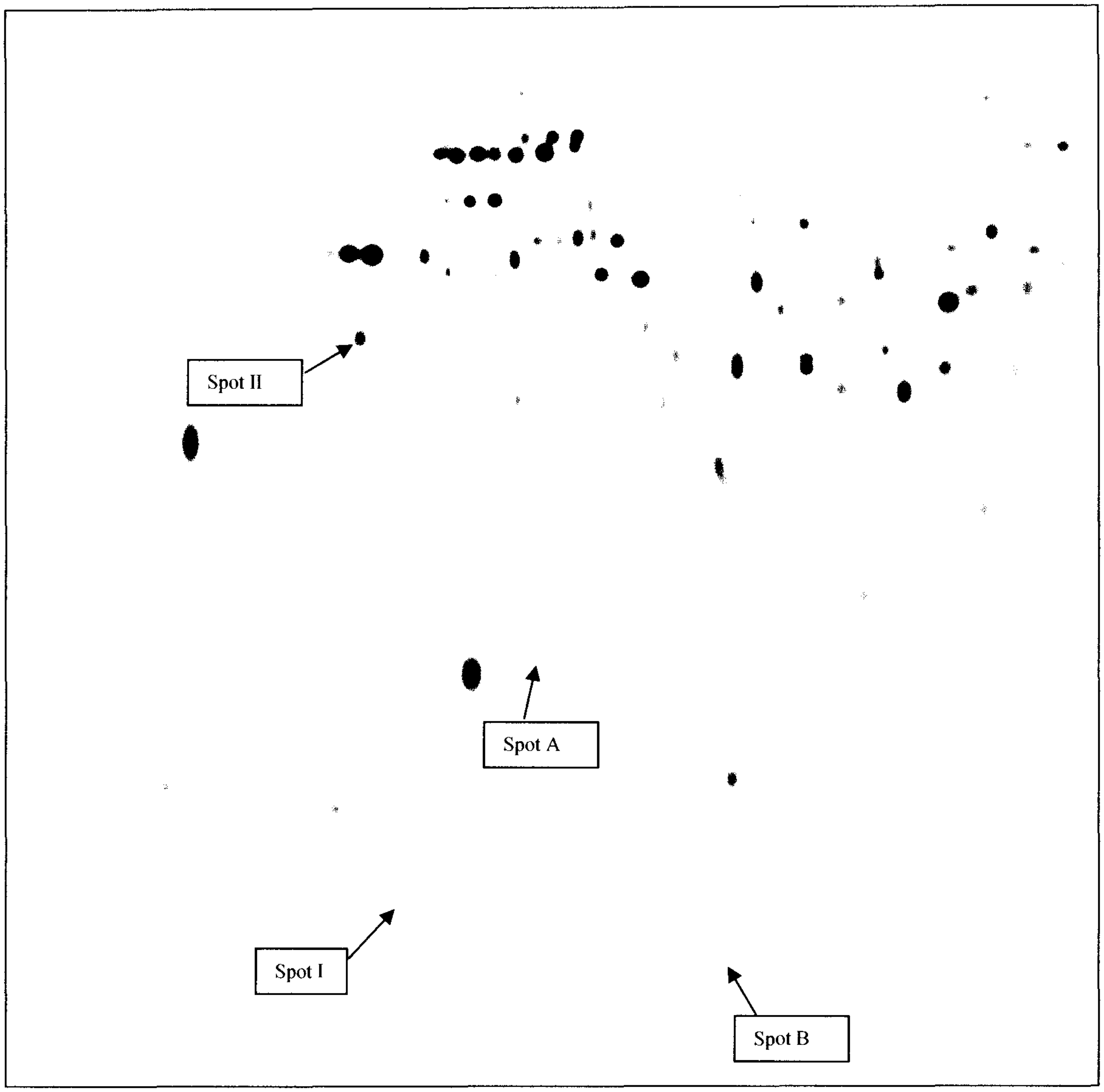
Spot A is present in the wild-type strain 8656 at 25°C. It is not present in the wild-type strain 8656 at 37°C. Spot A is missing in the mutant strain Mc3 at 37°C. It is over-expressed in the mutant strain Mc3 at 25°C.

Spot B is under-expressed in the wild-type strain 8656 at 25°C. It is also present in the wild-type strain 8656 at 37°C. Spot B is missing in the mutant strain Mc3 at 25°C. It is present in the mutant strain Mc3 at 37°C.

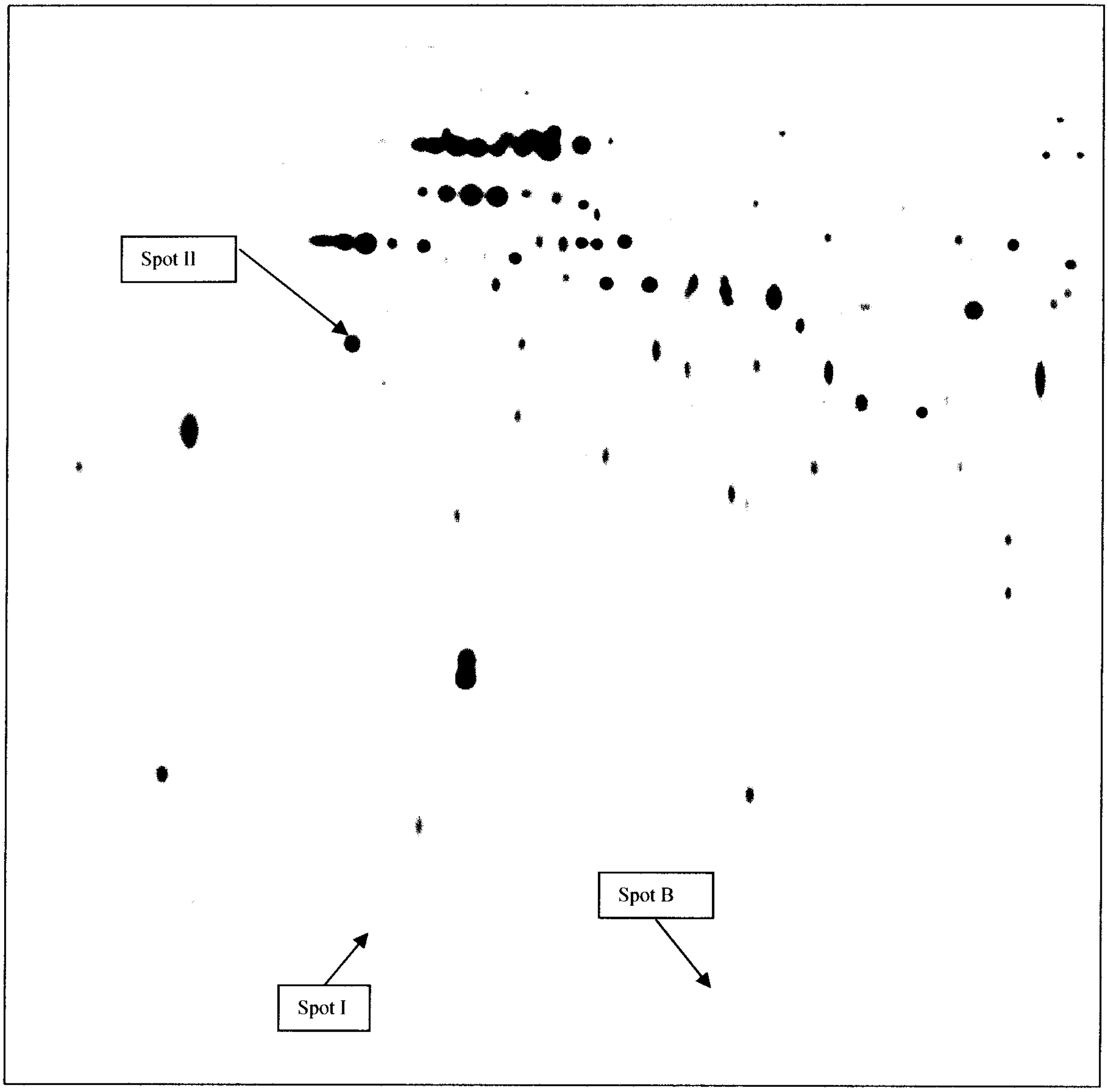
Spot C is missing in the wild-type strain 8656 at 25°C. It is also missing in the wild-type strain 8656 at 37°C. Spot C is expressed in the mutant strain Mc3 at 25°C. It is not present in the mutant strain Mc3 at 37°C.

Spot D is not present in the wild-type strain 8656 at 25°C. It is also missing in the wild-type strain 8656 at 37°C. Spot D is expressed in the mutant strain Mc3 at 37°C. It is not expressed in the mutant strain Mc3 at 25°C.

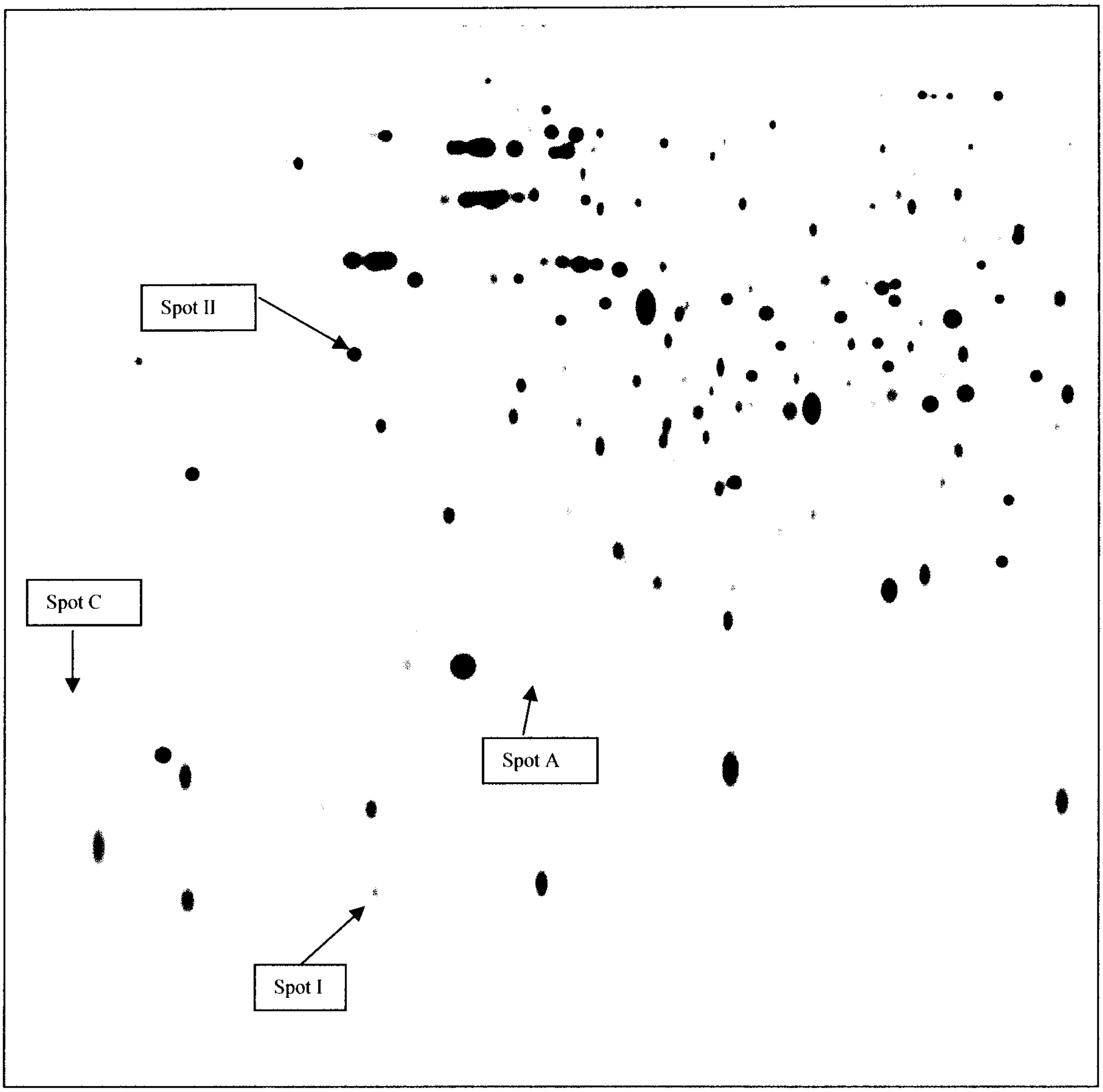
Filtered Composite Image of Wild-type Strain 8656 at 25°C


Spot II
Spot A
Spot I
Spot B

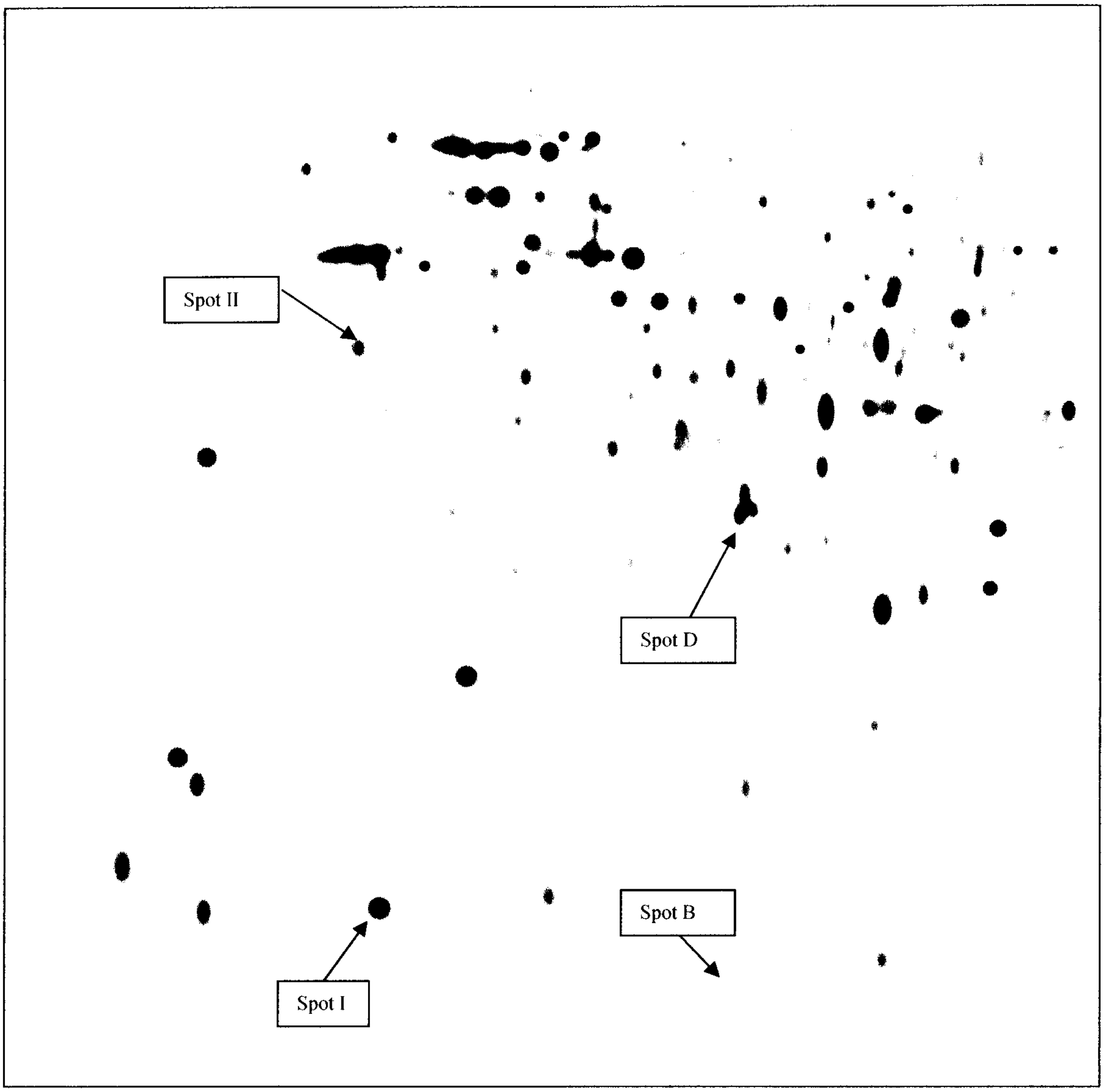
Filtered Composite Image of Wild-type Strain 8656 at 37°C


Spot II
Spot B
Spot I

Filtered Composite Image of Mutant Strain Mc3 at 25°C


Spot II
Spot C
Spot A
Spot I

Filtered Composite Image of Mutant Strain Mc3 at 37°C


Spot II
Spot D
Spot B
Spot I

Chapter IV

Discussion

Some of the differences between the proteomic profiles of the two growth temperatures elicited by the PD Quest 2-D Image Analysis software arose from variations in the exact resolution of the proteins. Dimensions between spots and spot clusters became slightly distorted by such factors as incomplete focusing in the first dimension and streaking in both dimensions. This was seen in the misalignment of the red and green spots in Figure 5. Differences in spot intensity of matched proteins between both growth temperatures were responsible for false mismatches.

We increased the resolution of proteins in several ways. First, IPG strips with a narrower pH range were used to resolve proteins. Second, manipulation of parameters in the analysis software allowed a more accurate correlation between both growth temperatures. Matches that were overlooked by the software were manually matched.

Manipulation of parameters in the software allowed for the detection of more protein spots than shown in Figures 3 and 4. Surprisingly, there were still a large number of proteins in the narrow pH range of 4-7. Manually tagging matches that were missed by the software greatly increased the number of matched spots between the growth temperatures in the wild type strain 8656 and the mutant strain Mc3.

The creation of the higher order matchsets listed in table 4 led to some discrepancies. The total number of proteins detected for a particular member in any given matchset was not the same number detected in the other member of the matchset. There are several reasons for this. The sensitivity of the software parameters for the selection of a representative faint spot, small spot, and large spot cluster can be adjusted.

The same parameters are used to detect spots in both members of the matchset. Spots that fall outside the range of the parameters due to differences in expression may not be detected.

Once the proteomic profile of the wild type strain 8656 was constructed in triplicate for both growth temperatures, the same process was carried out for the mutant strain Mc3. In essence, there were four master images from which the higher order matchsets were created. There were triplicate filtered master images for the wild type strain 8656 at both temperatures, and triplicate filtered master images for the mutant strain Mc3.

The higher order matchsets were created in order to assist in the selection of candidate proteins for sequencing that may have some application to the multicellular process in the mutant strain Mc3 and presumably in host infection. Therefore, proteins that were unique, over-expressed, or under-expressed in the mutant strain Mc3 at both growth temperatures were of particular interest. Proteins that were simply due to heat-shock were eliminated. Also, proteins which were ubiquitous in both strains and temperatures were ignored as they were unlikely to play a role in host infection.

Proteins were assumed to be heat-shock proteins if expression was increased in both the wild type strain 8656 and the mutant strain Mc3 at 37°C. Proteins also had to be expressed strongly enough to be visible for excision, as well as be isolated enough to avoid cross-contamination with other proteins.

Spot I was chosen as a good candidate for sequencing due to its over-expression in the mutant strain Mc3 at 37°C. It was isolated enough to be excised easily. It was also expressed strongly enough to be clearly seen for removal.

Spot II was over-expressed in the mutant strain Mc3 at 25°C as compared to 37°C. However, the difference was not deemed to be enough to merit selection. The slight over-expression may have been coincidental, as the protein spot was not weak in any of the samples, including the wild-type strain 8656 at both temperatures.

Spot A was of interest because it was not present in the mutant strain Mc3 or the wild type strain 8656 at 37°C. The spot was not expressed very strongly though. Even if it was intense enough to visualize for excision, it may not have yielded enough signal for sequencing.

Spot B was found to be missing only in the mutant strain Mc3 at 25°C. Preliminary visual analysis had hinted otherwise. There was no pattern detected to allude to some involvement in the multicellular process or host infection. Spot B was consequently eliminated as a candidate for sequencing.

Spot C was a unique protein to the mutant strain Mc3 at 25°C. It may thus lead to some pathway in the multicellular process simply by being shut off when the mutant strain Mc3 is exposed to a temperature of 37°C. Unfortunately the spot was too light to be visualized for excision or to be sequenced.

Spot D was found only in the mutant strain Mc3 at 37°C. This sole presence made it a very good selection for sequencing. The spot was very close to other spots in the gels however. While the spot was bright enough to easily visualize, it was probable that sequencing would be inconclusive. The proximity of the other protein spots likely would be enough to contaminate any sample sent for sequencing.

The only spot that was selected for further analysis by sequencing was spot I. It was sent to Cincinnati for sequencing on a mass spectrometer as the equipment was not available in the laboratory at YSU. The data that was returned was not sufficient to identify the protein. Work is being continued in this direction in an effort to identify the protein.

Further analysis may reveal more candidate proteins for sequencing. Increased resolution in the first dimension and the second dimension may sufficiently separate spots for sequencing if they were too proximal to each other. The increased resolution in the first dimension could be accomplished by using narrower pH ranges or by fractioning out the protein sample before running the first dimension. Different gel concentrations could be used to adjust the resolution in the second dimension.

The increase in resolution will yield more potential protein targets for sequencing. These proteins can then be isolated, physically excised, and sequenced by mass spectrometry. Studies of these proteins may contribute to the understanding of the pathogenicity of this fungus.

In summary, some of the goals of this study were accomplished. Proteomic profiles were created for each of the growth temperatures of the wild-type strain 8656. Likewise, proteomic profiles were created for the mutant strain Mc3. All of the work for these profiles was done in triplicate using a pH range of 4-7 in the first dimension of 2-dimensional gel electrophoresis.

Protein spots were selected for sequencing. However, no sequences have been obtained to this point. Some possible reasons for the failed sequencing may be the time-lapse between the trypsin digest of the excised spots and the sequencing experiment. A better method may be to excise the spots and allow the spots to be subjected to the trypsin digest in the facility where the sequencing will be performed, just prior to that experiment.

In the future, spots in the created profiles will continue to be analyzed and more candidate spots will be chosen for sequencing. Further experiments may be done to increase the resolution of the profiles already created. For example, more narrow pH ranges may be used for isoelectric focusing.

The investigation can expand in new directions once sequences for proteins are obtained. New mutant strains of *Wangiella dermatitidis* may be created in which the gene sequences of the identified proteins may be knocked out, under-expressed, or over-expressed. The phenotypes of these new mutants can then be analyzed to gain a better perspective of the pathways involved in the pathogenicity of *Wangiella dermatitidis*.

Chapter V

References

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

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. J Biol Chem. 72:248-254.

Cooper, C. R. and P. J. Szaniszlo. (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.

Geis, P. A., and C. W. Jacobs. (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.

Grove, S. N., K. B. Oujezdsky, and P. J. Szaniszlo. (1973). Budding in the dimorphic fungus Phialophora dermatitidis. J. Bacteriol. 115:323-329.

Harder, Alois, Wildgruber, Robert, Nawrocki, Arek, Fey, Stephen J., Larsen, Peter Mose, Gorg, Angelika. (1999). Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. Electrophoresis. 20:826-829.

Liebler, Daniel C. (2002). Proteomics and the New Biology, p. 3-14. In Introduction to Proteomics: Tools for the New Biology. Humana Press. New Jersey.

McGinnis, M. R. (1983). Chromomycosis and phaeohyphomyxosis: Concepts, diagnosis and mycology. J. Am. Acad. Dermatol. 8:1-16.

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

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

Szaniszlo, P. J., Hsieh, P. J., and Marlowe, J. D. (1976). Induction and ultrastructure of the multicellular (sclerotic) morphology in *Phialophora dermatitidis*. Mycologia 68:117-130.

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*. Curr Genet. 36:241-247.