The analysis of gene transcripts associated with conidiation in the insect pathogenic fungus, *Metarhizium anisopliae*

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1. ABSTRACT

Conidia of the insect pathogenic fungus, *Metarhizium anisopliae* play an important role in pathogenicity because they are the infective propagules that adhere to the surface of the insect, then germinate and give rise to hyphal penetration of the insect cuticle. Conidia are produced in the final stages of insect infection as the mycelia emerge from the insect cadaver. The genes associated with conidiation have not yet been studied in this fungus. In this study we used the PCR-based technique, suppression subtractive hybridization (SSH) to selectively amplify conidial–associated genes in *M. anisopliae*. We then identified the presence of these differentially expressed genes using the National Center for Biotechnology Information database. One of the transcripts encoded an extracellular subtilisin-like protease, Pr1, which plays a fundamental role in cuticular protein degradation. Analysis of the patterns of gene expression of the transcripts using RT-PCR indicated that conidial-associated cDNAs are expressed during the development of the mature conidium. RT-PCR analysis was also performed to examine *in vivo* expression of *Pr1* during infection of waxworm larvae (*Galleria mellonella*). Results showed expression of *Pr1* as mycelia emerge and produce conidia on the surface of the cadaver. It is well documented that Pr1 is produced during the initial stages of transcuticular penetration by *M. anisopliae*. We suggest that upregulation of *Pr1* is part of the mechanism by which reverse (from inside to the outside of the host) transcuticular penetration of the insect cuticle allows subsequent conidiation on the cadaver.

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6. INTRODUCTION

Metarhizium anisopliae is an anamorphic deuteromycetous fungus placed in the family Clavicipitaceae of the order Hypocreales in the class Sordariomycetes (Burnett, 1987; Samson *et al.*, 1988). This insect-infecting fungus has no known teleomorphic stages, however, it has been shown that *Cordeceps taii* is the teleomoph to *M. anisopliae* and is allied to other fungi of its class such as *Beauveria, Harposporium*, *Hirsutella* and *Verticillium* (Liang *et al.*, 1991). It is found in many parts of the world and has the ability to infect a wide range of insect hosts (Bidochka & Small, 2003 unpublished; Samson *et al.*, 1988). The mechanisms by which *M. anisopliae* infects the host insect are relatively well understood (St. Leger *et al.*, 1986a). Under appropriate conditions, conidia of the fungus adhere by non-specific hydrophobic mechanisms to the surface of insect (Boucias *et al.*, 1988). Once attached, the conidia can germinate and give rise to hyphal transgression of the insect cuticle (St. Leger *et al.*, 1986b, 1988a, 1991).

One of the major virulence factors produced by *M. anisopliae* is an extracellular subtilisin-like protease, Pr1 (St. Leger *et al.*, 1988a). This protease is upregulated in response to nutrient deprivation (St. Leger *et al.*, 1988b), hydrolyzes proteins in the insect cuticle that can be utilize (St. Leger *et al.*, 1988a) and is produced by hyphae transgressing the cuticle (Goettel *et al.*, 1989). Other cuticle-degrading enzymes produced during the initial stages of pathogenesis include esterases, lipases, N-acetylglucosaminidases and chitinases (St. Leger *et al.*, 1986b; St. Leger & Bidochka, 1996). Once the hyphae have breached the cuticle, the fungus grows as blastospores in the nutrient rich insect haemolymph where *Pr1* is downregulated by the presence of accessible carbon and nitrogen sources (St. Leger *et al.*, 1988a, 1996). Some strains of *M. anisopliae* produce a cyclodepsipeptide toxin called destruxin (St. Leger *et al.*, 1988c) in

combination with other cyclic depsipeptides and hydrophobins, which can interfere with the insect immune response (Griesch *et al.*, 2000). Within 3 to 7 days the insect dies (St. Leger *et al.*, 1988a) and the mycelia emerge from, and produce green conidia on the surface of the cadaver resulting in insect mummification in what is known as green muscardine disease (Goettel *et al.*, 1989).

Conidia play a very important role in pathogenicity because they are the infective propagules of insect pathogenic fungi. In many countries, the production and harvesting of conidia of *Metarhizium* strains are being commercially developed as an alternative to chemical pesticides (Goettel *et al.*, 1995; Schulze *et al.*, 2001). There are limitations, however, to using *Metarhizium* strains as a biological control agent even though they are known to be environmentally safe (Roberts & Hajek, 1991; Milner, 2000; Milner & Hunter, 2001). Some researchers have found that it is difficult to mass-produce conidia since there can be a natural decrease in production of these infective propagules during successive subculturing and some conidia can be ineffective and unstable under field conditions (St. Leger & Bidochka, 1996; Kamp & Bidochka, 2002).

Many researchers have tried to get a better understanding of insect pathogenic fungi by studying their life cycle since conidia are the primary means for dispersion in the environment. Asexual reproduction in *M. anisopliae* involves the differentiation of four cell structures, mycelia/vegetative hyphae, conidiophores, phialides and conidia (Samson *et al.,* 1988). Little is known of the genes upregulated during conidiation in *M. anisopliae*. Studies have been conducted with other Deuteromycetous fungi such as *Aspergillus nidulans*, where researchers have found three essential genes, *brlA, abaA* and *wetA* exclusively involved in conidiophore formation (reviewed in Adams *et al.*, 1998). Berlin and Yanofsky (1985) found *con* genes upregulated in *Neurospora crassa,* which

are involved in macroconidial morphogenesis. Both *A. nidulans* and *N. crassa* are but distantly related *Metarhizium* in their reproductive biology, host preference and phylogeny. Previous studies conducted by Kamp and Bidochka (2002) used PCR with primers designed from the regulatory pathway genes *brlA* and *wetA* involved in conidiation in *A. nidulans.* Sequence analysis showed no homology to *brlA* or *wetA* sequences indicating that the regulatory pathway involve in conidiation in *M. anisopliae* may be different than that of *A. nidulans*. If genes associated with conidiation could be identified in *M. anisopliae*, this would allow researchers to control conidiogenesis in this fungus.

In the present study, the upregulation of conidial-associated genes was analyzed in the insect pathogenic fungus, *M. anisopliae.* There are five objectives to this study. The first objective was to determine the earliest day there was a significant increase in conidium production and abundance in mycelial growth of *M. anisopliae* grown on different agar media. The second objective was to use suppression subtractive hybridization (SSH) to selectively amplify genes expressed during conidiation, while simultaneously suppressing mycelial cDNA genes. SSH is an efficient method for detecting differentially expressed genes (Diatchenko *et al.,* 1996), and has been successfully applied to isolation of cellulose–growth specific genes in *Agaricus bisporus* (Morales & Thurston, 2002), and genes enriched in conidia of *A. nidulans* (Osherov *et al.*, 2002). This technique was also used to compare differences between virulent and avirulent strains of *Escherichia coli* (Stocki *et al.,* 2002).

The third objective was to identify differentially expressed genes using nucleic acid homology searches such as the BLAST program at the National Center for Biotechnology Information (NCBI). The fourth objective was to profile gene expression

patterns in genes during conidiation by using reverse-transcriptase polymerase chain reaction (RT-PCR). Finally, a conidiation-associated gene (*Pr1*) was profiled using RT-PCR during insect infection.

7. LITERATURE REVIEW

7.1. *Metarhizium* as a Biocontrol Agent

M. anisopliae is one of the most widely studied entomogenous fungi and is naturally found in the soil (Zimmerman, 1993). A member of the Hyphomycetes class of fungi, *M. anisopliae* is categorized as a green muscardine fungus due to the green colour of sporulating colonies (Goettel *et al.*, 1995)(Fig. 7.1b). The first use of this fungus as a microbial agent against insects was in 1879, when Elie Metchnikoff and colleagues used it in experimental tests to control wheat grain beetle, *Anisoplia autriaca* (reviewed in Milani *et al.*, 1995). More recently, *M. anisopliae* has been used on a commercial scale for the biological control of several insect pests in many countries (Table 7.1). For example, Lutte Biologique contre les Locustes et Sauteriaux (LUBILOSA) developed a mycopesicide called Green Muscle that has been registered in many African countries (Jenkins *et al.*, 2001). This biological pesticide is based on the ability of the conidia of *M anisopliae* var. *acridium* to specifically infect and kill various species of grasshoppers. Green Muscle is an oil suspension that is applied to infected fields by spraying as seen in Fig. 7.1a (Jenkins *et al.*, 2001).

In Australia, a mycopesticide called BioGreen based on the conidia of another species, *Metarhizium flavoviride* was registered (Milner, 2000). *M. flavoviride* was used against a wide range of pests including termites and locusts. Later that year, Bio-Care Technology Pty Limited developed two other mycopesticides called BioCane and GreenGuard (reviewed in Milner, 2000) which are based on a strain of *M. anisopliae* var.

anisopliae and have been used commercially to control canegrubs and locusts. There are limitations, however, to using *M. anisopliae* as biological control agent since the fungus can take 3-7 days to kill the insect leaving plenty of time for serious damage to crops (St. Leger *et al.*, 1996). Many other questions are being asked about the persistence and effectiveness of the infective conidia of these fungal strains in relation to the environmental damage they may cause.

Table 7.1: Examples of most recently registered mycopesticides based on spores of the entomopathogen *Metarhizium* (Milner, 2000; Revised by Small, 2003).

Country	Name of mycopesticide	Fungus	Target Host	Crop
U.S.A	BioBlast	*M. anisopliae*	Termites	Houses
South Africa	Green Muscle	*M. anisopliae*	Locusts	Natural bushland
Australia	BioGreen	*M. flavoviride*	Red-headed cockchafer	Pasture/turf
Australia	Green Guard	*M. anisopliae* var. *acridium*	Locusts, grasshopper	Pasture
Australia	BioCane	*M. anisopliae*	Scarab beetles (canegrubs)	Sugarcane
U.S.A	Bay Bio	*M. anisopliae*	Termites	Houses

Fig. 7.1. (A) Shows the mycopesticide Green Muscle being dispersed through spraying in South Africa. (B) Shows a picture of a dead grasshopper sprayed by the mycopesticide, Green Muscle derived from the green spores of *M. anisopliae* var. *acridium* (Jenkins *et al.*, 2001).


A
B

7.2. Infection Cycle of *Metarhizium*

7.2.1. Conidial Attachment and Cuticle Invasion

Conidia are the infective propagules of *Metarhizium* and under appropriate conditions, they land on the surface of a compatible host mainly via wind dispersal (Boucias *et al.*, 1988). The site at which infection takes place is highly dependent on the environmental conditions (Boucias *et al.*, 1988; St. Leger *et al.*, 1991). Conidia of the fungus adhere by non-specific hydrophobic mechanisms to the surface of insect (Boucias *et al.*, 1988). Upon contact with the host's surface, the conidium swells and forms a germ tube, which differentiates into an appressorium (St. Leger *et al.*, 1991) (Fig. 7.2). Appressorium formation is reliant on factors including temperature and humidity and can be mediated by phosphorylation of various Ca^{2+} activated kinases and calmodulin-dependent compounds (St. Leger *et al.*, 1986a).

In order for the fungus to penetrate the epicuticle of its host, a penetration peg forms below the appressorium, applies mechanical pressure and secretes extracellular enzymes (proteases, esterases, N. acetylglucosaminidases, lipases and chitinases) to force and digest its way through the complex structure. During the early phases of infection, the cuticle-degrading enzymes degrade major compounds of the insect cuticle (St. Leger *et al.*, 1986a, b, 1987). Since the cuticle is mainly composed of proteins, the subtilisin-like protease, Pr1 plays a vital role in transcuticular penetration (St. Leger *et al.*, 1986b). This protease is the most widely studied and best understood determinant of insect pathogenesis (St. Leger *et al.*, 1987). Many studies have been conducted to provide evidence of Pr1 involvement in cuticle degradation by *Metarhizium* (St. Leger *et al.*, 1986a, b, 1989a; Goettel *et al.*, 1989). *In vitro* studies have shown that Pr1 hydrolyzes about 30% of proteins from the locust cuticle, releasing soluble peptides containing

amino acids and amino sugars required for fungal growth (St. Leger *et al.*, 1986a). Further investigations have shown that treating *Manduca Sexta* (tobacco hornworm) with a protease inhibitor (turkey egg white) delays fungal penetration of the cuticle (St. Leger *et al.*, 1988a).

Various levels of carbon and nitrogen in the environment transcriptionally regulate the synthesis of Pr1. An excess of readily utilized metabolites such as alanine in host tissues can inhibit the synthesis of Pr1. (St. Leger *et al.*, 1989b). The presence of proteases like Pr1 not only provides a nutritional relationship between the fungus and its host, but also affects the pH of that interaction. The release of proteolytic byproducts such as ammonium can drive the pH of the environment as high as pH 8 that enhances Pr1 production and is also optimal for Pr1 enzyme activity (St. Leger *et al.*, 1998). Other proteases including metalloproteases, aminopeptidases and trypsins are also believed to play a supporting role in hydrolyzing cuticular proteins (St. Leger *et al.*, 1986a, b).

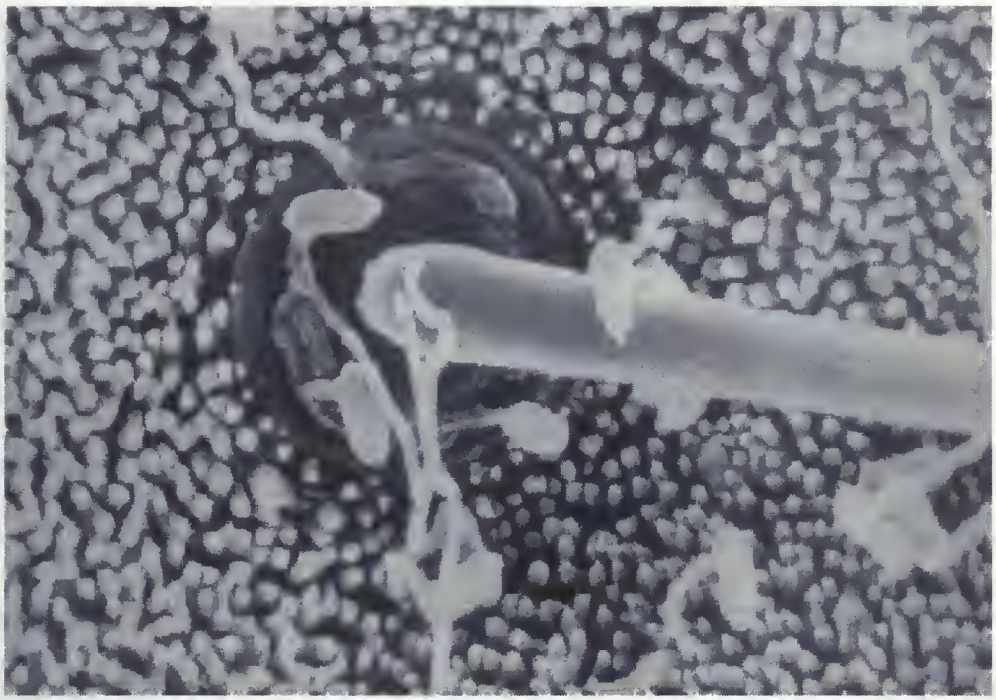

Fig. 7.2. Scanning electron micrograph showing the fungus germinating on caterpillar cuticle. An appressorium has formed at the base of the hair socket (www.bath.ac.uk/Depatments/BiolBioch/cham2.htm).

7.2.2. Host Defense and Haemolymph Invasion

Once the cuticle is breached, the fungus penetrates the procuticle and expands laterally to form penetration plates (St. Leger *et al.*, 1986a). As the fungus enters the haemolymph, it forms yeast-like bodies called blastospores, which are produce by budding and proliferate throughout the nutrient-rich haemolymph of the insect (St. Leger *et al.*, 1988a). The insect responds to fungal invasion by the induction of humoral and cellular immune responses (Vilcinskas & Wedde, 1997) that includes the production of phenolic compounds. The oxidation of these compounds produces quinones and melanin pigments, which can be directly toxic to the fungus or can partially inhibit protease activity (St. Leger *et al.*, 1988b). To combat the host's immune response, some strains of *M. anisopliae* produce cyclic peptide toxins called destruxins in combination with other cyclic depsipeptides, hydrophobins and proteases that can interfere with the insect's immune responses (St. Leger *et al.*, 1988c; Griesch *et al.*, 1998, 2000). Successful infection leads to death, which usually occurs between 3 and 7 days as a result of mechanical damage caused by a combinations of factors: fungal invasion, massive mycelial colonization, nutrient depletion, and the reaction to fungal toxins (St. Leger *et al.*, 1988c, reviewed in Charnley & St. Leger, 1991).

7.2.3. Conidial Outgrowth

In a moist, warm environment, the fungus exits the cuticle a few days after death and mycelia emerge to produce conidia on the cadaver (reviewed by Charnley & St. Leger, 1991). These mature conidia can then spread to other insects causing a population epidemic. The infection cycle of *Metarhizium* is shown in Fig. 7.3.

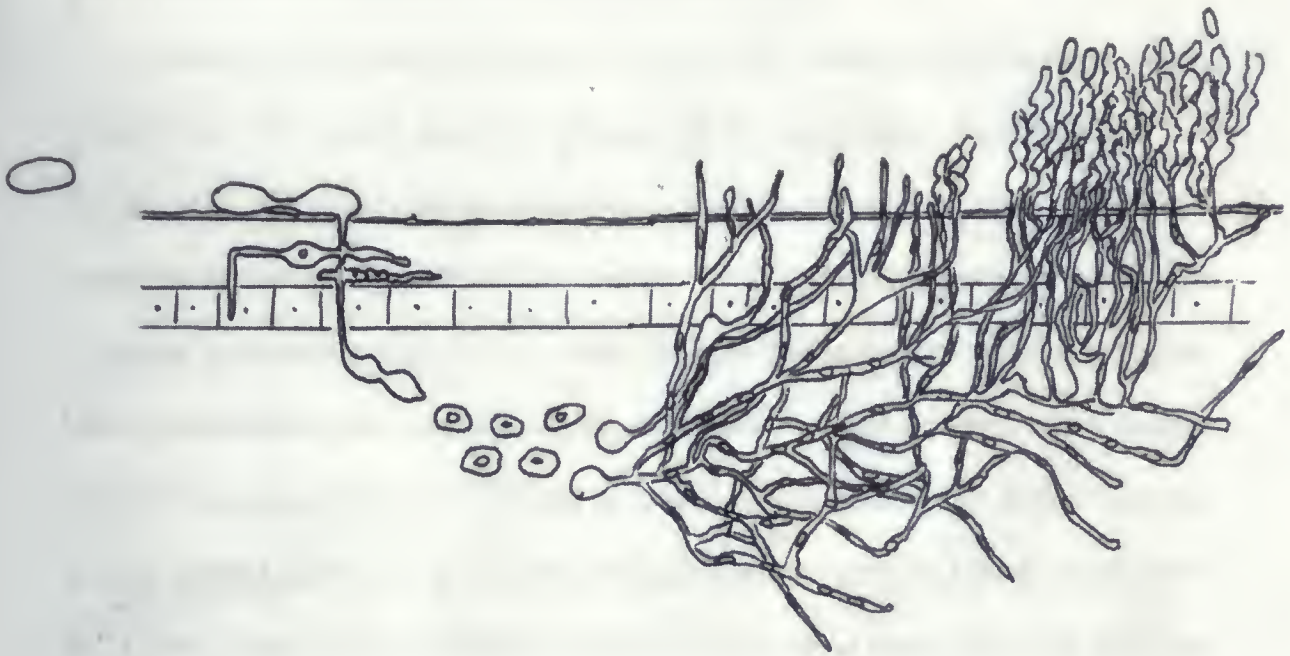

Fig. 7.3. A diagrammatic representation of the infection cycle of the insect pathogenic fungus, *Metarhizium anisopliae* (Small et al., 2004).

7.3. Conidiation

7.3.1. Conidium Development of *Metarhizium anisopliae*

Conidia play a vital role in the life cycle of many entomopathogenic fungi and are known to be the primary means of dispersal in the environment for infecting a host (Boylan *et al.*, 1987) Conidia can survive in a non-growing state for longer periods than vegetative hyphae (Ebbole, 1996). The saprophytic life cycle of *Metarhizium* begins with a conidial germination (Fig. 7.4a-c), which leads to the formations of tubular hyphae. These morphological structures then further differentiate into a network of interconnected cells known as mycelia (Ebbole, 1996)(Fig. 7.4d–f), which subsequently differentiate into conidial bearing structures called conidiophores. When the growth of these conidiophore stalks cease, the tip swells to form a specialized cell known as a phialide, which gives rise to dense masses of columns of chains of conidia (Fig. 7.5). The mature conidium of *M. anisopliae* is usually 5-8 µm long by 1.5-3.5 µm wide and is green in colour (Samson *et al.*, 1988).

M. anisopliae can be grown under conditions that promote either vegetative growth or induce conidiation. *In vitro* studies have shown that media high in nutrients activate vegetative growth, whereas conidial growth can be induced when these nutrients become limited (St. Leger *et al.*, 1988b, 1991b, 1992). It is still not clear the exact role that starvation and nutritional deprivation play in the developmental activation of conidiation.

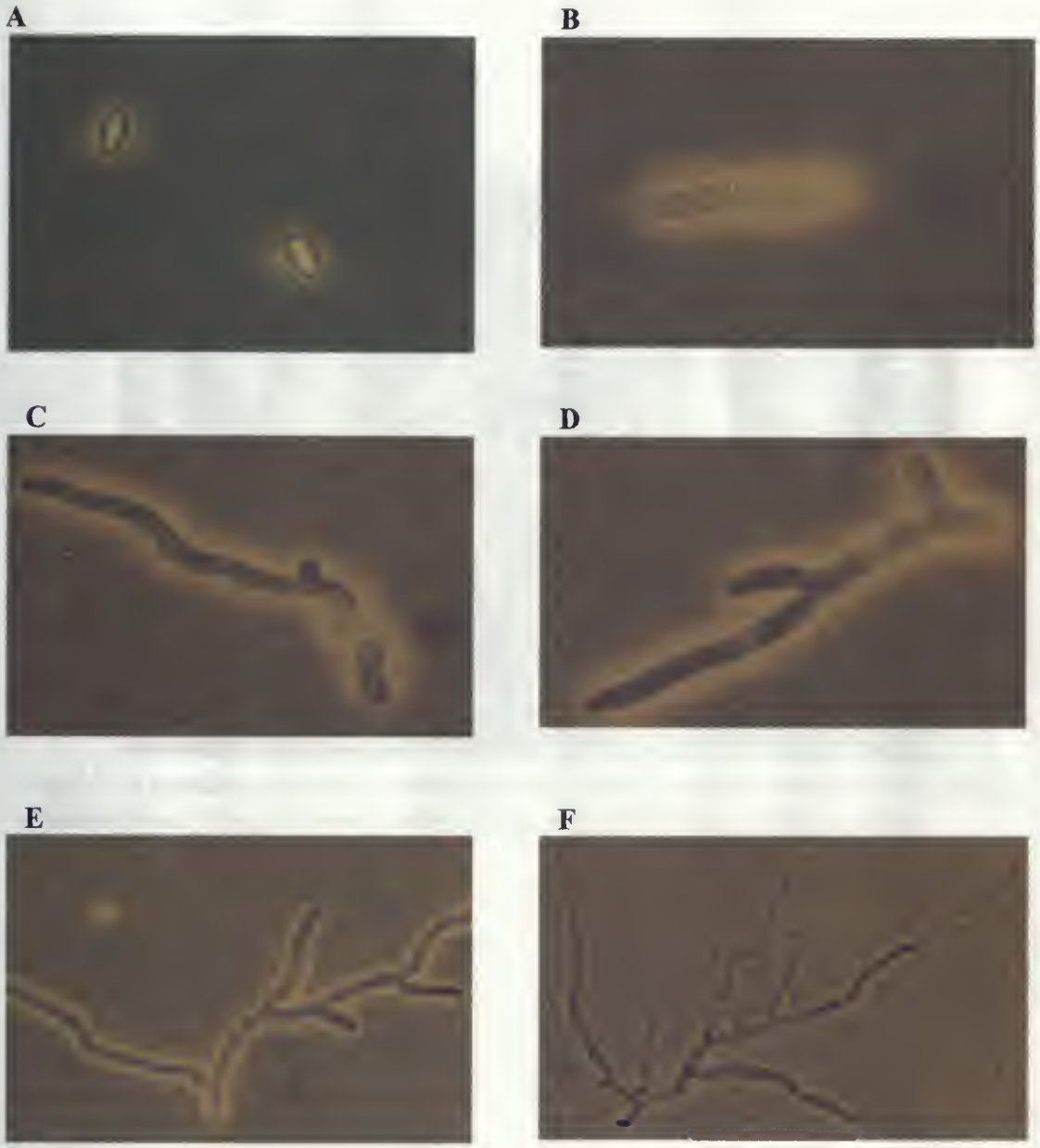

Fig 7.4. Developmental stages of conidiation in *M. anisopliae*. (A) Mature conidia. (B) Conidial germination. (C) Formation of tubular hyphae. (D) (E) and (F) Apical extension and branching of hyphae Pictures courtesy of S. Condotta, 2002 (400X).

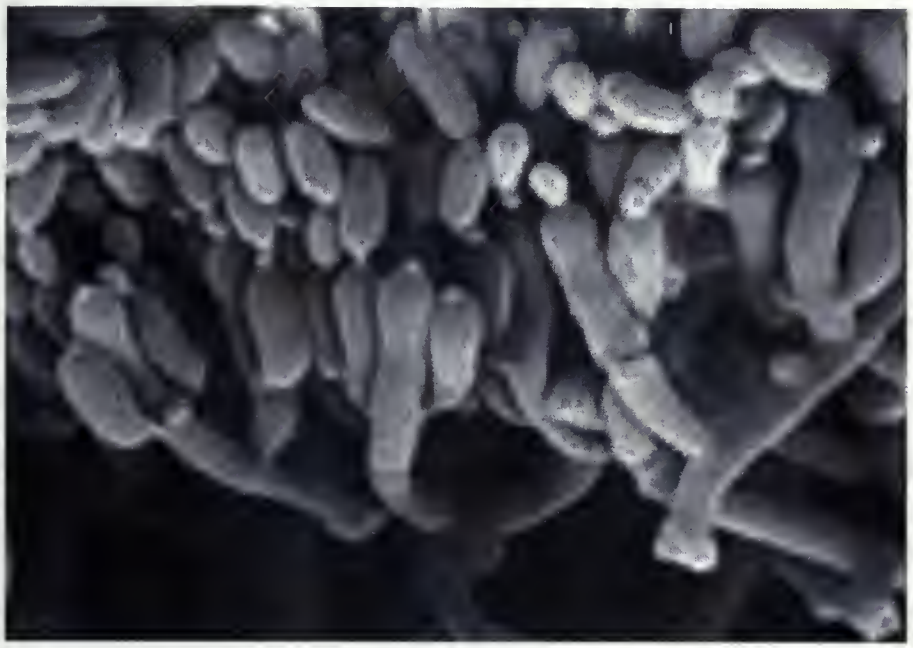

Fig 7.5. A scanning electron micrograph of phialides and conidia of the fungus *Metarhizium*. (http://www.publish.csiro.au/helix/cf/issues/th46a2.cfm).

7.3.2. Genetics of Conidiation in *Aspergillus nidulans* and *Neurospora crassa*

Conidiation occurs due to temporal and spatial regulation of gene expression that activates cell specialization and intercellular communication of cells during the life stages of many fungi (Adams *et al.*, 1998). The genetic mechanisms controlling and regulating these processes have been extensively studied and examined in the greatest detail in two filamentous fungi, *A. nidulans* and *N. crassa*.

A. nidulans

Genetic and biochemical studies have been very helpful to understand the genetic regulation of development of conidiation of *A. nidulans*. There are approximately 1,200 diverse mRNAs that appear to be associated with conidiation (Timberlake, 1990), but so far, researchers have only determined the function of a few of these genes (reviewed in Adams *et al.*, 1998). In *A. nidulans*, researchers have identified three conidial-specific genes. The best characterized of the three is *brlA*, which encodes a zinc-finger transcription factor and is required as early in the development of conidial formation as the time of conidiophore formation (Boylan *et al.*, 1987). Analyzing conidiophores from *brlA* mutants demonstrated how this gene controls conidial development. The structures grew indeterminately and failed to further differentiate into conidial-bearing cells (Boylan *et al.*, 1987; Timberlake, 1990). Overexpression of *brlA* further showed that this gene is necessary for conidiophore development. Transformed *A. nidulans* expressing *brlA* under control of an alcohol-inducible promoter, *alcA*, this construct produced viable conidia after it was switched from a medium containing glucose to one with threonine (Adams *et al.*, 1998). The *brlA* gene is required for full activation of other central regulators involved in the process of conidiation. The *abaA*

gene encodes a developmental regulator that is activated by *brlA* during phialide differentiation in conidiophore development (Boylan *et al.*, 1987, Adams *et al.*, 1998). Phenotypically, a*baA* mutants form normal conidiophore stalks and vesicles, but do not differentiate into sporogenous phialides (Boylan *et al.*, 1987).

Another well-studied conidial-specific gene is *wetA*, which is required late in conidial development (Boylan *et al.*, 1987; Timberlake, 1990). Phenotypes of *wetA* mutants make a normal conidiophore, but produce defective conidia (Boylan *et al.*, 1987). There are also two other developmentally regulated genes; *stuA* and *medA*, which are involved in establishing spatial organization of conidiophore growth, but are not directly required for conidiation (reviewed in Adams *et al.*, 1998). Mutational analysis showed that these two mutants still had the ability to produce some viable conidia.

Osherov *et al.* (2002) used SSH to identify conidial-specific transcripts in *A. nidulans*. Conducting a search on protein homology, researchers found that one of the genes was similar to a plant thaumatin-like protein and four genes encoded metabolic enzymes. The other six genes found in this study showed no significant homology to any matches and are believed to be novel genes.

N. crassa

Several genes specifically involved in conidiation have been characterized in the ascomycetous fungus, *N. crassa.* Berlin and Yanofsky (1985) found eleven transcripts, *con-1* to *con-11a* that hybridized to DNA clones containing genes expressed preferentially in conidiating cultures and not during mycelial growth. Roberts *et al.* (1988) found that *con-10* and *con-13* genes were induced coordinately during the later stages of conidiation. More specifically, *con-10* gene was weakly expressed in mycelia and induction occurred due to light, while it is upregulated during macroconidiation

(Roberts *et al.*, 1988). Homology searches showed sequence similarities to a starvation and stress-induced gene found in *Bacillus subtilus*. Mutants of *con-10* indicated that the gene plays a vital role after hyphal development (Roberts *et al.*, 1988).

Further studies have shown that *con-6* is upregulated in the latter stages of conidiation and in mature conidia, but degraded upon spore germination (White & Yanofsky, 1993). Researchers believe that it is possible that *con-6* may protect conidia from desiccation, but mutational analyses of *con-6* have shown no effect on conidia viability or germination (White & Yanofsky, 1993).

8. MATERIALS AND METHODS

8.1. Fungal strains, insect species and growth conditions

M. anisopliae strain, Ma2575 was obtained from USDA-ARS Collection of Entomopathogenic Fungi (Ithaca, New York) and grown on potato dextrose agar (PDA)(appendix B) medium at 27 °C. The cultures containing conidiating fungi were stored at 4 °C. A loopful of the fungus was suspended in 3 ml of 0.01 % Triton-X-100. Fifty microlitres of the conidia suspension (10^7 conidia ml^{-1}) was spread-plated onto PDA and Yeast Peptone Dextrose (YPD) agar media (appendix A) and incubated at 27 °C. The waxworm larvae, *Galleria mellonella* were obtained from Peterborough Live Bait (Peterborough, Ontario).

8.2. Determination of growth and conidiation

Conidium production by cultures grown on agar media was assessed daily over an 8-day period. A loopful of the fungus was taken daily from the YPD and PDA media to check for the presence of conidia using a microscope. To quantify conidium production, a 2.54 cm^2 plug was pressed down halfway from the center of the colony. The contents of the plug were then placed in 5 ml 0.01 % Triton X-100 in a 50 ml polystyrene tube and homogenized with an electric homogenizer (Greiner Scientific Corporation New York, N.Y., 110 volts, A.C-D.C) for 1 minute. Conidia were counted using a haemacytometer and the number of conidia per cm^2 calculated. At least four replicates were counted per sample (Appendix F & G). T-tests and ANOVA analysis with Scheffe's post-hoc comparison were performed.

8.3. RNA Isolation.

RNA was prepared from 3- and 4-day old cultures of mycelia grown on YPD and conidia grown on PDA. The fungal material was scraped from the agar media and ground

with liquid nitrogen to a powder in a prechilled mortar. Total RNA was extracted using TRI REAGENT and methods previously described (Sigma) except that during RNA precipitation, the sample with 2.5 ml isopropanol was placed at –20 °C for one hour. The total RNA pellet was then resuspended in 500 µl of 0.1 % DEPC (diethyl pyrocarbonate)-treated water. Poly $(A)^+$ RNA was isolated from total RNA using mRNA purification kit (Qiagen) according to manufacturer's instructions and eluted in 100 µl of preheated (70 °C) elution buffer. Concentration and purity of both total RNA and Poly $(A)^+$ RNA were determined by measuring the absorbance $A_{260/280nm}$ and by electrophoresis of RNA samples on a 1 % native agarose gel. All RNA samples were stored at –80 °C.

8.4. cDNA synthesis

Single-stranded conidial (tester) and mycelial (driver) cDNA were prepared from 2 µg of poly $(A)^+$ RNA using Omniscript RT (Qiagen) and 1 ng of oligonucleotide cDNA synthesis primer Pr16: 5′- $TTTTGTACAAGCTT_{30}$-3′ (Diatchenko *et al.*, 1996). This oligo dT primer is hybridized with the poly $(A)^+$ tail of the mRNA. Second-strand cDNA was then synthesized according to the PCR-Select cDNA subtraction kit (BD Biosciences, Clontech). Twenty micrograms of total RNA from both conditions were also reverse transcribed to ds cDNA and the reaction was terminated with EDTA/NaOH. The resulting cDNA pellet was dissolved in 10 µl of distilled, deoinized water (ddH_2O) and stored at –20 °C.

8.5. Suppression subtraction hybridization (SSH)

SSH was performed using PCR-Select cDNA Subtractive protocol (BD Biosciences, Clontech) and methods previously described (Diatchenko *et al.*, 1996). Fig. 8.1 gives an outline of the steps involved in subtractive hybridization; however, modifications to the methods are outlined below. Oligonucleotides used in SSH are

shown in Table 8.1. Driver and tester cDNA were digested overnight at 37 °C using 15 units of *RsaI* enzyme (New England Biolabs) in a 50-µl-reaction mixture. The digested cDNA was phenol-extracted and ethanol precipitated according to PCR-Select cDNA subtraction protocol and resuspended in 7 µl of ddH_2O. This above step was repeated if the digestion was incomplete. One microlitre of *RsaI* digested tester cDNA was diluted in 5 µl of ddH_2O and then 2 µl of this diluted mixture was ligated to 2 µl of adaptor 1 and adaptor 2 in separate reactions with 0.5 units of T_4 DNA ligase and ligase buffer (New England Biolabs) in a total volume of 10 µl. A parallel reverse subtraction using the driver cDNA was used as a control. The ligation reaction mixtures were incubated at rat 27°C for 2 hours and subsequently placed at 8 °C overnight. After ligation, 1 µl of 0.2M EDTA was added and ligation samples were heated at 70 °C for 5 minutes to inactivate the ligase and stored at –20 °C. A ligation efficiency test was also performed to verify that the cDNAs had both adaptors on both ends.

RsaI digested driver cDNA (1.5 µl) was added to two separate tubes containing 1.5 µl of adaptor 1- and adaptor 2- ligated tester cDNA samples in 1 µl of hybridization buffer. The samples were overlaid with a drop of mineral oil and denatured at 98 °C for 1.5 minutes, then annealed for 7 hours at 68 °C. Following the first hybridization, 1 µl of *Rsa*I digested driver cDNA was added to 1 µl of hybridization buffer and incubated at 98 °C for 1.5 minutes. The two hybridized samples were immediately mixed simultaneously in the freshly heat-denatured driver, overlaid with mineral oil and incubated overnight at 68 °C. After the second hybridization, the samples were diluted in 200 µl of dilution buffer, heated at 72 °C for 7 minutes and stored at –20 °C.

Table 8.1. Oligonucleotides used in SSH (Diatchenko *et al.*, 1996).

Adapters:	
Adapters-1	5'-GTAATACGACTCACTATAGGGCTCGAGCGG- CCGCCCGGGCAGGT-3' 3'-CCCGTCCA-5'
Adapters-2	5'-TGTAGCGTGAAGACGACAGAAAGGGCG- TGGTGCGGAGGGCGGT-3' 3'-GCCTCCCGCCA-5'
PCR primers:	
P1	5'-GTAATACGACTCACTATAGGGC-3'
P2	5'-TGTAGCGTGAAGACGACAGAA-3'
PN1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
PN2	5'-AGGGCGTGGTGCGGAGGGCGGT-3'

8.6. PCR amplification

Following subtraction, conidial- and mycelial-associated cDNAs were subjected to two rounds of PCR amplification. For the primary PCR, 4 µl of hybridized sample was added to a total volume of 25 µl containing 0.5 µl of primer P1 (10 µM) and P2 (10 µM) and 20 µl of PCR master mix prepared according to the Advantage cDNA PCR kit (BD Biosciences, Clontech). Samples were incubated at 75 °C for 7 min to extend adaptors and PCR was subsequently performed with the following parameters: 30 cycles at 91 °C for 30 sec, 68 °C for 30 sec, 72 °C for 2.5 min followed by a final extension at 68 °C for 7 min. One microlitre of the amplified product was used as a template for secondary PCR under the same protocol, except that primers PN1 and PN2 were used in the reaction mix and only 12 cycles were performed. Eight microlitres of the secondary PCR products were analyzed by 1 % agarose gel electrophoresis, viewed under UV light to visualize any bands and photographed using Multianalyst software and BioRad Gel Doc 1000.

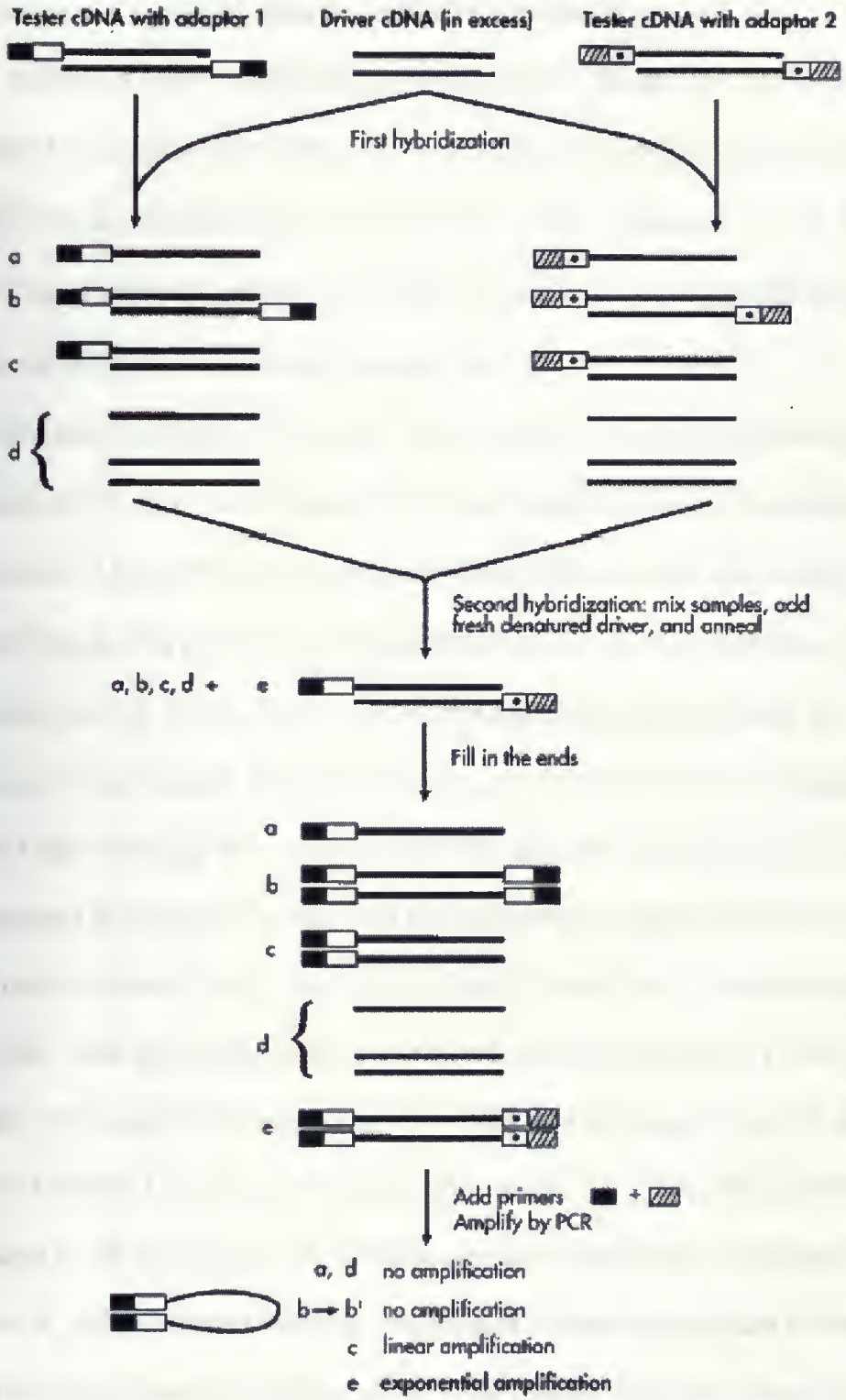

Fig. 8.1. Shows an overview of the suppression subtractive hybridization (SSH) method. The solid lines represent the *Rsa* I digested tester or driver cDNA. Solid boxes represent the outer part of the adaptor 1 longer strand and corresponding PCR primer SHPR1 sequence. Clear boxes represent the inner part adapters and corresponding nested PCR primers SHPR3 and SHPR4. Note that after filling in the recessed 3` ends with DNA polymerase, types a, b, and c molecules having adapter 2 are also present but are not shown (Diatchenko *et al.*, 1996).

8.7. Cloning and screening of subtracted cDNA products.

Subtracted cDNA fragments were excised from the gel and purified using a gel extraction kit (Qiagen) and eluted with 50 µl of ddH_2O. The cDNAs were ligated into pGEM-T vector (Promega) in a total volume of 10 µl containing 5 µl of 2X ligation buffer, 1 µl of pGEM-T vector, 1 µl T4 DNA ligase and 3 µl of purified PCR product. The ligation mixture was incubated overnight at 4 °C.

To make competent cells, XL1 Blue *Escherichia coli* cells (Strategene) were inoculated into 5 ml of Luria Bertani (LB) broth (appendix C) and incubated at 37 °C, 250 rpm for 16 hours (New Brunswick G24 Environmental Incubator Shaker). After 16 hours incubation, 200 µl of the culture was added to 5 ml of fresh LB broth and incubated for another 3 hours, 250 rpm at 37 °C. The remaining culture was placed at 4 °C. After the 3-hour growth period, the culture was placed on ice for 5 min. The culture was aliquoted and centrifuge for 1 min at max speed (Qualitron Centrifuge). The supernatant was discarded and the pellet in each tube resuspended by pipeting up and down in 1 ml of cold Buffer A (100mM NaCl, 5mM $MgCl_2$, 5mM Tris-HCl, pH 7.5) and incubated on ice for 10 min. After the 10-min incubation, the cells were centrifuged at 13200 rpm for 10 min (IEC, Micromax). The supernatant from each tube was again discarded and 1 ml of Buffer B (100mM $CaCl_2$, 5mM $MgCl_2$, 5mM Tris-HCl, pH 7.5) was added to the tube and placed on ice for 30 min. One hundred and ninety microlitres of the now competent XL Blue *E. coli* cells were added to the plasmid ligation mixture and incubated for 1 hour. The mixture was heat shocked at 42 °C for exactly 90 sec and placed immediately on ice for 5 min. Two hundred microlitres of the mixture (competent cells + ligation mixture) was added to 800µl of LB broth and incubated for 1 hour at 37 °C, 250 rpm. The transformed bacterial cells (2 x 25 µl, 50 µl, 100 µl, 150 µl) were spread-plated onto

LB agar plates containing ampicillin (100μg ml^{-1}), IPTG (50 μl of 100nM stock) and X-Gal (20 μl of 50mg ml^{-1} stock)(appendix D & E).

Bacterial cells that were transformed were visible after overnight incubation at 37 °C. Individual white recombinant clones were randomly picked, inoculated to 5 ml of LB broth with 10 μl of 100 μg ml^{-1} ampicillin and incubated for 16 hours at 37 °C. Plasmid DNA was isolated from recombinant clones using plasmid miniprep (Qiagen) and checked for putative subtracted cDNA inserts by digestion with 0.5 units of *Eag1* in a 10 μl reaction mixture. The samples were incubated overnight at 37 °C. After incubation, both undigested and digested plasmids were analyzed on a 1 % agarose gel.

8.8. Sequence analysis

Plasmids with inserts were sequenced by Core Molecular Biology Facility at York University (Toronto, Ontario) using T7 and/or SP6 sequencing primers. Nucleotide sequences derived were used to search the nucleic acid databases for homology using the BLAST program at the National Center for Biotechnology Information (NCBI, Bathesda, MD).

8.9. RT-PCR analysis of conidial-associated transcripts

Total RNA was prepared from day 2, 3, 4, 5 and 6 mycelial and conidiating cultures grown on YPD agar and PDA respectively at 27 °C. RNA was extracted from frozen fungus using RNeasy mini-kit (Qiagen) and all samples were DNase-treated using RNase-Free DNase I (Qiagen). RT-PCR analysis was performed to compare transcript abundance across multiple samples using One-Step RT-PCR Kit (Qiagen) with modifications. Thirteen nanograms of total RNA was added to a 50 μl reaction mixture with 10 μl 5X One-Step RT-PCR buffer, 2 μl dNTP mix (containing10 mM of each dNTP), 0.5 μl each of the forward and reverse primers, 2 μl of enzyme mix and 34 μl

RNase-free H_20. Also RT-PCR was prepared using a co-amplified internal control for sample normalization. The quantity of reagents used was the same as stated before, however, 0.5 µl each of the internal control primers were added to the reaction mix with a total volume of 50 µl. The primers were designed from the sequences obtained from SSH and using the program PRIMER3 (Rozen & Helen, 2000). The primers used are shown in Table 8.2. Annealing temperature of the primers was determined by gradient PCR at 55 °C +/- 5 °C. All samples were subjected to One-Step RT-PCR under the thermal cycling conditions shown in Table 8.3. RT-PCR was not done to saturation, and was tested with the control, β-actin in order to determine the exponential phase of the amplification. The exponential phase of amplification occurs in the PCR cycles when reaction components are still in excess and the PCR products are accumulating at a constant rate. PCR amplification was performed using *Taq*-Master Mix (Qiagen) and aliquoted into 15 µl volumes. The following PCR amplification conditions were used: 95 °C for 8 min; 31 cycles at 95 °C for 15 sec; 61 °C for 20 sec; 72 °C for 1 min; and a final extension at 72 °C for 5 min. Each 15 µl volume aliquot was removed from the thermal cycler after cycle 10, 12,14,16, 18, 20, 24, 26, 30 and 31 and resolved by electrophoresis on 1 % agarose gel.

Table 8.2. Primers used in RT-PCR. The primer sequences used to assay gene expression by RT-PCR are shown with the optimal annealing temperatures.

Homolog	Primer	Primer Sequence (5'-3')	Optimal annealing temp (°C)	Predicted Product size (bp)
Control: β-actin	ACT-512F ACT-783R	ATGTGCAAGGCCGGTTTCGC TACGAGTCCTTCTGGCCCAT	61	150
Subtilisin-like protease 1	MET-PR2 MET-PR5	AGGTAGGCAGCCAGACCGGC TGCCACTATTGGCCGGCGCG	58	800
Catalase	CAT-F CAT-R	CTTACCCGACACGATGGACGT AGCTTGGCGTAATCATGGTC	46	410
Cell-wall plasma membrane linker protein	CELL-F CELL-R	GTGAGTGAGGAAGCGGAAGA AGCGCTTAAGGGTTAGGGTC	50	395
Wingless protein	WING-F WING-R	TGGTACTGCGAGGACAAGTG AAGGATTCCGGGTGGATTAG	58	480
Replication initiation gene A protein	REP-F REP-R	CGAGGTCCATTAGCTGTACCA GGCAGGTCCTCCACTATGA	56	315
Putative protein	STR-F STR-R	ACAACGTGGAGGGCAGAGT GCCGAGGTCCTCTATAAAGCT	46	259
Envelope protein	XYL-F XYL-R	CGACCACGCTAACCCTATGA CGGTGGAATATGGATGGTCTT	48	244

Table 8.3. Thermal cycling conditions used in the One-Step RT-PCR for various transcripts

PCR steps	ACT	PR1	CAT	CELL	WING	REP	STR	XYL
Reverse transcription	50 °C 30 min	50 °C 30 min	50 °C 30 min	50 °C 30 min	50 °C 30 min	50 °C 30 min	50 °C 30 min	50 °C 30 min
Initial PCR activation step	95 °C 15 min	95 °C 15 min	95 °C 15 min	95 °C 15 min	95 °C 15 min	95 °C 15 min	95 °C 15 min	95 °C 15 min
Initial Denaturation	95 °C 8 min	94 °C 4 min	NA	NA	NA	NA	NA	NA
Denaturation	95 °C 15 sec	94 °C 1 min	94 °C 1 min	94 °C 1 min	94 °C 1 min	94 °C 30 sec	94 °C 30 sec	94 °C 30 sec
Annealation	61 °C 20 sec	58 °C 1 min	46 °C 1 min	50 °C 1 min	58 °C 1 min	58 °C 30 sec	46 °C 30 sec	48 °C 30 sec
Extension	72 °C 1 min	72 °C 2 min	72 °C 2 min	72 °C 2 min	72 °C 2 min	72 °C 2 min	72°C 2 min	72°C 2 min
Number of cycles	26	30	35	35	35	34	34	34
Final extension	72 °C 5 min	72 °C 6 min	72 °C 5 min	72 °C 5 min	72 °C 5 min	72 °C 5 min	72°C 5 min	72°C 5 min

Symbols: ACT, control β-actin; PR1, subtilisin-like protease 1; CAT, catalase; CELL, cell wall plasma membrane linker protein; STR, putative protein; XYL, envelope protein; REP, replication initiation gene A protein; WING, wingless protein; NA, not applicable.

8.10. *In vivo* expression of *Pr1* gene of infected waxworm larvae (*Galleria mellonella*)

G. mellonella was topically infected with conidia of *M. anisopliae*. One hundred microlitres of 3.55 x 10^8 conidia ml^{-1} suspension of the fungus was spread plated onto several PDA plates and then incubated at 27 °C for 10 days. One hundred insects were rolled on the conidiating culture, individually placed into sterile plastic vials and incubated at 27 °C. Different stages of infection of the insect were monitored over a 14-day period. At death, the infected insects were surface sterilized by immersing the dead insect for 1 min in 1ml of 1 % NaOCl followed by 2 washings of each in 1 ml of sterile distilled H_2O. After surface sterilization, the insects were placed in clean sterile plastic vials with sterilized moist paper. The infection stages were categorized as follows: 1) 24-hour post infection, 2) melanization of the infected insect (24-48 hours), 3) death (48 – 96 hours), 4) growth of mycelia on the surface of the cadaver (96 – 216 hours) and 5) conidial growth on the surface of the cadaver (+216 hours). At each of the observed infection stages, total RNA was extracted from 7 – 10 insects comminuted in liquid nitrogen using RNeasy mini-kit (Qiagen) and all samples were DNase-treated using RNase-Free DNase I (Qiagen). RNA was also extracted from fungal material scraped from the surface of the cadaver. RT-PCR analysis of the *Pr1* gene was performed to compare transcript abundance across infection stages of the insects. Total RNA was added to a 50µl reaction mixture containing: 10 µl 5X One-Step RT-PCR buffer, 2 µl dNTP mix (containing 10 mM of each dNTP), 0.5 µl each of primers MetPr 2 and MetPr 5 (Table 8.2), 2 µl of enzyme mix and 34 µl RNase-free H_20. An uninfected insect was used as a control and RT-PCR was also carried out as previously described.

9. RESULTS

9.1. Qualitative and Quantitative Aspects of Conidium Production

Metarhizium strain Ma2575 was grown on two different solid substrates, YPD and PDA to observe conidiation over time. When grown on YPD, a nitrogen and carbohydrate-rich substrate, thick, white mycelial fuzz formed (Fig. 9.1a). However, when the fungus was grown on PDA, a nutrient-limiting medium, conidia were induced and seen dark in colour (Fig. 9.1b).

Qualitative analysis was carried out to determine the earliest day of substantial increase in conidium production or mycelial growth when cultures were grown on PDA and YPD respectively. Microscopically, on the YPD medium, mycelia grew in long hyphal-branched extensions and were abundant by day 4, however no conidial growth was observed. In contrast, cultures grown on PDA had large amounts of conidia (oval-shaped) by Day 3 compared to the previous days (Fig. 9.2).

Fig. 9.3 shows conidium production by *M. anisopliae* when grown on YPD and PDA. Conidium production was significantly greater on PDA than on YPD each day up to day 8 ($t= 0.000312$, $P=<0.001$) (appendix I). Conidial counts were performed to quantitatively determine the earliest day that there was a significant increase in conidium production on PDA. Conidial growth was observed in the first four days; therefore only this time period was taken into account for statistical analysis. ANOVA analysis indicated the first significant difference in conidium production was between days 2 and 3 on PDA ($F= 251.48$, $p< 0.0001$) (Scheffe's post-hoc comparison) (appendix H). RNA extraction was therefore performed for day 3 cultures on PDA, which was the earliest day of significant conidium production. RNA was also extracted from day 4 cultures grown on YPD where mycelial growth was abundant.

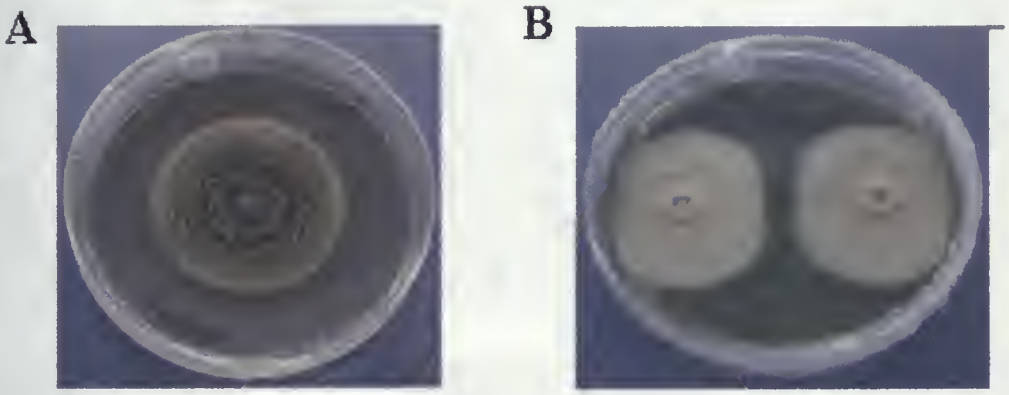

Fig. 9.1. 10-day old *Metarhizium anisopliae* strain Ma2575 grown on (A) PDA and (B) YPD agar.

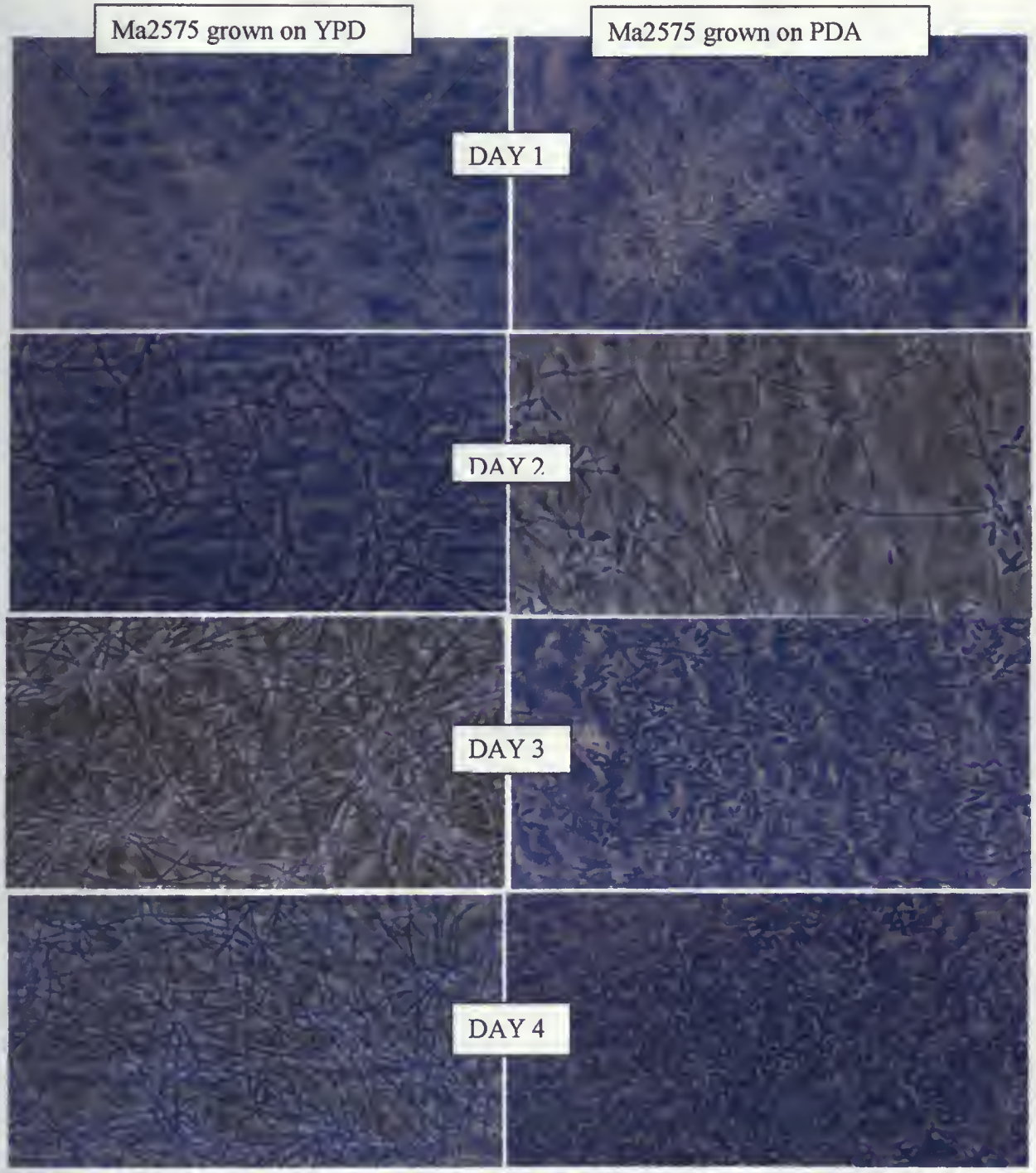

Fig. 9.2. Observations made under a microscope of Ma2575, which was grown on YPD agar and PDA over a 4-day period. The long hyphal extensions represent mycelial growth and the oval shape structures represent conidial development.

Fig. 9.3. A comparison of conidium production of *Metarhizium anisopliae* grown on PDA and YPD media over an 8-day period □ PDA ■ YPD

9.2. RNA isolation analysis

Total RNA was extracted on day 3 (Fig 9.4A) and on day 4 (Fig 9.4A) of Ma 2575 grown on PDA and YPD plates respectively. The quality of the total RNA was examined by electrophoresis on 1 % native agarose gel. Usually, when assessing the quality of total RNA, a denaturing agarose gel with formaldehyde is used, since most RNAs have secondary structures, which prevent it from migrating according to size as with DNA. These gels are primarily used with Northern Blots, however, this study, only concerned with the quality of the total RNA in terms of observing the possibility of extensive degradation and bands corresponding to ribosomal RNA. In addition, numerous RNA isolations were performed so it was easier to use native agarose gels since it was less time consuming and safer. Also, these gels are sufficient in assessing the quality of total RNA. Total RNA exhibited distinct bright smears, which correspond to 28S and 18S ribosomal RNA. The purity and concentration of RNA was determined on GeneQuant.

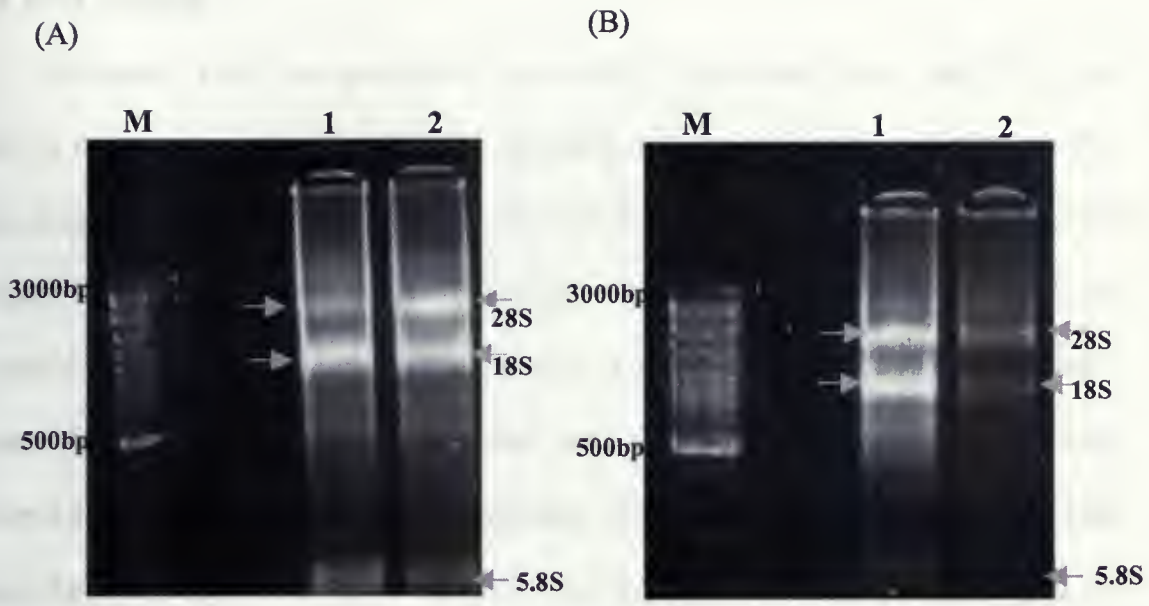

Fig. 9.4. 1 % agarose gel of Ma2575 total RNA extracted from conidia and mycelia and mRNA isolated from total RNA on Day 3 (A) and Day 4 (B). Lane M: Lambda DNA/*Eco*471 Marker. (A) Lane 1: Total RNA extracted from conidia with a ratio $A_{260/280nm}$ of 0.912, RNA concentration of 755.4µg/ml. Lane 2: Total RNA extracted from mycelia with a ratio $A_{260/280nm}$ of 0.265, RNA concentration of 502.4µg/ml. (B) Lane 1: Total RNA extracted from mycelia with a ratio $A_{260/280nm}$ of 1.464, RNA concentration of 27371.4µg/ml. Lane 2: Total RNA extracted from conidia with a ratio $A_{260/280nm}$ of 0.1687, RNA concentration of 6148.4µg/ml. The arrows shown correspond to the ribosomal bands 28S and 18S and 5.8S.

9.3. SSH Analysis

Secondary PCR was performed from cDNA synthesized from total RNA and mRNA isolated from day 3 and day 4. Mycelial and conidial subtracted cDNAs were cloned into pGEM-T vector and transformed into *E. coli* XL1 Blue competent cells. PCR amplification showed the presence of multiple bands, which represented conidial and mycelial subtracted cDNA transcripts. A total of 9 conidial cDNA subtractions and 3 mycelial cDNA subtractions were carried out. One to 7 well-defined bands were observed on a 1% agarose gel and co-migrated with sizes ranging from 50 to 1500 base pairs (bp) (Fig. 9.5 – 9.8). Several conidial and mycelial subtractions were carried out with different RNA isolations each time to determine whether there was any consistency to the number of fragment sizes derived from the secondary PCR or whether more rare transcripts could be generated. Some fragments that were observed to have the same size in different subtractions represented the same PCR product when sequenced. For example, transcripts C2-3, C3-3, C4-2, C6-1, C7-70* which were generated from C2, C3, C4, C6 and C7 conidial subtractions respectively, when sequenced, blast search results showed homology to a small subunit rRNA sequence of *Cordyceps taii*. A summary of all fragments excised is shown in Table 9.1.

Over 700 clones were screened for transformants, only two examples of the recombinant plasmids digested with *Eag*I and inserts were shown in Fig. 9.9 - 9.10. There was a total of 736 transformants randomly selected and 91 plasmids were present. From these, 10 plasmids with inserts were sequenced for mycelial-subtracted cDNA clones (Table 9.2 - 9.4) and 26 for putative conidial-associated transcripts (Table 9.5 – 9.7). Summaries of all screening results for both conditions are shown in Table 9.8.

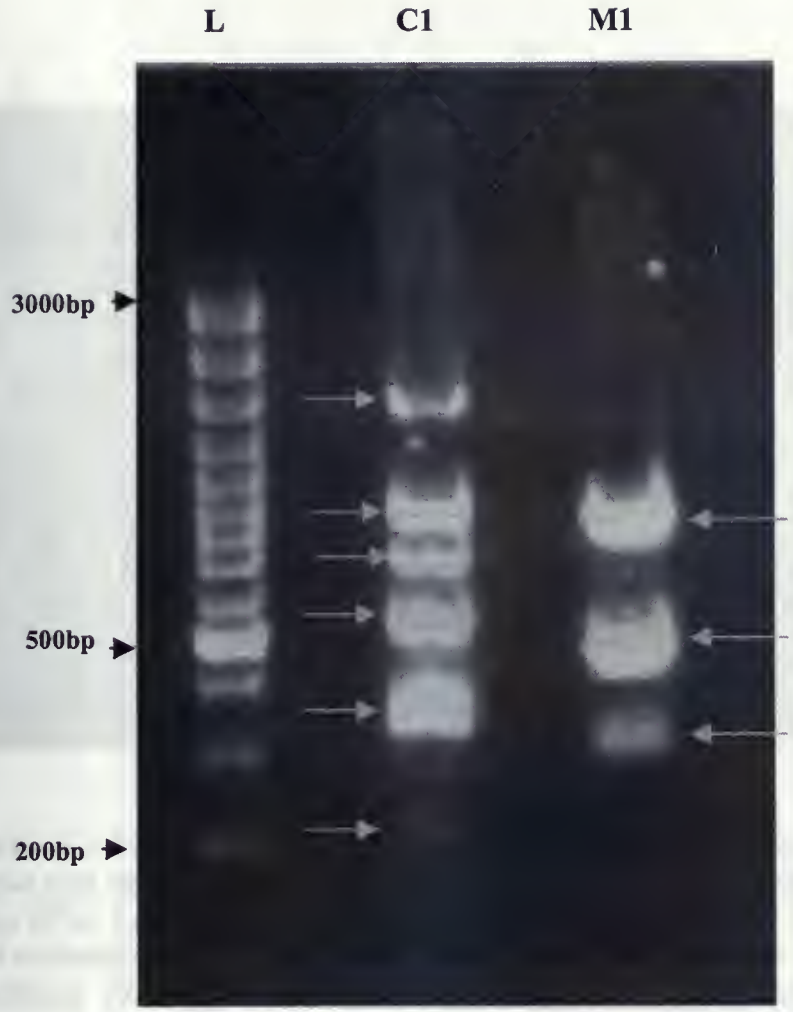

Fig. 9.5. A 1 % agarose gel of secondary PCR product of the subtracted Ma2575 conidial and mycelial-associated cDNA from total RNA extracted on Day 3 and 4 respectively. Lane C1: Ma2575 subtracted conidial-associated cDNA Day 3. Lane M1: Ma2575 subtracted mycelial-associated cDNA, Day 4. Bands from both lanes represent differentially expressed sequences. C= conidia and M= mycelia. The numbers represent the number of conidial or mycelial cDNA subtractions performed. Lane L: 100 bp DNA Ladder Plus. Arrows represent bands excised, purified and cloned.

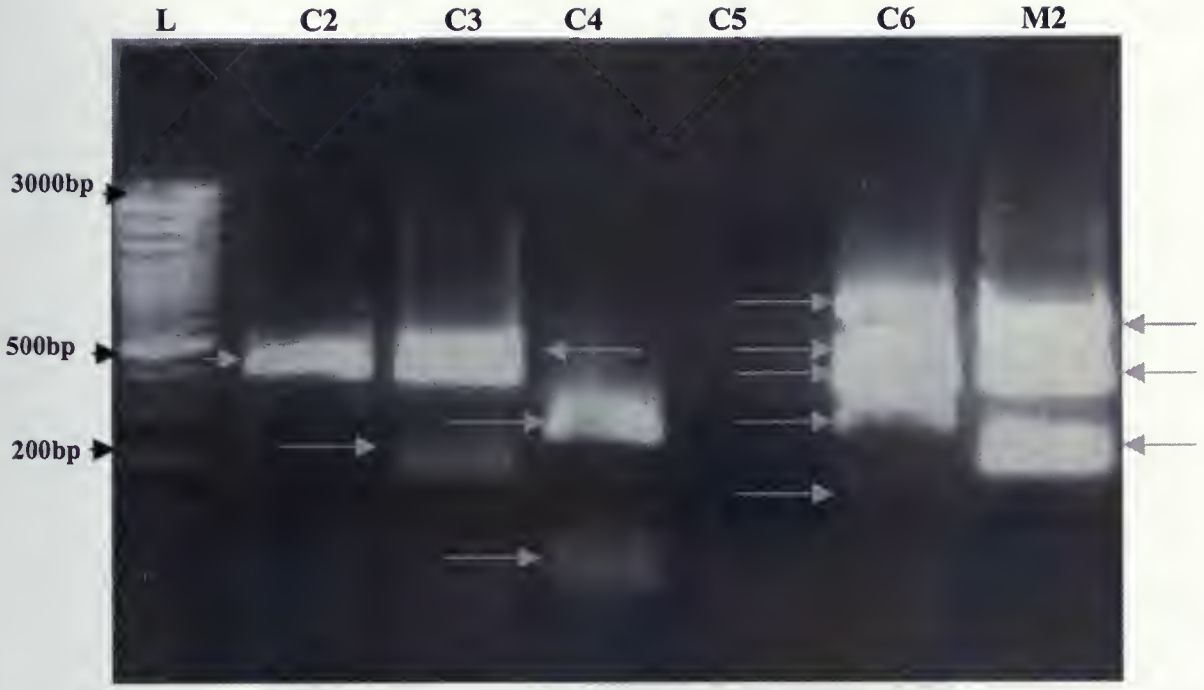

Fig. 9.6. A 1 % agarose gel of secondary PCR product of the subtracted Ma2575 conidial and mycelial cDNA (from total RNA) from various subtractions. Lane L: 100 bp DNA Ladder Plus. Lane C2: Ma2575 subtracted conidial-associated cDNA Day 3 (hybridized 02/06/23). Lane C3: subtracted conidial-associated cDNA, Day 3 (hybridized 02/07/09). Lane C4: subtracted conidial-associated cDNA, Day 3 (hybridized 02/07/30). Lane C5: subtracted conidial-associated cDNA, Day 4 (hybridized 02/07/30). Lane C6: subtracted conidial-associated cDNA, Day 3 (hybridized 02/10/29). Lane M2: subtracted mycelial-associated cDNA, Day 4 (hybridized 02/07/09). C= conidia and M= mycelia. The numbers represent the number of conidial or mycelial cDNA subtractions performed. Bands from both lanes represent differentially expressed sequences. Arrows represent bands excised, purified and cloned.

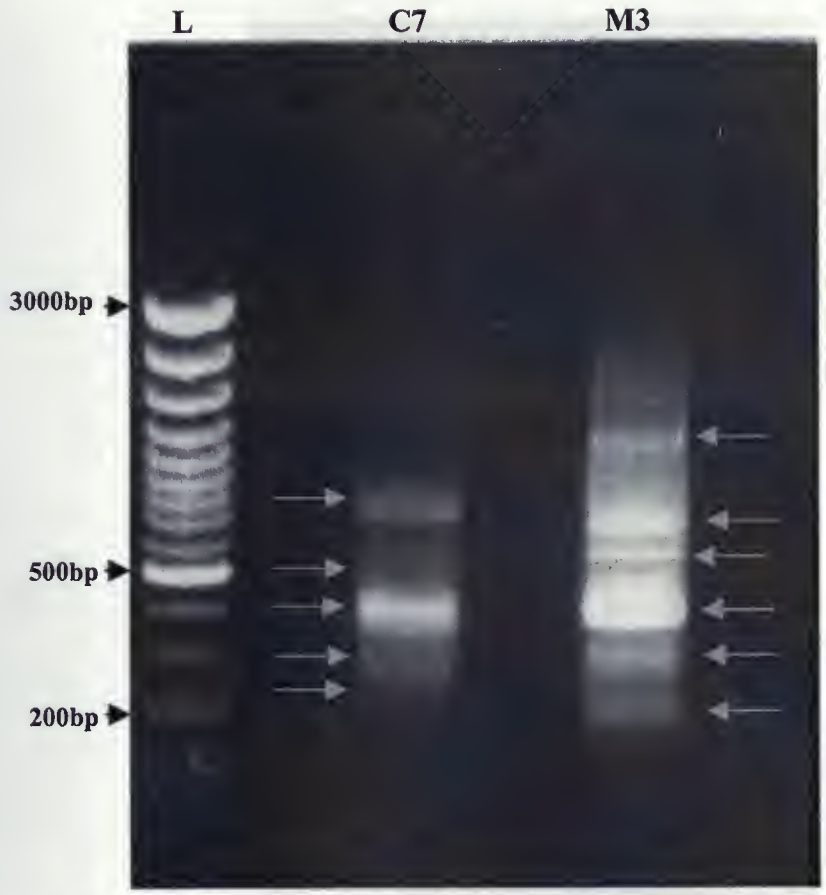

Fig. 9.7. A 1 % agarose gel of secondary PCR product of the subtracted Ma2575 conidial and mycelial-associated cDNA from mRNA extracted on Day 3 and 4 respectively. Lane L: 100 bp DNA Ladder Plus. Lane C7: Ma2575 subtracted conidial-associated cDNA Day 3. Lane M3: Ma2575 subtracted mycelial-associated cDNA, Day 4. C= conidia and M= mycelia. The numbers represent the number of conidial or mycelial cDNA subtractions performed. Bands from both lanes represent differentially expressed sequences. Arrows represent bands excised, purified and cloned.

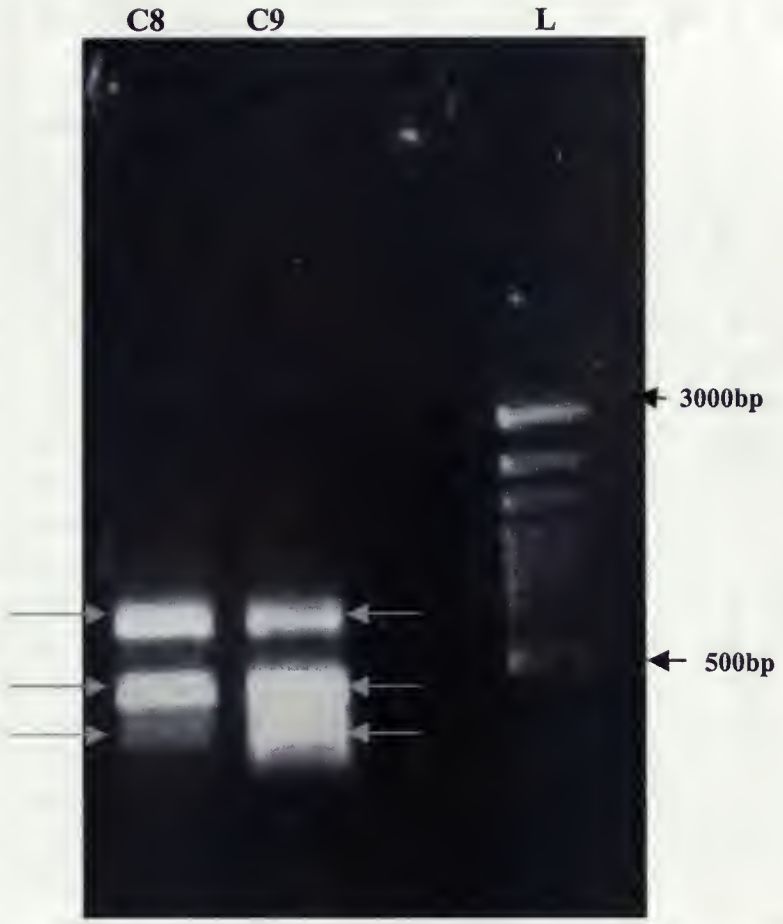

Fig. 9.8. A 1 % agarose gel of secondary PCR product of the subtracted Ma2575 conidial-associated cDNA from mRNA and total RNA extracted on Day 3. Lane C8: Ma2575 subtracted conidial-associated cDNA from mRNA extracted Day 3. Lane C9: Ma2575 subtracted conidial-associated cDNA from total RNA Day 3. Lane L: 100 bp DNA Ladder Plus. Bands from both lanes represent differentially expressed sequences. C= conidia and M= mycelia. The numbers represent the number of conidial or mycelial cDNA subtractions performed.

Table 9.1. A Summary of all fragment sizes from conidial and mycelial cDNA subtractions after secondary PCR amplification.

# of subtractions / Approximate fragment size (bp)	C1	C2	C3	C4	C6	C7*	C8*	C9	M1	M2	M3*
50	-	-	-	+	-	-	-	-	-	-	-
150	-	-	-	-	+	-	-	-	-	-	-
200	-	-	+	-	-	-	-	-	-	+	+
225	+										
250	-	-	-	+	+	+	-	+	-	-	-
300	-	-	-	-	-	+	+	-	-	-	+
325	-	-	-	-	-	-	-	-	+	-	-
350	+	-	-	-	-	-	-	-	-	-	-
400	-	-	-	-	+	+	+	+	-	-	+
450	-	+	+	-	+	-	-	-	-	+	-
500	-	-	-	-	-	+	-	-	+	-	-
550	+	-	-	-	-	-	-	-	-	-	-
600	-	-	-	-	-	-	-	-	-	+	+
650	-	-	-	-	+	-	-	-	-	-	-
700		-	-	-	-	-	-	-	-	-	+
750	+	-	-	-	-	-	-	-	-	-	-
800	-	-	-	-	-	+	+	+	-	-	-
900	-	-	-	-	+	-	-	-	+	-	-
950	+	-	-	-	-	-	-	-	-	-	-
1200	-	-	-	-	-	-	-	-	-	-	+
1500	+	-	-	-	-	-	-	-	-	-	-

Symbols;
C represents conidial cDNA subtraction
M represents Mycelial cDNA subtraction
Numbers 1 to 9 represent the number of subtractions performed
+ represents fragment present after amplification
- represents no fragment after amplification
* represents cDNA subtraction using mRNA

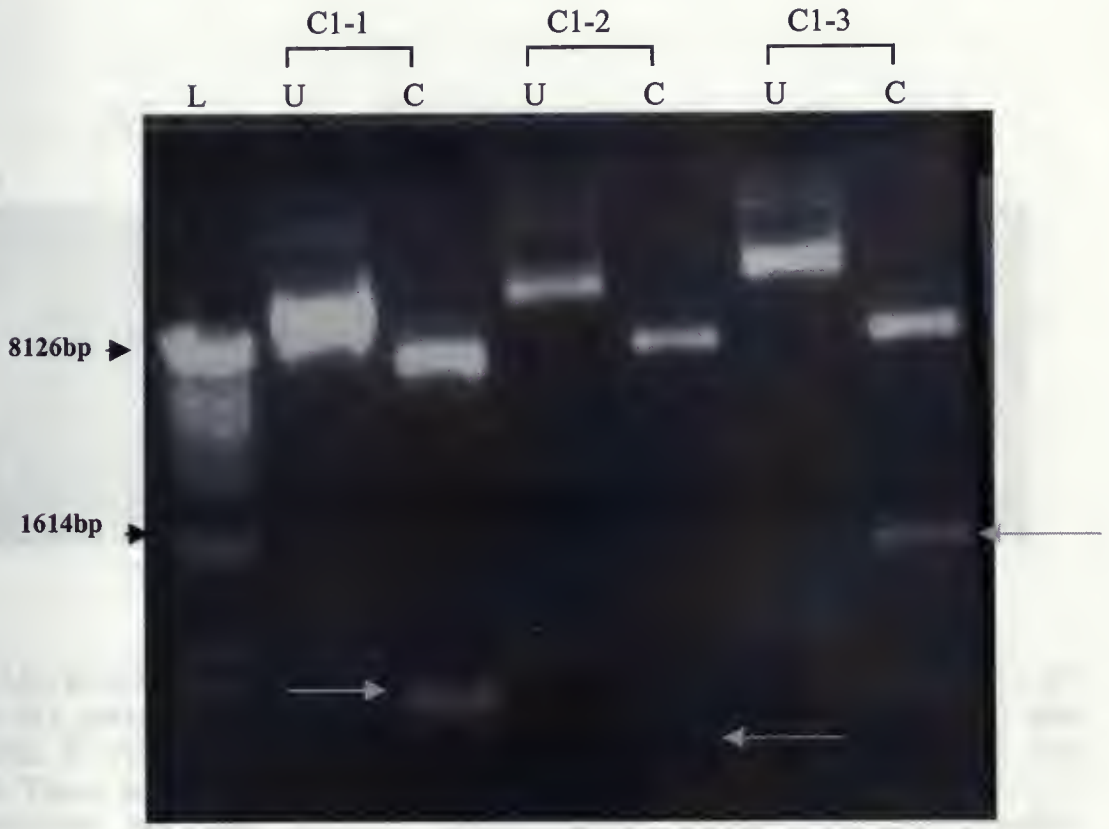

Fig. 9.9. pGEM-T plasmids containing the putative conidial-associated transcripts from C1 subtraction (from total RNA; shotgun cloning). Lane M: Lambda DNA/Eco471 Marker. U represents undigested plasmids. C represents plasmids digested with *Eag*I. Arrows indicate conidial-associated transcripts, which are named C1-1, C1-2, C1-3. These transcripts were given designated names according to number of subtractions and clone number. Inserts co-migrated to approximate sizes (bp): C1-1: 500, C1-2: 350, C1-3: 900.

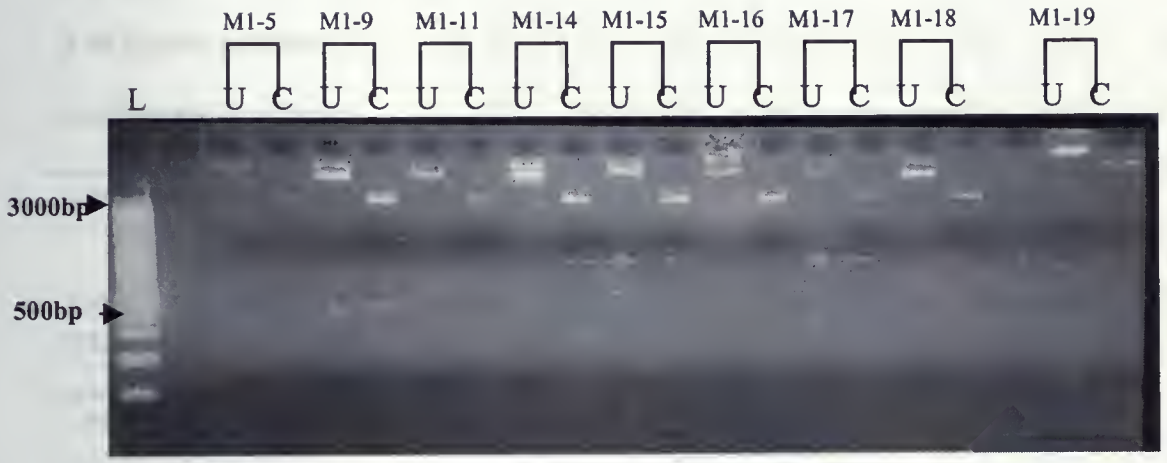

Fig. 9.10. pGEM-T plasmids containing the putative mycelial-associated transcripts from M1 subtraction (from total RNA; shotgun cloning). Lane L: 100 bp plus Marker. U represents undigested plasmids. C represents plasmids digested with *Eag*I. These transcripts were given designated names according to number of subtractions and clone number. Arrows indicate putative mycelial-transcripts. Inserts co-migrated to approximate sizes (bp): M1-9: 500, M1-12: 900, M1-15: 1000, M1-16: 1000.

Table 9.2. Shotgun cloning of putative conidial subtracted cDNA transcripts (from total RNA and mRNA)

Conidial subtracted cDNA transcripts	C1	C2	C3	C5	C6	C7*	C8*	C9
# of putative transformants	4	7	7	7	7	165	4	4
# of inserts present	3	3	2	0	3	22	4	4
# of inserts sequenced	3	3	1	0	3	5	0	0

* represents clones that were generated from mRNA for subtractive hybridization

Table 9.3. Individual fragment cloning of putative conidial subtracted cDNA transcripts (from total RNA and mRNA)

Conidial subtracted cDNA transcripts	C1	C7 *
# of putative transformants	170	82
# of inserts present	15	10
# of inserts sequenced	3	8

*represents clones that were generated from mRNA for subtractive hybridization

Table 9.4. Shotgun cloning of putative mycelial subtracted cDNA transcripts (from total RNA and mRNA)

Mycelial subtracted cDNA transcripts	M1	M2	M3 *
# of putative transformants	122	6	84
# of inserts present	17	0	2
# of inserts sequenced	4	0	1

*represents clones that were generated from mRNA for subtractive hybridization

Table 9.5. Individual fragment cloning putative mycelial subtracted cDNA transcripts (from total RNA and mRNA)

Mycelial subtracted cDNA transcripts	M2	M3*
# of putative transformants	2	65
# of inserts present	0	6
# of inserts sequenced	0	5

*represents clones that were generated from mRNA for subtractive hybridization

Table 9.6. Shows overall cloning results for conidial subtracted cDNA transcripts

# of putative transformants	457
# of inserts present	66 (14%)
# of inserts sequenced	26 (6%)

Table 9.7. Shows overall cloning results for mycelial subtracted cDNA transcripts

# of putative transformants	279
# of inserts present	25 (9%)
# of inserts sequenced	10 (4%)

Table 9.8. Shows summary of results of putative mycelial and conidial subtracted cDNA transcripts

# of putative transformants	736
# of inserts present	91(12%)
# of inserts sequenced	36 (5%)

9.4. Sequence Analysis

All sequences were used to search the DNA databases using the blast program (BLASTN) at the National Center of Biotechnology Information (NCBI). This tool is designed to explore all of the available nucleotide sequence databases. The program then selects possible sequences in a ranking system. The scores assigned in the search have a well-defined statistical interpretation and therefore detect relationships among sequences, which share only isolated regions of similarity. In this study, BLASTN was performed to detect and identify any ribosomal RNA clones and if found, no further searches would be conducted on those clones.

A total of 36 sequences were obtained from the transformants and, of these, 26 clones were found in the conidial-subtracted library, but not from mRNA transcripts from *M. anisopliae*. Of the 26 clones, (appendix J) 11 (44%) were identical to mitochondrial or small subunit ribosomal RNA sequences that are expressed in all tissues in *M. anisopliae*. Such a percentage of these matches may indicate that not all of the highly redundant clones were removed from the subtracted library as we expected.

Six clones showed very significant homology (99%) to *Homo sapiens*. For example, clone C1-1 was 485 bp in size and had an e-value of 0.0 with a high identity of 452/454. Numerous reasons could be explained for this high homology to human sequences. One could be that because of the biased nature of genebank, in which there is a high percentage of information on *Homo sapiens* in comparison to fungal genomes. Another reason could be contamination. However, questions can then arise regarding the possible sources of contamination and if it is probable that they are of my own experimental origin. Assessing the steps carried out in the SSH technique, it is very improbable that the contamination came from RNA since ubiquitous RNases would

degrade the RNA. DNA contamination could be another possibility, but again this is unlikely since the adaptors are specific for the primers used in the PCR and the probability of amplification with similar primer sequences is low. Therefore the contamination could come from external sources such as the enzymes, the Taq polymerase or the primers, which would then question the quality of the reagents and products used in the study. Another likely source could be the sequencing facility itself. In addition, it is difficult to assess these results since other publications do not report BLASTN results and negative findings. In conclusion, due to the questionable sources of these clones, they were not studied further. Nevertheless, additional studies like Northern, Southern analysis or full-length cDNA sequences could help us determine if these clones are indeed of fungal or human origin. However, since very specific fungal genes were amplified we do have credible evidence that the technique is valid.

Eight clones were from various mRNA species, however 1 clone, C7-52 was found to have the highest homology, 99.7% to a subtilisin-like serine protease, pr1 of *M. anisopliae*, but of a different strain (ARSEF 3540) to the one used in my study (2575) (Table 9.9) which could be the reason why search results was not 100% homology. In addition, a nucleotide search was performed for homology to known fungal genes for conidial subtracted cDNA clones and is shown in Table 9.10. However this search as with the BLASTN searches with all organisms showed clones with the highest homology to rRNA sequences and the clone C7-52 to be similar to a protease from *M. anisopliae* as was found in Table 9.9. Some of the other clones were found to have very low e-value, but the identities were small in comparison to the fragment size of the clone. Mycelial-subtracted cDNA clones (appendix K) were searched in the database, but only one of the clones showed homology to a *Homo sapiens* estrogen receptor gene, which was not

significant since it showed no similarity to any fungal species and the other 9 showed identical matches to *M. anisopliae* ribosomal RNA expressed in all tissues (Table 9.11).

Table 9.9. Summary of BLASTN search of putative conidial-associated transcripts that were generated by suppression subtractive hybridization.

Transcript	Size (bp)	Putative Homology[a] with Accession numbers in bold	E-value [b]	Score [c] (bits)	Identities[d]
C1-1	485	**BC013346** *Homo sapiens*, similar to FLJ00066 protein,	0.0	884	452/454
C1-2	315	**AF299314** Coliphage phiX174 isolate CMMloC, complete genome	e-145	521	269/271
C1-3	730	**AC096677** *Homo sapiens* chromosome 1 clone RP11-134G8, complete sequence	0.0	1392	711/714
		AL832006 *Homo sapiens* mRNA; cDNA	0.0	1368	708/714
C1 (1)-13	138	**AL928893** Mouse DNA sequence from clone RP23-55I14 on chromosome 2, complete sequence	0.23	42.1	22/23
C1 (1)-14	480	**AY128458** *Drosophila melanogaster wingless CG4889-PB (wg)* mRNA	4e-27	129	80/85
C1 (3)-2	259	**BX161388** Human full-length cDNA clone CS0DC006YH13 of Neuroblastoma of Homo sapiens (human)	2e-59	236	151/159
C2-2	470	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	0.0	731	384/389
C2-3	430	**AF543763** *Cordyceps taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396

C2-4	38	**AF079314** *Rattus norvegicus* nuclear factor kappa B subunit p65 (NFkB) mRNA, partial cds	6e-04	48.1	30/32
C3-3	430	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	0.0	767	390/391
C4-2	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C6-1	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C6-3	360	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	7e-93	347	198/202
C6-7	373	**AF218207** Small subunit ribosomal RNA *(M. anisopliae*)	3e-93	355	189/191
C7-70*	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C7-76*	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C7(1)-2*	747	**AB027344** *Cordyceps inegoensis* mitochondrial DNA for small subunit ribosomal RNA	0.0	1043	670/714
C7(1)-4*	825	**AC096677** Homo *sapiens* chromosome 1 clone RP11-134G8, complete sequence	0.0	1503	764/766
C7 (1)-5*	811	**AC096677** *Homo sapiens* chromosome 1 clone RP11-134G8, complete sequence	0.0	1522	771/772

C7 (1)-6*	1519	**AL832006** *Homo sapiens* mRNA; cDNA DKFZp451O223	0.0	1475	756/772
C7 (3)-3*	410	**AJ489197** Beta nana Ty1-copia-like retrotransposon partial pol pseudogene, clone Tbn-2	3e-65	265	179/193
C7 (3)-4*	395	**AB042431** Shuttle vector pSET3 DNA, complete sequence	3e-12	80	43/44
C7 (4)-8*	244	**AF180731** *Klebsiella pneumoniae* dihydrofolate reductase	1e-25	123	81/86
		S. salar microsatellite DNA, CA-repeat (AC)11.5	1e-25	123	81/86
C7-40*	88	**BT009458** *Triticum aestivum* clone wlsu2.pk0001.h3:fis, full insert mRNA	6e-04	50.1	25/25
C7-52*	800	**AY389134** *M. anisopliae var. anisopliae* pr1A gene for subtilisin-like serine protease, exons 1-4	0.0	1455	740/742
C7-97*	515	**AB027361** *M. anisopliae* mitochondrial DNA for small subunit ribosomal RNA	0.0	854	479/490

Symbols

* represents clones that were generated from mRNA for subtractive hybridization

'a' represents sequences with the highest homology to the fragments listed above. The homology is not usually the entire length of the fragment.

'b' represents E-values, which represent the number of hits one can "expect" to see just by chance. The database sequences were divided into three categories: highly significant (e-values ≤ e-20), moderately significant (e-values e-4 to e-19), and weakly significant (e-values e-2 to e-3). E-values greater than e-2 was considered not a statistically significant match.

'c' Score represents the value calculated by BLAST search of the transcripts. The score increases as the level and length of homology increases.

'd' The identity indicates the similarity of the conidial transcripts over the potential match in the base pairs.

Table 9.10. Summary of BLASTN search of fungal origin of putative conidial-associated transcripts that were generated by suppression subtractive hybridization

Transcript	Size (bp)	Putative Homology[a]	E-value [b]	Score [c] (bits)	Identities [d]
C1-2	315	**ABI293759** *A. bisporus* mRNA for putative chloroperoxidase (cpo gene)	5e-04	46.1	23/23
C1 (1)-13	138	**XM_326648** *Neurospora crassa* strain OR74A	3.6	32.1	19/20
C1 (1)-14	480	**BX295540** *N. crassa* DNA linkage group VI Cosmid contig 49D12	0.19	38.1	19/19
C1 (3)-2	259	**ABI534349** *A. bisporus* partial mRNA for putative stress protein (sp gene)	1e-04	48.1	24/24
C2-2	470	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	0.0	731	384/389
C2-3	430	**AF543763** *Cordyceps taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C2-4	38	**AJ293759** *A. bisporus* mRNA for putative chloroperoxidase (cpo gene), strain D649	0.003	40.1	20/20
C3-3	430	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	0.0	767	390/391
C4-2	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C6-1	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396

C6-3	360	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	7e-93	347	198/202
C6-7	373	**AF218207** Small subunit ribosomal RNA (*M. anisopliae*)	3e-93	355	189/191
C7-70*	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C7-76*	430	**AF543763** *C. taii* small subunit ribosomal RNA gene, partial sequence	e-144	517	368/396
C7 (1)-2*	747	**AB027344** *Cordyceps inegoensis* mitochondrial DNA for small subunit ribosomal RNA	0.0	1043	670/714
C7 (3)-3*	410	**AC068564** *Filobasidiella neoformans*, complete sequence	7e-32	139	117/130
C7 (3)-4*	395	**AY337720** *Trichoderma asperellum* isolate T203 mutant exc2y genomic region	8e-14	75.8	41/42
C7 (4)-8*	244	**AJ307598** *Armillaria ostoyae* microsatellite DNA, locus AoSSR84	4e-27	123	81/86
C7-40*	88	**AJ534345** *A. bisporus* partial mRNA for putative glyoxylate pathway regulator (gpr gene), clone pm166	3e-05	44.1	24/24
C7-52*	800	**AY389134** *M. anisopliae* var. *anisopliae* pr1A gene for subtilisin-like serine protease, exons 1-4	0.0	1462	740/742
C7-97*	515	**AB027361** *M. anisopliae* mitochondrial DNA for small subunit ribosomal RNA	0.0	854	479/490

Symbols

* represents clones that were generated from mRNA for subtractive hybridization

'a' represents sequences with the highest homology to the fragments listed above. The homology is not usually the entire length of the fragment.

'b' is E-values, which represent the number of hits one can "expect" to see just by chance. The database sequences were divided into three categories: highly significant (e-values ≤ e-20), moderately significant (e-values e-4 to e-19), and weakly significant (e-values e-2 to e-3). E-values greater than e-2 was considered not a statistically significant match.

'c' Score represents the value calculated by BLAST search of the transcripts. The score increases as the level and length of homology increases.

'd' The identity indicates the similarity of the conidial transcripts over the potential match in the base pairs.

Table 9.11. Summary of BLASTN search of putative mycelial-associated transcripts that were generated by suppression subtractive hybridization

Transcript	Size (bp)	Putative Homology[a] with Accession numbers in bold	E-value [b]	Score [c] (bits)	Identities [d]
M1-54	748	**AB027361** *M. anisopliae* mitochondrial DNA for small subunit ribosomal RNA, strain:IFO5940	0.0	870	471/485
M1-102	431	**AY425004** Homo sapiens estrogen receptor 1 (ESR1) gene, complete cds	8e-04	52.2	26/26
M1-105	238	**AB027361** *M. anisopliae* mitochondrial DNA for small subunit ribosomal RNA, strain:IFO5940	e-135	488	246/246
M1-117	728	**AB027361** *M. anisopliae* mitochondrial DNA for small subunit ribosomal RNA, strain:IFO5940	0.0	1312	697/709
M3-6*	672	**AB027361** *M. anisopliae* mitochondrial DNA for small subunit ribosomal RNA, strain:IFO5940	0.0	1172	637/645
M3-12*, M3(2)-4* M3(2)-6* M3(4)-1* M3(4)-6*	430 567 783 428 435	**AF218207** *M. anisopliae* ribosomal RNA gene, complete sequence	0.0	767	390/391

Symbols

* represents clones that were generated from mRNA for subtractive hybridization

'a' represents sequences with the highest homology to the fragments listed above. The homology is not usually the entire length of the fragment.

'b' is E-values, which represent the number of hits one can "expect" to see just by chance. The database sequences were divided into three categories: highly significant (e-values ≤ e-20), moderately significant (e-values e-4 to e-19), and weakly significant (e-values e-2 to e-3). E-values greater than e-2 was considered not a statistically significant match.

'c' Score represents the value calculated by BLAST search of the transcripts. The score increases as the level and length of homology increases.

'd' The identity indicates the similarity of the conidial transcripts over the potential match in the base pairs.

The sequences derived were analyzed for possible gene function through homology with known genes using the BLASTX that compares nucleotide sequences to proteins in all six reading frames. The values presented for putative homologs derived from the results obtained showed sequences with known function as well as those of unknown function. The database sequences were divided into three categories: highly significant (e-values ≤ e-20), moderately significant (e-values e-4 to e-19), and weakly significant (e-values e-2 to e-3). E-values greater than e-2 were considered not a statistically significant match (Anderson & Brass, 1998). The number of putative homologues identified in the BLASTX analyses is summarized in Table 9.12. There was one clone which showed similarity to a replication initiation gene A protein of phage 13, while clone C7 (3)-3 encoded a catalase. Another insert was similar to a cell wall plasma membrane protein from *Arabidopsis thaliana*. Clone C7-52 had the lowest e-value, e-129 to a subtilisin-like serine protease *Pr1*A with the highest amino acid percentage identity of 93%. The rest of the sequences were either homologous to genes of unknown function or had no significant homology to any sequences in the database (defined as those with e-values greater than e-2). Only one mycelial- subtracted cDNA clone had an insignificant match (e-value of 1.5) to a flagellar biosynthetic protein (Table 9.13).

Table 9.12. Summary of BLASTX search of putative conidial-associated transcripts that were generated by suppression subtractive hybridization.

Transcripts	Putative Homology with Accession numbers in bold	E-value
C1-2	**AAG29971** replication initiation gene A protein –phage 13	7e-59
C1(1)-13	**NP_919988** hypothetical protein (*Oryza sativa*)	4.7
C1(1)-14	**AAB29369** wingless protein (*Drosophila* sp.)	7e-07
C1(3)-2	**NP_499780** putative protein (3O549) (*Caenorhabditis elegans*)	1.6
C7(3)-3*	**CAA59444** catalase (*Campylobacter jejuni*)	2e-06
C7(3)-4*	**T52340** cell wall-plasma membrane linker protein homolog (*Arabidopsis thaliana*)	1e-05
C7(4)-8*	**CAD42938** manganese superoxide dismutase (*A. camphorata*)	2e-07
C7-40*	**NP_252980** probable chemotaxis transducer [*Pseudomonas aeruginosa* PA01]	3.7
C7-52*	**CAC95049** subtilisin-like serine protease PR1A (*M. anisopliae* var. *anisopliae*)	e-129

* Represents clones that were generated from mRNA for subtractive hybridization

Table 9.13. Summary of BLASTX search of putative mycelial-enriched transcripts with homology to proteins that were generated by suppression subtractive hybridization

Transcript	Size (bp)	Putative Homology with Accession numbers in bold	E-value
M1-102	431	**NP_240069** flagellar biosynthetic protein FlhB [Buchnera aphidicola str. APS (*Acyrthosiphon pisum*)]	1.5

9.5. RT-PCR analysis of conidial-associated transcripts on agar media

RT-PCR analysis was performed on seven clones to verify that these transcripts were associated with mature conidium development. Table 9.14 shows a summary of the clones used for this analysis and their possible gene function. Total RNA was prepared from day 2, 3, 4, 5 and 6 cultures grown on PDA and YPD. All samples were treated with RNase-free DNase I prior to RT-PCR (appendix M). This step was performed during RNA extraction to remove any possible genomic DNA contamination. RT-PCR for all samples was not done to saturation, however amplification of the control gene β-actin was carried out to detect and determine the exponential phase of the PCR product (appendix L).

For RT-PCR analysis, the same concentration of total RNA from various stages of growth was used. The amplification of the control, β-actin showed that similar amounts of RNA were in each sample with a constant amount of transcript present throughout the developmental stages of mycelia and conidia. A gradient PCR was performed on the primers designed specific for seven possible conidial-associated transcripts (Appendix N for one example of a gradient PCR on primers designed for clones C1 (3)-2 and C7 (4)-8). The annealing temperatures were then chosen where a band was observed at the expected size. Since the primers annealed at various temperatures and one band was observed, one temperature was chosen for each of the clones tested.

During the developmental stages of conidial growth, there was either small amount of transcripts or none detected on day 2, however by day 3, there was an upregulation of the genes (Fig. 9.21). The results indicated that the conidial-associated cDNAs are highly expressed during the development of mature conidia.

Expression of the clones varied throughout the mycelial growth where there were large amounts of transcripts detected by day 3, a decrease in expression at day 4, and then an increase by day 5. Except for clone C7-52, all the clones showed expression in the mycelia stages of development, which may indicate false positives or contamination. However a method that would be used to verify this could be Northern analysis. This technique not only detects mRNA levels between samples as with RT-PCR, but it allows the researcher to probe specifically for the transcript of the gene of interest after the RNA is transferred to a membrane. Therefore if the gene of interest derives from mycelial RNA, a signal would be detected.

All clones were enriched in the early stages of mature conidium development (day 3) when compared to the mycelial stage (day 4) at which SSH was performed (Fig. 9.11). Only clone C7-52 was highly enriched throughout the stages of mature conidial growth in contrast to being expressed only in the conidiating mycelial growth stages on YPD at days 5 and 6.

Table 9.14. Conidial-associated transcripts that were generated by suppression subtractive hybridization and used in RT-PCR analysis.

Gene	Putative homologue	E-value	Function of Homologue
C7-52*	Subtilisin-like serine protease Pr1A (*M. anisopliae*)	e-129	Protein degradation
C7(3)-3*	Catalase (*Campylobacter jejuni*)	2e-06	Intracellular survival
C1-2	Replication initiation gene A protein (phage-13)	7e-59	Replication initiation protein
C7(4)-8*	Envelope protein (Bovine immunodeficiency virus)	2e-04	Glycoprotein
C1(3)-2*	Putative protein (3O549) (*Caenorhabditis elegans*)	1.6	Unknown
C7(3)-4*	Cell wall plasma membrane linker protein (*Arabidopsis thaliana*)	1e-05	Secreted & plasma membrane protein
C1(1)-14	Wingless protein (*Drosophila* sp)	8e-07	Soluble extracellular signaling gene

*represents clones that were generated from mRNA for subtractive hybridization

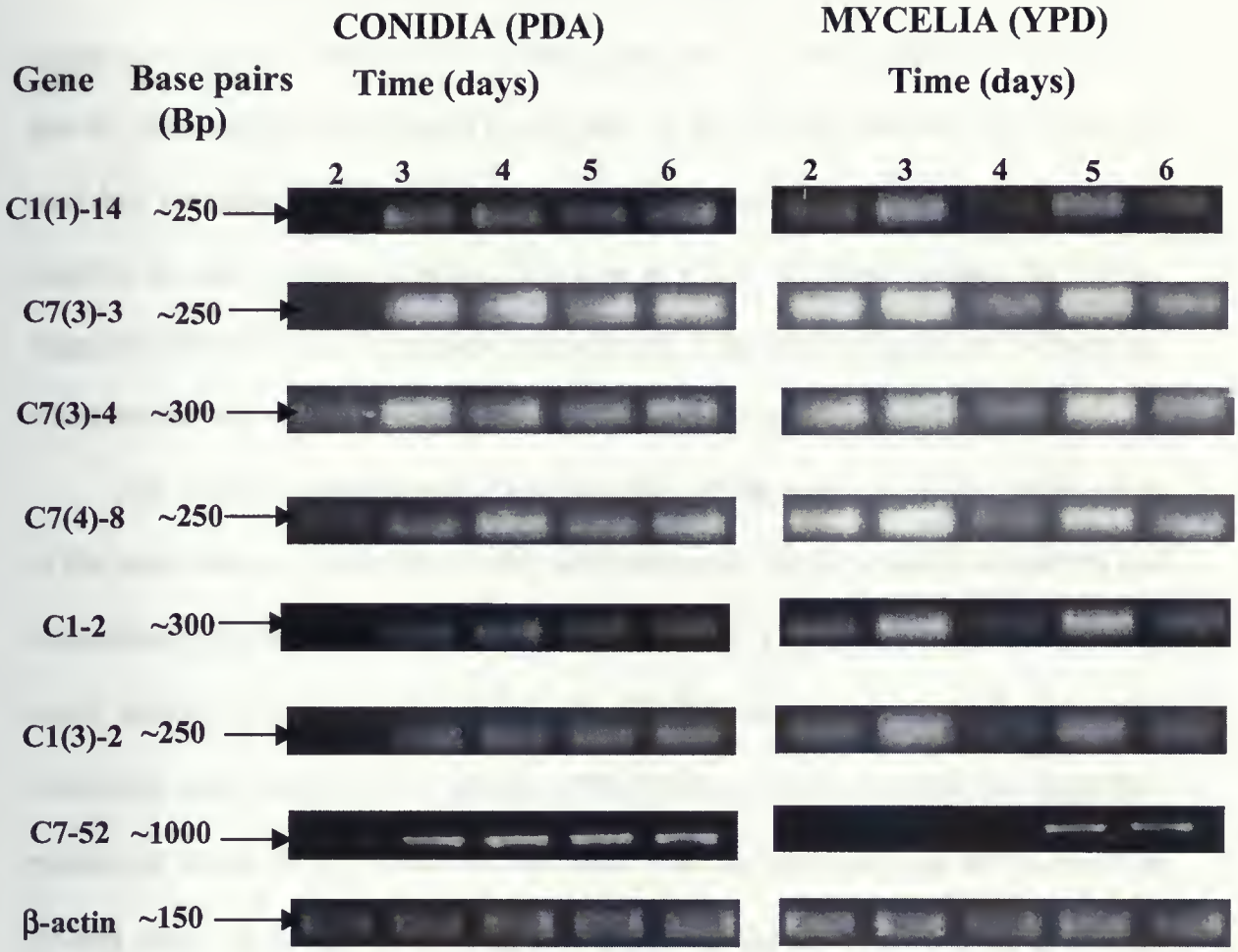

Fig. 9.11. RT-PCR analysis of control (β-actin) and putative conidial-associated transcripts using total RNA from conidia and mycelia grown on PDA and YPD plates respectively from day 2 to day 6.

9.6. RT-PCR analysis of *Pr1* during insect infection

G. mellonella was infected with the *M. anisopliae* strain, Ma2575 and different stages of infection of the insect were monitored and observations made over a 14-day period. Ninety percent of infected insects died by day 3, while the other 10 % died the next day. The latter were discarded since we could not absolutely determine the cause of death to be due to fungal infection. The time at which the insect reached the various stages of infection varied. To maintain consistency, RNA was isolated from 7-10 insects, which were observed to have similar progressive infection at each stage (Fig. 9.12a).

Fig. 9.12c is a diagrammatic representation of the stages of fungal transgression of the insect cuticle, proliferation within the haemocoel, and the mycelial outgrowth and conidiation on the insect surface. RT-PCR analysis of *Pr1* and β-actin expression during insect infection is shown in Fig. 9.12b. No RT-PCR product was amplified from the uninfected insect using the *Pr1* primers. The β-actin primers, however, did amplify a product of about 100bp, which was different from the expected size of β-actin PCR product using fungal cDNA as template (150bp). In the RT-PCR analysis of β-actin expression, it was difficult to distinguish between fungal and insect β-actin, because the transcripts were very close in size. If time was permitting, a more appropriate technique that could have been used was *in situ* RT-PCR, which detects gene expression directly in cells and the cellular localizaton of these gene transcripts.

RT-PCR analysis of *Pr1* was carried out to compare transcript abundance across multiple samples at each progressive stage of infection of waxworm larvae. Results showed a Pr1 product of approximately 1000bp during the infection stages of death (48-96h), lots of mycelial growth (96 – 216h) and conidial growth (+216h) on the surface of the cadaver. *Pr1* and fungal-specific actin were found in fungal material scraped from an

infected insect cadaver (Fig. 9.12b). Expression of *Pr1* or fungal β-actin was not detected post-24h infection or melanization.

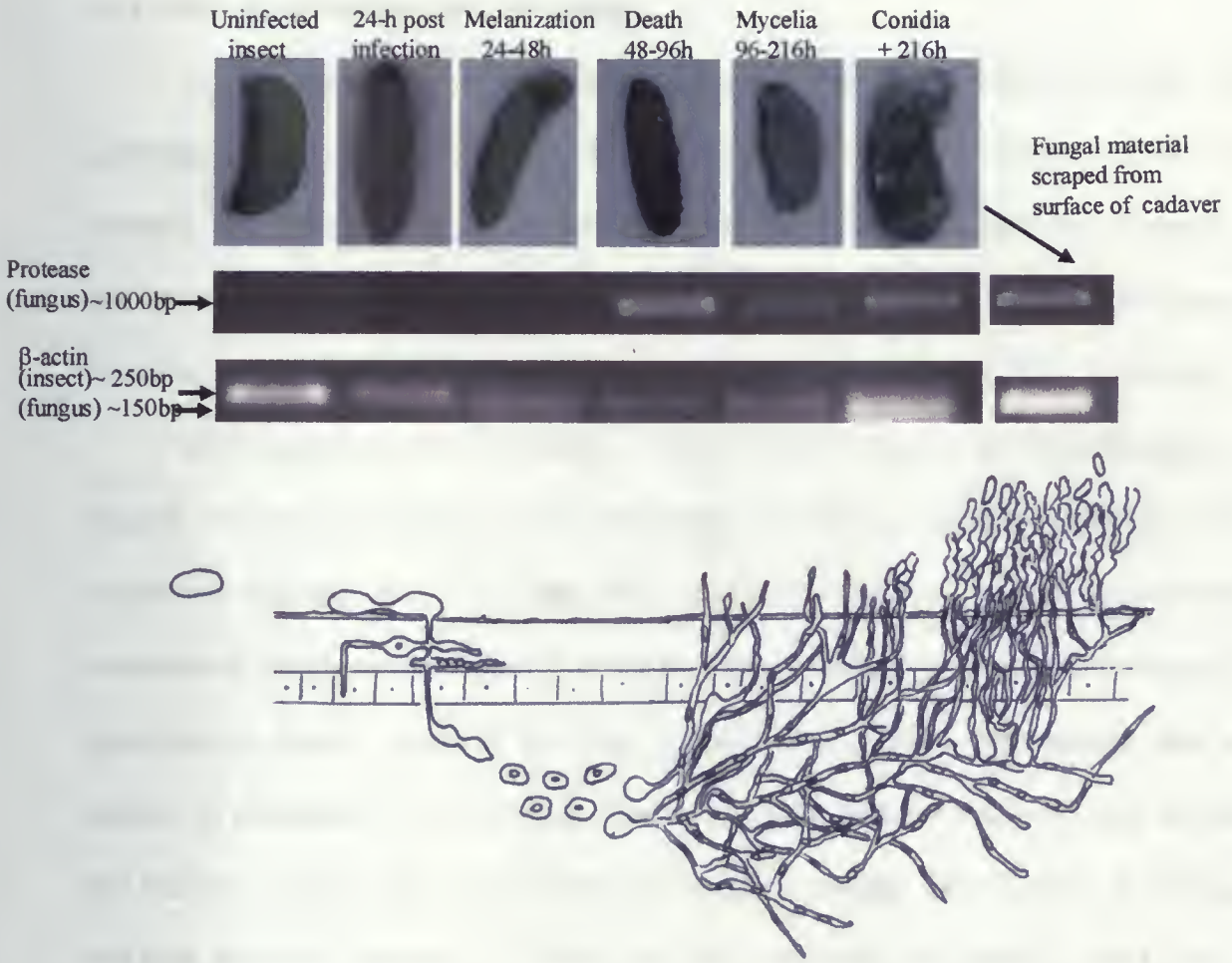

Fig. 9.12. RT-PCR analysis of *Pr1* and β-actin expression in the progressive infection of waxworm larvae (*Galleria mellonella*) with Ma2575. (A) Progression of infection of infected waxworm larvae at 24-hour post infection, melanization of the infected insect (24-48 hours), death (48 – 96 hours, growth of mycelia on surface of cadaver (96-216h) and conidial growth on surface of cadaver (+216h). Uninfected insect shown as a control. (B) RT-PCR analysis of β-actin and protease gene expression during progressive infection of the waxworm larvae. (C) An artistic impression of infection cycle in waxworm larvae with Ma2575, which corresponds to panel A.

10. DISCUSSION

10.1. SSH: disadvantages and advantages

Over the years, there have been strategic approaches aimed at characterizing and identifying differentially expressed genes in order to understand the molecular nature of diseases, cell systems and various other developmental processes. Most tissue-specific mRNAs are believed to be expressed at very low levels; therefore the principal issue would be the effectiveness that these techniques have at detecting rare mRNA transcripts.

cDNA microarray is a developing technology that allows for high throughput analysis of expression patterns of gene, comparing them among multiple conditions. This chip-based approach uses RNA from two conditions, which is labelled by reversed transcription incorporation of two different fluorescent-labelled probes and subsequently hybridized to known clones in the array (Schena *et al.*, 1995). Microarrays offer a number of advantages over other high capacity methods. They are relatively easy to use and the use of cDNA clones and fluorescent labelling provides hybridization specificity and high detection (Schena *et al.*, 1996). On the other hand, this approach can be very costly, requires a large amount of RNA and cannot be employed to identify novel genes since microarrays are constructed with known cDNAs.

Expressed sequence tags (ESTs) have also been used on a wide scale for identification of differentially expressed genes. This approach is based on sequencing random cDNA clones from libraries that represent genes expressed in different cells at various stages of development (Adams, 1991; Boventius & Weller, 1994). As with other methods, the sequences, which represent differentially expressed genes, are used to search existing databases for homology to known sequences. The results from a number of ESTs projects that are now publicly available have been essential to identify genes not

previously discovered using traditional methods. Usually screening is performed on a large number of clones (+1000) and although sequencing costs has been considerably reduced; it is still relatively expensive to carry out EST projects in standard laboratories.

mRNA differential display (mRNA-DD), developed by Liang and Pardee (1992), is a technique based on the use of an anchored oligo-dT primer and designed to bind to a subpopulation of the poly (A)+ tail of mRNAs for reverse transcription. PCR amplification is subsequently followed using these 3'-anchored primers in conjunction with 5'-arbitrary primers. The advantages of this technique are that it requires very small amounts of RNA and permits multiple comparisons amongst various experimental conditions. However, some studies have found that even though this is a quick and sensitive method, there is a high incidence of false positives and redundant identification, (Liang & Pardee, 1992).

Another PCR-based technique is SSH, which was used in the present study. SSH has also been successfully applied to the characterization and identification of differentially expressed genes of both high and low abundance. More recently, this technique has been applied to fungal systems to isolate cellulose–growth specific genes in *Agaricus bisporus* (Morales & Thurston, 2002) and also to identify differentially expressed genes during conidiation in *A. nidulans* (Osherov *et al.*, 2002). In the present study, this technique based on the model system, that one cDNA population (control condition or driver) is hybridized in excess to another cDNA population (experimental condition or tester) to suppress common sequences between the two populations, was used to isolate transcripts unique to the tester population (Diatchenko *et al.*, 1996). However, as with other techniques, there are disadvantages to using SSH. When using this method, two distinct cDNA populations are needed, and one of these (the control) is

expected to be subtracted in the hybridization process. It is not always possible to achieve two distinct populations. In the present study, we attempted to separate cDNA enriched in conidial development from cDNAs present in mycelial development. To actually separate these two morphological changes posed a difficult task. During the saprophytic life cycle of *Metarhizium*, mycelia precede the growth of conidia; therefore both of these stages of development could be present. Despite the variations, second order hybridization kinetics are believed to rectify these problems, however, it may not always be efficient.

Another disadvantage to using SSH is lack of complete reproducibility. In this study, several conidial and mycelial subtractions were carried out with different RNA isolations each time to determine if there was any consistency to the number of fragment sizes derived from the secondary PCR and to identify as many differentially expressed genes from the subtractions. No fragments were seen repeatedly in all conidial or mycelia cDNA subtractions performed. Failure or inconsistencies at any one stage of SSH could lead to either false positives or losses in rare transcripts. During the 1st hybridization, the presence of driver cDNA in two separate tester cDNA samples is expected to successfully (a) form single-stranded tester cDNA, (b) produce homo hybrids cDNAs (annealing of tester to tester cDNA), and (c) produce common sequences which occur between tester and driver cDNA (Diatchenko *et al.*, 1996). For these various conditions to co-occur the developmental time factor is critical. Moreover, an equal abundance of both driver and tester cDNAs in both reaction mixtures could result in a loss of rare transcripts. Morales *et al.* (2003) modified the technique by performing 3 hybridizations instead of 2 to further ensure a higher probability that common sequences will be suppressed. Also Gurskaya *et al.* (1996) introduced the normalization of specific transcripts during subtraction, and also increased their hybridization times from 7 to 20

hours, which would affect the tester-specific fraction. These researchers were able to identify 6 specific clones from the Jurkat cell subtracted cDNA not previously found in this well studied cellular system. Other studies have identified from 4 to 50 clones using SSH (Akopyants *et al.*, 1998; Yokota *et al.*, 2001; Robert *et al.*, 2001; Osherov *et al.*, 2002; Stocki *et al.*, 2002; Morales & Thurston; 2003).

Ribosomal RNA contamination has also been found in subtracted libraries in our study, and in other studies, which typically occur using this subtraction procedure. The conidial-associated subtracted cDNA library contained 11 clones identical to mitochondria ribosomal RNA (rRNA) sequences that were expressed in all tissues of *M. anisopliae*. Yokota and coworkers (2001) found six 28s rRNAs in their identification of differentially expressed genes in rat hippocampus after transient global cerebral ischemia. In a study to identify mRNA expressed in granulose cells associated with bovine oocyte developmental competence, 5 rRNAs were found (Robert *et al.*, 2001). Despite these disadvantages, when compared to other methods, SSH technique is a cost-effective tool for analyzing the genes associated with specific developmental conditions, diseases or cell systems. The following section is a discussion of genes isolated during conidiation in *M. anisopliae* using SSH.

10.2. Putative identification and analysis of conidial-associated genes

In this study, we identified seven conidial-associated genes in the insect pathogenic fungus, *M. anisopliae* using SSH. We also analyzed their expression patterns during development on different agar media and examined the *in vivo* expression of the transcript encoding a subtilisin-like protease during infection of *G. mellonella*.

Clone C7 (3)-3 was found to have homology to a gene encoding catalase of *Campylobacter jejuni*. Catalases are ubiquitous enzymes responsible for the

detoxification of H_2O_2, preventing the formation of a very reactive hydroxyl radical. They utilize H_2O_2 both as an electron acceptor and donor, yielding oxygen and water in the reaction (Navarro *et al.*, 1996). It has been suggested that the gene encoding catalase (*kat A*) in *C. jejuni* has the ability to inactivate hydrogen peroxide thus aiding in the persistence and survival of bacteria within host cells (Day *et al.*, 2000). Catalases have also been found in insect pathogenic fungi where they may also combat reactive oxygen species (St. Leger *et al.*, 1988c). A conidial-associated catalase found in *Aspergillus nidulans* not only provided protection from H_2O_2 generated by environmental stress and toxic effects, but also regulated cell growth and differentiation (Navarro *et al.*, 1996). Other studies have found that this catalase was upregulated before the induction of conidiation and during conidium production but not in isolated conidia (Kawasaki *et al.*, 1997). The *A. nidulans* catalase is believed to be a virulence factor since it degraded H_2O_2 *in vitro* and transiently protected the fungus from its host. Catalases have also been found in *Cladosporium fulvum*, a tomato pathogen, which has functions similar to catalases found in *Aspergillus* sp. (Bussink & Oliver, 2001). We speculate that during mature conidium production in *M. anisopliae*, catalase accumulates in conidia and is able to protect them from H_2O_2 toxicity. This transcript is expressed in both mature conidia and throughout the conidiating mycelial stages of growth.

C7(3)-4 showed similarity to a cell wall- plasma membrane linker protein, which is a proline-rich secreted protein isolated from *Arabidopsis thaliana*; however, its function is unknown, but it could possibly be involved in cold response (Goo *et al.*, 1999). In *Saccharomyces cerevisiae*, a cell wall protein (Cwp1p) has been isolated and this secretory protein may be involved in maintaining cell wall integrity (Ram *et al.*, 1998). It is possible that in *M. anisopliae* the transcript homologous to a cell wall-plasma

membrane protein encodes for proteins that participate in cell wall reinforcement localized at the site of attempted insect cuticle penetration by the fungi, thus allowing the fungus to adapt to various stress conditions.

There were also clones that showed similarities to viral and insect sequences. Clone C1-2 showed significant homology to a replication initiation gene A protein of phage 13. This viral protein aids in DNA replication of phage and is localized in the membrane in the cell (Lau & Spencer, 1985). There is a possibility that this putative homolog may behave similarly to fungal replication initiation proteins or may represent a novel protein since it was not homologous to any known sequences in the fungal databases. Another *M. anisopliae* clone, C1(1)-14, was similar to the wingless protein of *Drosophila*, which is involved in signaling pathways and controls differentiation and growth patterns during development (The & Perrimon, 2000). It is speculated that clone C1(1)-14 may have similar functions in *M. anisopliae* development. Fourteen percent of the clones had no significant homology to sequences in the databases; nevertheless further analyses such as target disruption could decipher the roles of these novel genes.

Of the transcripts isolated, six of the seven clones showed variable expression throughout vegetative growth before the onset of conidium development. It is expected that expression of these genes, would occur during the later stages of mycelial growth at the onset of conidiation. We speculate that these genes are associated, but not directly involved with the development of mature conidia. These findings were similar to Osherov *et al.* (2002) where *cetD*, a putative conidial-enriched gene, was also expressed during vegetative growth. Our results also showed that when *M. anisopliae* is grown on YPD, low levels of expression of transcripts occurred on day 4 in contrast to days 3 and 5 in six of the genes. The complete developmental life cycle from mycelia to the growth of

mature conidia of many fungi may occur very quickly (Adams *et al.*, 1998). These rapid switches in morphology, influences the processing of mRNA transcripts and gene expression. Moreover, mRNA transcripts can have a short lifespan since they accumulate and disappear at such a rapid rate (Adams *et al.*, 1998). In turn, different transcripts may be present at different times during growth.

Our SSH analysis results were similar to those found by Osherov *et al.* (2002) in that no transcripts were found to be homologous to genes that are directly involved in regulating conidiation, such as *brlA*, *abaA* or the *con* genes from *Aspergillus nidulans* and *Neurospora crassa*, respectively (Boylan *et al.,* 1987; Adams, Boylan & Timberlake, 1988; Roberts *et al.*, 1988; Adams, Weiser & Yu, 1998). They found 12 transcripts enriched in *A. nidulans* conidia which were similar to thaumatin-like proteins and metabolic proteins (Osherov *et al.*, 2002). Similar results were also found in previous studies of *M. anisopliae* were the *brlA* and *wetA* homologues were unable to be identified (Kamp & Bidochka, 2002a). It is speculated that the genes involved in regulating conidiation are transcription factors that are in very low abundance, which in turn would make the detection of these genes difficult. Since *Aspergillus* and *Neurospora* belong to two different classes of fungi their regulatory pathways for conidiation may be different thus the methodology employed in assessing the function of these genes may vary.

We found that clone C7-52 encoded a cuticle-degrading subtilisin-like protease (Pr1). Pr1 is the most extensively and best understood gene involved in entomopathogenicity and to date, eleven subtilisins have been identified in *M. anisopliae* during growth on the insect cuticle (Freimoser *et al.*, 2003; Bagga *et al.*, 2004). Subtilisin-like proteases have been found to play important roles in the early stages of

fungal infection since they degrade insect cuticular proteins during host penetration (St. Leger *et al.*, 1992).

In the presence of low levels of complex nitrogenous compounds, *M. anisopliae* synthesizes large amounts of *Pr1* (St. Leger *et al.*, 1988b, 1989a, 1991b, 1992; McIntyre *et al.*, 2000; Krieger de Moraes *et al.*, 2003). Our findings have shown that both conidiation and Pr1 production are induced by starvation of *M. anisopliae* after nutrient depletion of growth media containing carbon and low levels of nitrogen. *In vitro* analysis of *Pr1* in a tomato pathogen, *Colletotrichum coccodes* further illustrated the enzyme's involvement in generating metabolizable carbon during pathogenesis (Redman & Rodriguez, 2002). It can be suggested that *Pr1* is a catabolite repressor since an inverse relationship can be establish between protease activity and substrate levels over time.

10.3. *In vivo* expression of *Pr1* during insect infection

M. anisopliae has specific nutrient and thigmotropic requirements that affect differentiation and pathogenesis. For penetration to occur, Pr1 is produced by the appressoria, which form under low nitrogen conditions present on the hydrophobic cuticular surface. Pr1 subsequently degrades cuticular proteins, allowing hyphal transgression of the cuticle (St. Leger *et al.*, 1989a, b). Under similar nutrient-deprived conditions, but in the absence of a hydrophobic surface, conidiation can be induced as opposed to appressorium formation. Our *in vitro* results are a representative analogy of how conidiation occurs during the later stages of insect infection. Within the insect haemolymph, *M. anisopliae* utilizes the readily available nutrients. However as the nutrients become depleted, *Pr1* is upregulated and functions to breach the cuticle, allowing the fungus to emerge from, and conidiate in large masses on the surface of the

insect cadaver. Our *in vivo* findings showed *Pr1* to be upregulated in the later stages of insect infection as nutrients are being depleted and as the fungus envelops the cadaver and subsequently conidiates.

Expression of *Pr1* was detected less at 96-215 hours when there was lots of mycelial growth in comparison to during the infection stages of death. This could be because *Pr1* expression is decreased during mycelial growth, and as nutrients become limiting, there is an increase expression of the gene and the subsequent growth of conidia on the surface of the cadaver. In the infected larvae, *G. mellonella*, the *Pr1* transcript was not detected during post-24h infection in contrast to previous *in vitro* studies (St. Leger *et al.*, 1989a). Nonetheless, this could be due to the fact that there was low fungal biomass at that stage of infection. *Pr1* was not detected during the melanization of the insect.

11. CONCLUSION

This study is the first attempt at identifying genes associated with conidiation in the insect pathogenic fungus, *M. anisopliae*. Techniques such as gene disruption and over expression analysis would further define and determine the function and role of these possible conidial-associated transcripts. Using SSH, we have identified seven conidial-associated cDNA transcripts, one of which encoded a subtilisin-like protease, Pr1, and have found these to be expressed during the development of mature conidia and mycelia. In addition, this study gives insight into the multiple roles that Pr1 plays during pathogenesis. Not only is Pr1 produced during the initial stages of fungal invasion (St. Leger *et al.*, 1986b, 1988b, 1989a, b; Goettel *et al.*, 1989) but it is also produced during the later stages of pathogenesis. Pr1 may not be directly involved in the formation of conidia, but may be required for the completion of the pathogenic cycle to cadaver mummification and conidium production. The identification and further characterization of conidial-associated genes, in particular *Pr1*, may enhance our understanding of these specific-adaptive relationships that have evolved between conidiation and insect pathogenesis.

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APPENDICES

Appendix A

Media preparation of yeast potato dextrose (YPD) agar

0.2% yeast extract……..1.6g
1.0% peptone……..8.0g
2.0% dextrose (glucose)….16.0g
1.5% agar……...12.0g
Add up to 800ml of dH_2O. Mix and dissolve and then autoclave for 15 min. Cool to 50°C and pour 15ml of YPD agar to make 50 plates.
Allow cooling and upon solidification, store at 4°C.

Appendix B

Media preparation of PDA (potato dextrose agar)

Measure 31g and make up to a volume of 800ml dH_2O.
Mix and dissolve then autoclave fro 15 min.
Cool to 50°C and pour 10ml of PDA.
Allow plates to cool and solidified and store at 4°C.

Appendix C

Media preparation of LB (Luria bertani) broth

2 g tryptone
1 g yeast extract
1 g NaCl
Add up to 200ml of dH_2O. Mix and dissolve and then autoclave for 15 min. Allow to cool and store at 4°C.

Appendix D

LB plates

Use the same ingredients as appendix C but add 3 g Bacto-agar. After autoclaving pour agar into petri dishes (15ml per plate). Once solidified, store inverted at 4°C.

Appendix E

LB plates containing ampicillin with IPTG (Isopropyl-β-D- thiogalactopyranoside) and X-Gal

Make LB plates according to appendix D. After autoclaving, allow to cool to 50°C. Add 400 µl ampicillin (50 mg/ml) to the 200 ml LB agar resulting in a final concentration of 100 µg/ml ampicillin in LB. Pour 10 ml media on petri dishes and allow to solidify.

Spread 100 µl of mM IPTG and 20 µl of 50mg/µl X-Gal over LB plates. Allow to absorb 30 min at 37 °C before precipitation.

Appendix F

Conidial counts of *M. anisopliae* grown on PDA over an 8-day period.

	PDA						
	Sample 1	Sample 2	sample 3	sample 4	Days	Avg.Spores	Std. Dev.
Day 1	0.00E+00	0.00E+00	0	0.00E+00	1	0	0
Day 2	5.90E+04	3.93E+04	46300	51000	2	39120	23012
Day 3	3.09E+06	2.07E+06	2540000	2780000	3	2620000	430271
Day 4	3.63E+06	3.35E+06	3680000	3500000	4	3540000	147648
Day 5	5.68E+06	3.89E+06	4800000	4320000	5	4672500	767653
Day 6	1.62E+07	1.08E+07	13500000	11000000	6	12875000	2534265
Day 7	2.24E+07	2.23E+07	28900000	26000000	7	24900000	3173851
Day 8	2.68E+07	2.30E+07	27000000	28000000	8	26200000	2196968

Appendix G

Conidial counts of *M. anisopliae* grown on YPD over an 8-day period.

	YPD					
Days	Sample 1	Sample 2	Sample 3	Sample 4	Avg. Spores per cm2	Std. Dev.
1	0.00E+00	0.00E+00	0	0	0	0
2	0.00E+00	0.00E+00	0	0	0	0
3	5.90E+04	1.97E+04	35000	3.98E+04	38375	16203
4	4.92E+04	5.90E+04	45000	5.19E+04	51275	5881
5	6.89E+04	1.97E+04	67000	5.13E+04	51725	22760
6	3.93E+04	6.89E+04	51200	5.40E+04	53350	12169
7	5.90E+04	5.97E+04	62000	6.89E+04	62400	4519
8	7.87E+04	5.90E+04	68700	7.35E+04	69975	8379

Appendix H

ANOVA analysis with Scheffe's post-hoc comparison on the earliest day of conidium production of *M. anisopliae* strain, Ma 2575 on PDA in the first 4 days.

n	16

PDA	n	Mean	SD	SE
Day 1	4	0.000	0.000	0.0000
Day 2	4	48900.000	8273.250	4136.6250
Day 3	4	2620000.000	430271.232	215135.6161
Day 4	4	3540000.000	147648.231	73824.1153

Source of variation	SSq	DF	MSq	F	p
PDA	###########	3	###########	251.48	<0.0001
Within cells	###########	12	###########		
Total	###########	15			

		Scheffe	
Contrast	Difference	95% CI	
Day 1 v Day 2	-48900.000	-569415.534 to 471615.534	
Day 1 v Day 3	-2620000.000	-3140515.534 ###########	(significant)
Day 1 v Day 4	-3540000.000	-4060515.534 ###########	(significant)
Day 2 v Day 3	-2571100.000	-3091615.534 ###########	(significant)
Day 2 v Day 4	-3491100.000	-4011615.534 ###########	(significant)
Day 3 v Day 4	-920000.000	-1440515.534 to -399484.466	(significant)

Appendix I

T-Test analysis to determine which media (YPD or PDA) showed a more significant increase in conidium production by day 8.

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	24700000	69975
Variance	6.63E+12	70209167
Observations	4	4
Hypothesized Mean Difference	0	
df	3	
t Stat	19.13573	
P(T<=t) one-tail	0.000156	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.000312	
t Critical two-tail	3.182449	

Variable 1 – PDA
Variable 2-YPD

Appendix J

Sequences of conidial subtracted cDNA inserts using T7 promoter

C1-1

CGAGCGGCCGCCCGGGCAGGTACATCAGACTGGTTAGGAGGCATCCCAGAA
GGGGCAGCCTCATGCCCAGGTTTCAGCCCTAAAGGTAATGATTGTCTTGACTC
TGCCTTGGCATTTCGCTCAGAATCACGGCAGACTTGGAGTGTTTCTTCAAGGC
AGGCATCTGCTTATGAGCAAGGTCAAAGATTTTTCAAAATATTGTGCATTAAT
TCATTAAAGCTACTGTTAAATATTTGCTGTTTTTAGATCGGCGTCCGTGCTAA
TTCTGCAGTTGTAGCACTGTATATTTTATCTCATTTCTGTGCCAAGAAAGTTCA
TCTTTATGTTTTTCTAATACACAATCTTGATCATGTTTCCAAAATAAAGCTTCA
GCTCCTTGGTCAATAGAAGTAAGGGTGTAGCCATCCAGGGTCTCCCGGCTCT
AGGCAGACCGGATCCCGCAGTTCATCCCATGGTTGTTGAAATGTACCTCGGC
CGCGACCACGCTA

C1-2

AGCGTGGTCGCGGCCGAGGTCCATTAGCTGTACCATACTCAGGCACACAAAA
ATACTGATAGCAGTCGGCGTGTGAATCATTAGCCTTGCGACCCTCGGCAGCA
AGAACCATACGACCAATGTCACGAAAATAGTCACGCAAAGCATTGGGATTAT
CATAAAACGCCTCTAATCGGTCGTCAACCAACGTGAGAGTGTCAAAAACGAT
AAACCAACCATCAGCATGAGCCTGTCGCATTGCATTCATCAAACGCTGAATA
GTAAAGCCTCTACGCGATTTCATAGTGGAGGACCTGCCCGGGCGGCCGCTCG
AA

C1-3

AGCGTGGTCGCGGCCGAGGTACTAGTGCCTCCCAGATGCTCTTCTCCTGACTG
GCTCTTTGTGACCTAAATTTCAACTGCTCCACCCCACAAGGAGGAAACCAGG
GCCACAAATGTAGTGAGATTTTCATAAAGCTAAATGTATTCAGTGGAAACGA
CCTTCCTCTGATCTAAAAGCATGCATGTCTTTGTTTTATTACTGGAGTATTAAA
TTAGCCCTTACTTATGGAATAGGAGGACAGTAGGTGGCAATTGTTTTCTAATG
CTCTTACTTCACAAAATAAAATGGCGGCAGCACAATGTCAGTATTTATATTAC
ACAATATTATTAGTTTGTAAAAACCGAAAGCCAGGGAGCTTACTGCCCTGGA
TTCTTTAGGGCGTGGGTCTTATCACCTCATTCATTTCTTCCCTTCCCCTAAGAG
TGCCACAAGCATGGCAGTTGCTCAGCTGAGACACATGAATGGGAACAGAACT
GAAATATCAGGATGTGACCTCGAGCCAGCTGGTTGGGGCTGGGGATCCCCCT
GGATCTCCACTCTGTCCTGTCCGAGAAAGGGGAAGCTGCCCTATGAGTCTTTT
TTCTGGGGGATTCCAGAGGTTGAACGTCTGTTCTGAGGCTCCTCAGAACCTCG
AGGGAGGGGACGAGAGAGATGACCCCTTGCCCAACCACTGGCTGAAAAATC
TTGAAACTCCCTGACTTTGACCAGGGAATTCCCAGTTGTGCTAAGCCC

(C2-4)

AGCGGCCGCCCGGGCAGGTCTCGGCCGCGACCACGCTA

(C7-40)

AGATGCGATCGAATTCAAGTTGCTTCGGAGCCATGGACGAGGGATTAGCGTG
GTCGCGGCCGAGGTCCTGCCCGGGCGGACGCTCGAA

C7(1)-4

CTTCTGCTCGCTAGACGCGCATGCTCCCGGCCGCCATGGCCGCGGGATTAGC
GTGGTCGCGGCCGAGGTACTAGTGCCTCCCAGATGCTCTTCTCCTGACTGGCT
CTTTGTGACCTAAATTTCAACTGCTCCACCCCACAAGGAGGAAACCAGGGCC
ACAAATGTAGTGAGATTTTCATAAAGCTAAATGTATTCAGTGGAAACGACCT
TCCTCTGATCTAAAAGCATGCATGCCTTTGTTTTATTACTGGAGTATTAAATT
AGCCCTTACTTATGGAATAGGAGGACAGTAGGTGGCAATTGTTTTCTAATGCT
CTTACTTCACAAAATAAAATGGTGGCAGCACAATGTCAGTATTTATATTACAC
AATATTATTAGTTTGTAAAAACCGAAAGCCAGGGAGCTTACTGCCCTGGATT
CTTTAGGGCGTGGGTCTTATCACCTCATTCATTTCTTCCCTTCCCCTAAGAGTG
CCACAAGCATGGCAGTTGCTCAGCTGAGACACATGAATGGGAACAGAACTGA
AATATCAGGATGTGACCTCGAGCCAGCTGGTTGGGGCTGGGGATCCCCCTGG
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CTGGGGGATTCCAGAGGTTGAACGTCTGTTCTGAGGCTCCTCAGAACCTCCA
GGGAGGGGACGAGAGAGATGACCCCTTGCCCAACCACTGGCTGAAAAATCTT
GAAACTCCCTGACTTTGACCAGGGAATTCCCAGTTGTGCTAAGCCCTGCCTGG
TGGAATGTCCCTGGAGGAAGTAACCAGGACACGCATACGTTTTTATGTACTT
GCCCGGCGGCGCTCGAATCACTAATGGCGGCCGC

(C1-14)

AGAGTCCAAAGAGGATCCCAGGCGGGCGCCCGTGTAGAGTAGTGGAGCCGG
CCGTGGCTACCGAAGACGAGCTGGTGGTGGTCGAGCAGGCGCATCGCATTCC
ACTGGTACTGCGAGGACAAGTGCAAGCTGTGTCGGACGAAGAAGGTTATCTA
CACGTGTCTGTAATGGGGCATGCCCCACGCACTACGCCCTCTCTATAGCTTGC
CCCCGCCCGGCTTCACCCCACACTTTGTATGTGTTATGTATGTTGTCTACGCTT
AGCCTGTGTTATTAGCACTTTAAGAGCCTAGACTATAGCATAGTTAAAGATAC
AGCTAATCCACCCGGAATCCTTTAGGTGGCACTCGCTCTCTTGTAGCAGTATT
ACACAGGAGGAACATTAACGAGTAGAGTTCGAGAAGAAGTGTGAAAAGTAT
ATTAAGCCAGAACATGTGTGTGCCCAGTTTTTAGTCAAGTAAGCGCCATGAT
ATAAGTGGT

C1(3)-2

CCGACAGCACAACGTGGAGGGCAGAGTGCGTGCTAGGAAACTTGAGTTATAG
AGGAGAGAAGGGACTGGATGCCGGAGTCAGAGTTCTTGGCTTGGACTCTGGC
TTCTCTAACTCTGCGTAGCCTCTGGGTTAGGCAGCTGAACCTCTCAAGGCCAA
GGTACCTCGGCCGCGACCACGCTAGTCTCCATTGCGTCGTGGACCTCGGCCG
CGACCACGCTAGAATGAGCTTTATAGAGGACCTCGGCGCGACCACGCTAC

C1-13

ATATAACCGAGGGAGGATTCCCATATGGGCGAGAGGGGGGTGGACGTACT
AATGACAACAAGCGGCTATGGTTAGTAGGGAGCATGCGACGTAGGGGAGA
GAGAGCGCTCATAAGAGAGTTTTAAAAAACCCCCCCGG

C7-52

CATGTGCATATTGCTGGTAGTACCGAACATGGTGTTATGACCTTGCTTCACCT
GGTGCTGAAGCATAAGGGTGTCTCCGAGCTTATCGAGGTACAATACTTACTG
TGCGGCCACCAATCCAGGTGGAAAGAACATTGCTACCAGGAGCGAAAATATC

GACAACTCTGCCGTAGTTGGAGAAGGAAGATCGGCTGTCATTTTCCGCAGAG
GCACCAACAGTGCAGGCAGAAGGCTCGGAAGCGGGAGAGGTGTTCTGGGCA
TCCCGGTTATCGTTGCCAGCGGCGACGGCAAGGAAGACACCAGAATTGACCA
AAGCAGCAGCACCTTGGTTGACGGACGCCGAGTAGCCACCTCCCAGGCTCAT
GGAAGCAATGGCGCCGTTGGGGCAGCCGCGGGTCTTGGAGTCCTGTGCAACG
TAGTCCATGCCACTGATGATACCGGAGTAGGAACCACTGCCCTGGTTGTCAA
GAACCTTGACACCATAGAGCTTAGCCTTTTTGGCAACACCGTAGGTCTTGCTA
CCAATGGTACCAGCGCAGTGAGTCCCATGGCCGTGGCCATCAGTGTTTTGAC
CGCTGATGAAGCTCTTAAGAAAAGTGGCGCGACCCTCAAACTCCTGGATGAT
ATTGTTAAATTTCCTACTCCGCACTATGGAGTTTTGACGGCACAACTTACGGG
GTGGGAGGCCTCAATACCAGTGTCAATGATATATACGCAAGTACCCTGACCA
GCACTATCATCGTAGCGATAGGTGGTGCTTCCCTTACTGCGGTGAGAGATGC
GCCCAAGACCCAGGGGAG

C7(4)-8
CCTCGGCCGCGACCACGCTAACCCTATGAGACAGTATAAAATACTCCAGCTA
TGCATCCAACGCTGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCG
CACTAATGATTTCGAGCGGCCGGCCGGGGAAGTACTCGGTCGCAACCACAAT
ATACAAGGGCCGCCACGACAATACCCCCGACTCCAATAAACAAATTCGAAAG
TNTCCCATAAAGACCATCCATATTCCACCGCGCTAA

C7(1)-5)
TCGAGCGGCCGCCCGGGCAGGTACATTAAAAACGTATGCGTGTCCTGGTTAC
TTCCTCCAGGGACATTCCACCAGGCAGGGCTTAGCACAACTGGGAATTCCCT
GGTCAAAGTCAGGGAGTTTCAAGATTTTTCAGCCAGTGGTTGGGCAAGGGGT
CATCTCTCTCGTCCCCTCCCTCGAGGTTCTGAGGAGCCTCAGAACAGACGTTC
AACCTCTGGAATCCCCCAGAAAAAAGACTCATAGGGCAGCTTCCCCTTTCTC
GGACAGGACAGAGTGGAGATCCAGGGGGATCCCCAGCCCCAACCAGCTGGC
TCGAGGTCACATCCTGATATTTCAGTTCTGTTCCCATTCATGTGTCTCAGCTGA
GCAACTGCCATGCTTGTGGCACTCTTAGGGGAAGGGAAGAAATGAATGAGGT
GATAAGACCCACGCCCTAAAGAATCCAGGGCAGTAAGCTCCCTGGCTTTCGG
TTTTTACAAACTAATAATATTGTGTAATATAAATACTGACATTGTGCTGCCAC
CATTTTATTTTGTGAAGTAAGAGCATTAGAAAACAATTGCCACCTACTGTCCT
CCTATTCCATAAGTAAGGGCTAATTTAATACTCCAGTAATAAAACAAAGGCA
TGCATGCTTTTAGATCAGAGGAAGGTCGTTTCCACTGAATACATTTAGCTTTA
TGAAAATCTCACTACATTTGTGGCCCTGGTTTCCTCCTTGTGGGGTGGAGCAG
TTGAAATTTAGGTCACAAAGAGCCAGTCAGGAGAAGAGCATCTGGGAGGCA
CTAGTACCTCGGCCGCGACCACGCTA

C7(1)-6)
AGCGTGGTCGCGGCCGAGGTACTAGTGCCTCCCAGATGCTCTTCTCCTGACTG
GCTCTTTGTGACCTAAATTTCAACTGCTCCACCCCACAAGGAGGAAACCAGG
GCCACAAATGTAGTGAGATTTTCATAAAGCTAAATGTATTCAGTGGAAACGA
CCTTCCTCTGATCTAAAAGCATGCATGCCTTTGTTTTATTACTGGAGTATTAA
ATTAGCCCTTACTTATGGAATAGGAGGACAGTAGGTGGCAATTGTTTTCTAAT
GCTCTTACTTCACAAAATAAAATGGTGGCAGCACAATGTCAGTATTTATATTA
CACAATATTATTAGTTTGTAAAAACCGAAAGCCAGGGAGCTTACTGCCCTGG
ATTCTTTAGGGCGTGGGTCTTATCACCTCATTCATTTCTTCCCTTCCCCTAAGA

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CTGAAATATCAGGATGTGACCTCGAGCCAGCTGGTTGGGGCTGGGGATCCCC
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GGCAGCACAATGTCAGTATTTATATTACACAATATTATTAGTTTGTAAAAACC
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GTCTGTTCTGAGGCTCCTCAGAACCTCGAGGGAGGGGACGAGAGAGATGACC
CCTTGCCCAACCACTGGCTGAAAAATCTTGAAACTCCCTGACTTTGACCGGG
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ACTAGGACACGCATACGTTTTTAATGTACCTGCCCGGGCGGCCGCTCGAA

C7(3)-3

CCTCTGCCGCGACCACGCTTAAGGGGAGCATGGTGCTTAGCAGGCTTACCCG
ACACGATGGACTGGATGGGGATGCTCATGTCACGACACGATACCCGACTGCG
CAGGGATGTAAGGAGCGCAACGCATTGGGAGTATGATACTATCACTGGTAAT
GGACCCTCGGCTTTGCACTCTATGCTTCCGGCTCGTATGCTGCGTGGAATTGA
GAGCGTATAATTTTTCGCACAGGAAACAGGCTATGACCATGATTACGCCAAG
CTATATAGGAGACACTATAGAATACTCAAGCTATGCATCCAACGCGTGGGGA
GCTCTCCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTTCGAGAGCT
CCCTCGCTGATCCTGGCCGGGCGGACCTCGAACGCGACCACCGCT

C7(3)-4

CTAGTGAGTGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCCTCCGCA
CCACGCCCTTAAACTGCTGGTGCGCCCGGGGCCGAGACCTAAAAAAGCAAAC
CGCCCTCTGCGCCACGCCCTATATGTTCCCGCCCTCCGCACCGCGCCCTAAAA
CCGCAGTGACGCCCTCGCACCACGTCCCTTAAATAAGAAAGCCGCCCTCCGC
ACCACGCCCTAAATGCTACCGCCCTCCGCACCACGCCCTAACGCCGGGGTGG
TGCTCCGCGCCCTGTCCATACGCGACCCTAACCCTTAAGCGCTGGGGGCGGC
CACCGGCCCTACGCACCTAGTACCTAAACGGGCCTCCGTAACCACGTCCCTA
CCGTGATACCGCCCTACGCACCACGCCCTAA

C2-3

AGCGTGGTCGCGGCCGAGGTACCTTACTACTTGGATAACCGTGGTAATTCTA
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ACAAAACCAATGCCCCTCGGGGCTCTCTGGTGATTCATGATAACTCGTCGAAT
CGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTT
CGATGTTTGGGTAGTGGCCAAACATGGTGGCAACGGGTAACGGAGGGTTAGG

GCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGG
CAGTAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACAATAAA
TACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTGCCCGGG
CGGCCGCTCGAA

C3-2

AGCGTGGTCGCGGCCGAGGTACCTTACTACTTGGATAACCGTGGTAATTCTA
GAGCTAATACATGCTAAAAATCGCGACTTCGGAAGGGATGTATTTATTATAT
ACAAAAACCAATGCCCCTCGGGGCTCTCTGGTGATTCATGATAACTAGTCAAA
TCGTGCGTCCTTGCGCCGGCCGATGGTGTCATTCAAATTTCTTCCCTATAAAC
TTTAGGATGCTTGGGTATCGCCCAACTATGGTGTTTAACGGGATAACGGAGG
GTTAGGGATCGACCCCGGAGAAGCCCCTGAAAAACGGCTACTACATCCAAGG
AAGGCAGTAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACA
ATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTGC
CCGGGCGGCCGCTCGAA

C6-1

AGCGTGGTCGCGGCCGAGGTACCTTACTACTTGGATAACCGTGGTAATTCTA
GAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTATTAGAT
ACAAAACCAATGCCCCTCGGGGCTCTCTGGTGATTCATGATAACTCGTCGAAT
CGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTT
CGATGTTTGGGTAGTGGCCAAACATGGTGGCAACGGGTAACGGAGGGTTAGG
GCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGG
CAGTAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACAATAAA
TACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTGCCCGGG
CGGCCGCTCGAA

C7-76

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ACAAAACCAATGCCCCTCGGGGCTCTCTGGTGATTCATGATAACTCGTCGAAT
CGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATCTCTTCCCTATCAACTTT
CGATGTTTGGGTAGTGGCCAAACATGGTGGCAACGGGTAACGGAGGGTTAGG
GCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCCACTACATCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACAATAA
ATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTGCCCGG
GCGGCCGCTCGAA

C2-2

AGGGATCAAGCAAGCTCTCGGAAGCCATGGTTGCGGGATTAGCGTGGTCGCG
GTCGAGGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATG
CTAAAAATCCCGACTTCGGAAGGGATGTATTTATTAGATACAAAACCAATGC
CCCTCGGGGCTCTCTGGCGATTCATGATAACTCGTCGAATCGCACGGCCTTGC
GCCGGCGATGGTTCATTCCAATTTCTTCCCTATCAACTTTCGATGTTTGGGTA
GCGGCCAAACATGGTGGCAACGGGAAACGGAGGGTTAGGGCTCGACCCCGG
AGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGC
AAATTACCCAATCCCGATACGGGGAGGTACTGACAATAAATACTGATACAGG

GCTCTTTTGGGTCTTGTAATTGGAATGAGTTACCTGCCCGGGCGGCCGCTCGA
A

C3-3

TCGAGCGGCCGCCCGGGCAGGTACTCATTCCAATTACAAGACCCAAAAGAGC
CCTGTATCAGTATTTATTGTCACTACCTCCCCGTATCGGGATTGGGTAATTTG
CGCGCCTACTGCCTTCCTTGGATGTAGTAGCCGTTTCTCAGGCTCCTTCTCCG
GGGTCGAGCCCTAACCCTCCGTTACCCGTTGCCACCATGTTTGGCCACTACCC
AAACATCGAAAGTTGATAGGGAAGAAATTTGAATGAACCATCGCCGGCGCA
AGGCCGTGCGATTCGACGAGTTATCATGAATCACCAGAGAGCCCCGAGGGGC
ATTGGTTTTGTATCTAATAAATACATCCCTTCCGAAGTCGGGATTTTTAGCAT
GTATTAGCTCTAGAATTACCACGGTTATCCAAGTAGTAAGGTACCTCGGCCGC
GACCACGCTA

C6-3

ATACGATCCAATATAGTCCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGC
CATGGGCGGGATTAGCGTGGTCGCGGCCGAGGTCCGGCGATGGTTCATTCAA
ATTTCTTCCCCATCAACTTTCGATGTTTGGGTAGTGGCCAAACATCGAAAGTT
GATAGGGAAGAAATTTGAATGAACCATCGCGCGCAAGGCGTGCGATTCGACG
AGTTATCATGAATCACCAGAGAGCCCCGAGGGCATTGGTTTTGTATCTAATA
AATACATCCCTTCCGAAGTCGGGATTTTTAGCATGTATTAGCTCTAGAATTAC
CACGGTTATCCAAGTAGTAAGGTACCTGCCCGGGCGGCCGCTCGAA

C6-7

CCTTAGATCCAAGGGATCCAAGGAGGGCGAATTGGGCCCGACGTCGCATGCT
CCCGGCCGCCATGGCGCGGGATTAGCGTGGTCGCGGCCGAGGTCCGGCGATG
GTTCATTCAAATTTCTTCCCCATCAACTTTCGATGTTTGGGTAGTGGCCAAAC
ATCGAAAGTTGATAGGGAAGAAATTTGAATGAACCATCGCCGGCGCAAGGCC
GTGCGATTCGACGAGTTATCATGAATCACCAGAGAGCCCCGAGGGCATTGGT
TTTGTATCTAATAAATACATCCCTTCCGAAGTCGGGATTTTTAGCATGTATTA
GCTCTAGAATTACCATGGTTATCCAAGCAGCAAGAGTACCTGCCCGGGCGGC
CGCTCGAA

C7-97

AGCGTGGTCGCGGCCGAGGTACTTGAGGAGGAGAGATGAAATTCTATTATAC
CAAAGGGACTCGGTAAAGGCGAAGGCAGCCCTCTATGTAAAAACTGACGTTG
AAGGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTG
CAGTAAATGATGAATGTCATAGGTTAGATTAAGCTTTAATTAATTAGTTTATC
AGTAATGAACTAACTATTAAACAATATTTAGTCTATAAATGAAAGTGTAAGC
ATTCCACCTCAAGAGTAATGTGGCAACGTAGGAACTGAAATCCCTAGACCGT
TTCTGACACCAGTAGTGAAGTATGTTGTTTAATTCGATGATCCACGAAAACCT
TACCCTAATTTGAATATTTTACAGGAGTTGCACGGCTGTTTTCAGTTAATGTT
GTGAAACTGCGGTTTCCATGAAATTAACGCAATCCCTGGCTTTATTTTTTTTTT
CTATTACCGATAAAGCATTTGATCCACGGATTTGGCAAAGAAA

C7-70

AGCGTGGTCGCGGCCGAGGTACCTTACTACTTGGATAACCGTGGTAATTCTA
GAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTATTTGAT
ACAAAACCAATGCCCCTCGGGGCTCTCTGGTGATTCATGATAACTCGTCGAAT

CGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATCTCTTCCCTATCAACTTT
CGATGTTTGGGTAGTGGCCAAACATGGTGGCAACGGGTAACGGAGGGTTAGG
GCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCCACTACATCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACAATAA
ATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTGCCCGG
GCGGCCGCTCGAA

C7(1)-2

TCGAGCGGCCGCCCGGGCAGGTACCAATCCAAGGTAAATTCACCGTGACATG
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AATCCTTAAAATTTATATCCTATTTTTAGAGATTAGCTTAAATTCACATTTTTG
CATCTCTTTGTGTAGGAATATGTGGCACGTCTATAGCCCACAATATCAAGGCC
ATGATGACTTGTCTTTGTCCCCTTTTTCTTTCCAAATCCTGGATCAAATGCTTT
ATCGTAATAAAAAAAAAAAATAAAGCCAGGGATTGCGTTAATTTCATGGAAAC
CACAGTTTCACAACATTAACTGAAAACAGCCGTGCAACTCCTGTAAAATATT
CAAATTATGGTAAGGTTTTCGTGGATCATCGAATTAAACAACATACTTCACTA
CTGGTGTCAGAAACGGTCTAGTGATTTCAGTTCCCGCGTTGCCGCATTACTCT
TGAGGTGGAATGCTTACACTTTCATTTATAGACTAAATATTGTTTAATAGTTA
GTTCATTACTGATAAACTAATTAATTAAAGCTTAATCTACCTATGGACATTCA
TCATTTACTGCAAAGACTACTTGGGTATCTAATCCTGTTTGTGCTCTGTGCCTT
CGTCCTTCAACGTCAGTTTTTACATAGAGGGCTGCCTTCGCCTTTACCGAGTC
CCTTTGGTATAATAGAATTCCTCTCTCCTCCTCAAGTACCTCGGCCGCGACCA
CGCTA

Appendix K

Sequences of mycelial subtracted cDNA inserts using T7 promoter

M1-54

AGACTAAATATTGTTTAATAGTTAGTTCATTACTGATAAACTAATTAATTAAA
GCTTAATCTAACTCGAGCGGCCGCCCGGGCAGGTACCAATCCAAGGTAAATT
CACCGTGACATAGTGATTCACGATTACTAGTAATTACTTATTCATATAGTCGA
GTTTCAGACTATAATCCTTAAAATTTATATCCTATTTTTAGAGATTAGCTTAA
ATTCACATTTTTGCATCTCTTTGTGTAGGAATATGTGGCACGTCTATAGCTCA
CAATATCAAGGCCATGATGACTTGTCTTTGTCCCCTTTTTCTTTCCAAATCCTG
GATCAAATGCTTTATCGTAATAAAAAAAAAAATAAAGCCAGGGATTGCGTTAA
TTTCATGGAAACCACAGTTTCACAACATTAACTGAAAACAGCCGTGCAACTC
CTGTAAAATATTCAAATTATGGTAAGGTTTTTCGTGGATCATCGAATTAAACA
ACATACTTCACTACTGGTGTCAGAAATGGTCTAGTGATTTCAGTTCCTGCGTT
GCCACATTACTCTTGAGGTGGAATGCTTACACTTTCATTTATCTATGACATTC
ATCATTTACTGCAAAGACTACTTGGGTATCTAATCCTGTTTGTGCTCTGTGCCT
TCGTCCTTCAACGTCAGTTTTTACATAGAGGGCTGCCTTCGCCTTTACCGAGT
CCCTTTGGTATAATAGAATTTCATCTCTCCTCCTCAAGTACCTCGGCCGCGAC
CACGCTA

M1-102

ACCATCGTCTCCCCCAACATGCACCAATACGAGGATATAAACTTCCATACTTG
CAGATGCACTCTTACTAGGATTTACTTATTCATATAGTAGAGACTCACACTAT
AATCCTAAAGCTCTATATACTATCCCCTATAAAATACCTTAGTTTCCTATTCTC
GCATTCTATTTATGTCATAAATATGTATTAACCCTCTATATACTACAATAATA
AGGACTTAACTACTTATTATTCTCCATTTATTCTATCCACCTACTTGATAACAT
GCTATATCCTAATCAATGTACCTACACCCCAATATAGCCTTCTTTTACCTGTA
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ATATTCAAATTATTCCAAAATTTTTTCCGAGTATCCACCGGTGGGATCCATAT
AATTT

M1-105

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TGCCCGGGCGGCCGCTCGAA

M1-117

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GAGGTGGAATGCTTACACTTTCATTTATAGACTAAATATTGTTTAATAGTTAG
TTCATTACTGATAAACTAATTAATTAAAGCTTAATCTAACCTATGACATTCAT
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GTCCTTCACGTCAGTTTTTACATAGAGGGCTGCCTTCGCCTTTACCGAGTCCC
TTTGGTATAATAGAATTTCATCTCTCCTCCTCAAGTACCTCGGCCGCGACCAG
CTA

M3-12*

TCGAGCGGCCGCCCGGGCAGGTACTCATTCCAATTACAAGACCCAAAAGAGC
CCTGTATCAGTATTTATTGTCACTACCTCCCCGTATCGGGATTGGGTAATTTG
CGCGCCTGCTGCCTTCCTTGGATGTAGTAGCCGCTTCTCAGGCTCCTTCTCCG
GGGTCGAGCCCTAACCCTCCGTTACCCGTTGCCACCATGTTTGGCCACTACCC
AAACATCGAAAGTTGATAGGGAAGAAATTTGAATGAACCATCGCCGGCGCA
AGGCCGTGCGATTCGACGAGTTATCATGAATCACCAGAGAGCCCCGAGGGGC
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GACCACGCTA

M3-6*

TCGAGCGGCCGCCCGGGCAGGTACCAATCCAAGGTAAATTCACCGTGACATG
GTGATTCACGATTACTAGTAATTACTTATTCATATAGTCGAGTTTCAGACTAT
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ATCGTAATAAAAAAAAAATAAAGCCAGGGATTGCGTTAATTTCATGGAAACC
ACAGTTTCACAACATTAACTGAAAACAGCCGTGCAACTCCTGTAAAATATTC
AAATTATGGTAAGGTTTTTCGTGGATCATCGAATTAAACAACATACTTCACTA
CTGGTGTCAGAAACGGTCTAGTGATTTCAGTTCCTGCGTTGCCGCATTACTCT
TGAGGTGGAATGCTTACACTTTCATTTATAGACTAAATATTGTTTAATAGTTA
GTTCATTACTGATAAACTAATTAATTAAAGCTTAATCTAACCTATGACATTTC
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M3(2)-4

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CGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTT
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CAGCAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACAATAA
ATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTGCCCGG
GCGGCCGCTCGGGCAGGTACAATGTCTGTATAGAGACGGAGGTGGACCACGA
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M3(2)-6*

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CCGCCATGGCGCGGGATTAGCGTGGTCGCGGCCGAGGTCCGGCGATGGTTCA
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AAGTTGATAGGGAAGAAATTTGAATGAACCATCGCCGGCGCAAGGCCGTGCG
ATTCGACGAGTTATCATGAATCACCAGAGAGCCCCGAGGGCATTGGTTTTGT
ATCTAATAAATACATCCCTTCCGAAGTCGGGATTTTTAGCGTGTATTAGCTCT
AGAATTACCACGGTTATCCAAGTAGTAAGGTACCTGCCCGGGCGGCCGCTCG
GGCAGGTACTCATTCCAATTACAAGACCCAAAAGAGCCCTGTATCAGTATTT
ATTGTCACTACCTCCCCGTATCGGGATTGGGTAATTTGCGCGCCTGCTGCCTT
CCTTGGATGTAGTAGCCGTTTCTCAGGCTCCTTCTCCGGGGTCGAGCCCTAAC
CCTCCGTTACCCGTTGCCGCCATGTTTGGCCACTACCCAAACATCGAAAGTTG
ATAGGGAAGAAATTTGAATGAACCATCGCCGGCGCAAGGCCGTGCGATTCGA
CGAGTTATCATGAATCACCAGAGAGCCCCGAGGGGCATTGGTTTTGTATCTA
ATAAATACATCCCTTCCGAAGTCGGGATTTTTAGCATGTATTAGCTCTAGAAT
TACCACGGTTATCCAAGTAGTAAGGTACCTCGGCCGCGACCACGCTA

M3(4)-1*

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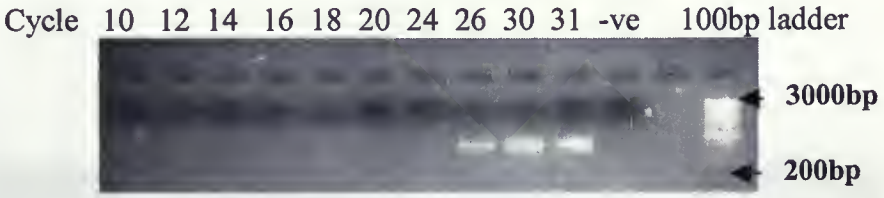
ACAAAACCAATGCCCCTCGGGGCTCTCTGGTGATTCATGATAACTCGTCGAAT
CGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTT
CGATGTTTGGGTAGTGGCCAAACATGGTGGCAACGGGTAACGGAGGGTTAGG
GCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGG
CAGCAGGCGCGCAAATTACCCAATCCCGATACGGGGAGGTAGTGACAATAA
ATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACCTCGGCCG
CGACCACGCTA

M3(4)-6*

AGCGTGGTCGCGGCCGAGGTACTCATTCCAATTACAAGACCCAAAAGAGCCC
TGTATCAGTATTTATTGTCACTACCTCCCCGTATCGGGATTGGGTAATTTGCG
CGCCTGCTGCCTTCCTTGGATGTAGTAGCCGTTTCTCAGGCTCCTTCTCCGGG
GTCGAGCCCTAACCCTCCGTTACCCGTTGCCACCATGTTTGGCCACTACCCAA
ACATCGAAAGTTGATAGGGAAGAAATTTGAATGAACCATCGCCGGCGCAAG
ACCGTGCGATTCGACGAGTTATCATGAATCACCAGAGAGCCCCGAGGGGCAT
TGGTTTTGTATCTAATAAATACATCCCTTCCGAAGTCGGGATTTTTAGCATGT
ATTAGCTCTAGAATTACCACGGTTATCCAAGTAGTAAGGTACCTGCCCGGGC
GGCCGCGACCACGCTA

Appendix L

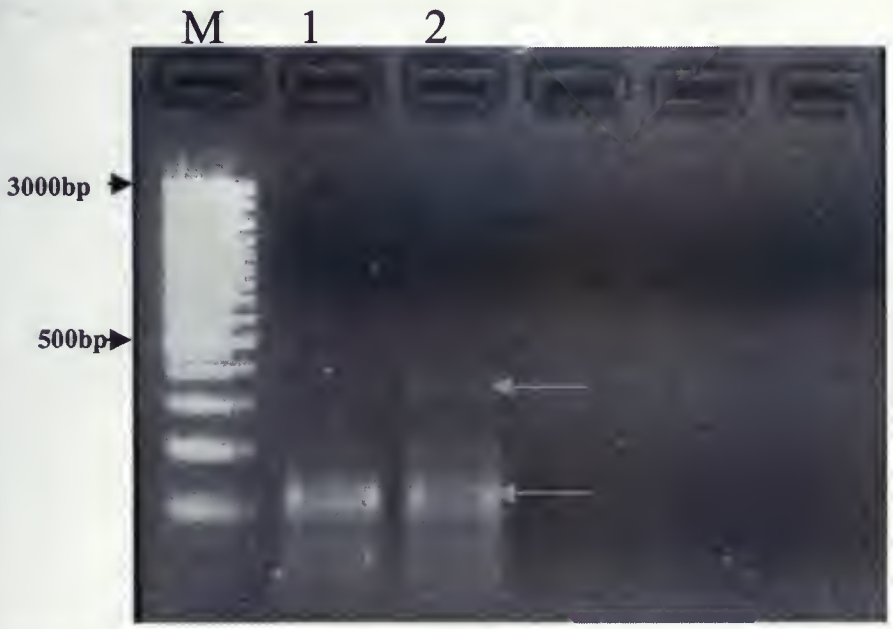
PCR Amplification of β-actin in order to determine the exponential phase of amplification



Shows 1 % agarose gel of PCR amplification of β-actin which shows the exponential phase of amplification at the end of cycle 10, 12, 14, 16, 18, 20, 24, 30 and 31.

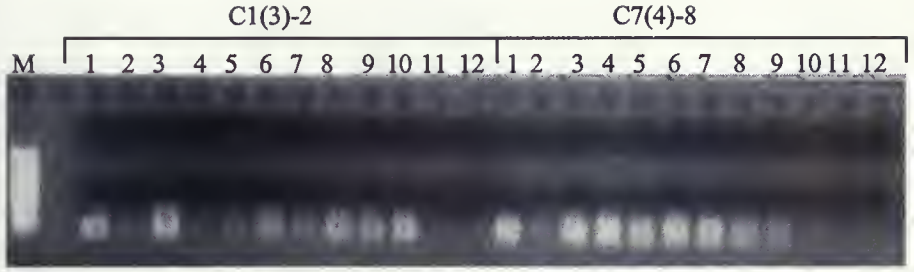
Appendix M

Shows RT-PCR with β-actin primers to determine if there was genomic contamination present.



Shows 1% agarose gel of RT-PCR with β-actin primers from total RNA extracted using RNeasy kit. Lane M, 100bp plus ladder. Lane 1, RT-PCR using total RNA from mycelia DNase treated shows a 150bp sized band which represents amplified cDNA. Lane 2, RT-PCR using total RNA from mycelia not DNase treated shows two bands: 150 bp representing amplified cDNA and 350bp representing amplified Genomic DNA.

Appendix N



1% agarose gel of Gradient PCR using primers designed from sequences of putative conidial-enriched transcripts C1 (3)-2 and C7 (4)-8 Amplification was performed at annealing temperatures of 55 C +/-10°C at 35 cycles. Lane M: 100bp plus ladder. Lanes numbered 1-12 in °C: #1= 45.0, #2= 45.3, #3= 46.4, #4= 48.2, #5=50.4, #6=53.0, #7=55.8, #8=58.5, #9=61.0, #10= 63.0, #11, =64.7, #12=65