DEVELOPMENT OF A PCR-BASED SPECIFIC METHOD FOR THE DETECTION OF *ASPERGILLUS FUMIGATUS* BY RANDOM CDNA CLONING

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Approval of the Graduate School of Natural and Applied Sciences

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

DEVELOPMENT OF A PCR-BASED SPECIFIC METHOD FOR THE DETECTION OF *ASPERGILLUS FUMIGATUS* BY RANDOM CDNA CLONING

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Aspergillus fumigatus is a foodborne and airborne human pathogen which produces mycotoxins such as gliotoxin, helvolic acid, fumigallin, and fumigaclavin. The most common disease caused by *A. fumigatus* is aspergillosis, which is often fatal, especially among AIDS, cancer, and organ-transplant patients. In this study, random cDNA cloning was performed from a cDNA library of *A. fumigatus* (IMI 385708) constructed on λ ZAP Express. Some of these clones were selected according to their insert sizes and were further subjected to sequence analysis. The sequences were then analyzed by a BLAST search (NCBI Genome Database) to determine the possible functions of these genes. Two of the clones were identified as the primase and 60S ribosomal protein L1-b genes. These genes and a third gene corresponding to the antigenic cell wall galactomannoprotein gene of *A. fumigatus*

were used for the design of three distinct primer pairs. For the primer design, a software was written to differentiate nonconserved regions by multiple sequence alignment. Designed primers were employed in PCR experiments against genomic DNAs of different *Aspergillus* species. Unique bands were obtained only against *A. fumigatus* genomic DNA. It was concluded that this PCR-based detection method can be further developed for the rapid detection of *A. fumigatus* spores from air and food samples.

Keywords: *Aspergillus fumigatus*, molecular diagnosis, PCR, cDNA cloning, ribosomal protein, primase, galactomannoprotein

ÖZ

RASTLANTISAL CDNA KLONLANMASIYLA *ASPERGILLUS FUMIGATUS* İÇİN PCR-BAZLI ÖZGÜL BİR TANI YÖNTEMİNİN GELİŞTİRİLMESİ

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Aspergillus fumigatus gıda ve hava ile bulaşan bir insan patojeni olup gliotoksin, helvolik asit, fumigallin, fumigaklavin gibi mikotoksinler üretmektedir. *A. fumigatus*'un en sık sebep olduğu hastalık olan aspergillosis genellikle öldürücüdür ve özellikle bağışıklık sistemi zayıf düşmüş, AIDS, kanser ve organ nakli hastalarında görülür. Bu çalışmada, *Aspergillus fumigatus* IMI 385708'in λ ZAP Express'le kurulan cDNA kütüphanesinden rastlantısal cDNA klonlaması yapılmış, bu klonlardan bazıları insert boyutlarına göre seçilip nükleotid dizilim analizleri elde edilmiştir. Dizilimler daha sonra Gen Bankası ve ilgili yazılımlarla, genlerin olası fonksiyonlarının bulunması amacıyla analiz edilmiştir. Klonlardan ikisi primaz ve 60S ribozomal protein L1-b genleri olarak tanımlanmıştır. Bu iki gen ve *A. fumigatus*'un antijenik hücre duvarı galaktomannoprotein geni üç ayrı çift primerin

tasarlanmasında kullanılmıştır. Primer tasarlanabilmesi için, çoklu dizilim analiziyle, korunmayan bölgeleri ayırt edebilecek bir yazılım geliştirilmiştir. Tasarlanan primerler, değişik *Aspergillus* türlerinde PCR aracılığı ile denenmiş ve sadece *A. fumigatus* DNA'sına karşılık tek ve özgül bir bant elde edilmiştir. Buna göre, bu çalışmada geliştirilen PCR bazlı tanı yönteminin *Aspergillus fumigatus*'un gıdalardan ve havadaki sporlardan hızlı bir şekilde tayini amacı ile daha fazla geliştirilerek kullanılabileceği sonucuna varılmıştır.

Anahtar Sözcükler: *Aspergillus fumigatus*, moleküler tanı, PCR, cDNA klonlaması, ribozomal protein, primaz, galaktomannoprotein

To My Family

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At last, but not the least, I deeply thank to my wife who have been there with me not only in the roads full of roses but also full of thorns. She made my life meaningful.

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INTRODUCTION

1.1 The Genus *Aspergillus*

Aspergillus is a genus of fungi found worldwide; over 180 species are officially recognized (Pitt et al., 2000), some of which are of medical or industrial importance. The fungus *Aspergillus* derives its name from its resemblance to the brush, called an "aspergillum", used for sprinkling holy water (Shah and Panjabi, 2002). *Aspergillus fumigatus* is the most common mould pathogen of humans, causing both life-threatening invasive disease in immunocompromised patients and allergic disease in patients with atopic immune systems (Marr et al., 2002). *Aspergillus nidulans*, an occasional human pathogen, is a model organism that has contributed to our understanding of genetics, gene regulation and cellular biology (Pontecorvo et al., 1953; Martinelli and Kinghorn, 1994), while *Aspergillus niger* (Bennett and Klich, 1999; Ruijter et al., 2002) and *Aspergillus oryzae* (Nout and Aidoo, 2002) are both used in industrial processes. Several other *Aspergillus* species are known to be significant allergens or to be responsible for mycotoxin production on stored food (Blyth et al, 1977; Jarvis and Morey, 2001; Pitt, 2000).

1.2 *Aspergillus fumigatus*

A. fumigatus is a saprophytic thermotolerant fungus that plays an essential role in recycling environmental carbon and nitrogen (Haines, 1995; Pitt, 1994; Vanden Bossche et al., 1988). Its natural ecological niche is the soil, wherein it

survives and grows on organic debris such as mushroom compost. Although this species is not the most prevalent fungus in the world, it is one of the most ubiquitous of those with airborne conidia (Mullins et al., 1976; Mullins et al., 1984; Nolard, 1994). It sporulates abundantly, with every conidial head producing thousands of conidia. The conidia released into the atmosphere have a diameter small enough (2 to 3 μm) to reach the lung alveoli (Raper and Fennell, 1965; Samson and Van Reenen-Hoekstra, 1988). *A. fumigatus* does not have an elaborate mechanism for releasing its conidia into the air; dissemination simply relies on disturbances of the environment and strong air currents. Once the conidia are in the air, their small size makes them buoyant, tending to keep them airborne both indoors and outdoors. Environmental surveys indicate that all humans will inhale at least several hundred *A. fumigatus* conidia per day (Chazalet et al., 1998; Goodley et al., 1994; Hospenthal et al., 1998).

Inhalation of conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are eliminated relatively efficiently by innate immune mechanisms. Thus, until recent years, *A. fumigatus* was viewed as a weak pathogen responsible for allergic forms of the disease, such as farmer's lung, a clinical condition observed among individuals exposed repeatedly to conidia, or aspergilloma, an overgrowth of the fungus on the surface of preexisting cavities in the lungs of patients treated successfully for tuberculosis (Disch et al., 1995; Kwon-Chung and Bennett, 1992; Pennington, 1988). Because of the increase in the number of immunosuppressed patients, however, and the degree of severity of modern immunosuppressive therapies, the situation has changed dramatically in recent years (Cohen et al., 1993; Rogers, 1995; Ruchlemer et al., 1996). Over the past 10 years, *A. fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunocompromised hosts in developed countries (Andriole, 1993; Beck-Sagué and Jarvis, 1993; Bodey and Vartivarian, 1989; Denning, 1998; Dixon et al., 1996; Groll et al., 1996). A fourfold increase in invasive aspergillosis (IA) has been observed in the last 12 years. In 1992, IA was responsible for approximately 30% of fungal infections in patients dying of cancer, and it is estimated that IA occurs in 10 to 25% of all leukemia patients, in whom the mortality rate is 80 to 90%, even when treated (Bodey et al., 1992; Denning, 1995; Denning, 1996; Groll et al., 1996; Verweij and Denning, 1997). IA is now a major

cause of death at leukemia treatment centers and bone marrow transplantation (BMT) and solid-organ transplantation units (Cordonnier et al., 1996; Derouin, 1994; Patel and Paya, 1997; Salonen and Nikoskelainen, 1993). Although *A. fumigatus* is the most common etiologic agent, being responsible for approximately 90% of human infections (Bodey and Vartivarian, 1989; Derouin, 1994; Dixon and Walsh, 1992; Kurup and Kumar, 1991; Latgé et al., 1997; Schaffner, 1992; Vanden Bossche et al., 1988), it is not the only pathogen in this genus. *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* can also cause human infections (Latgé, 1999).

Aspergillus spp. are frequently present on food and thus can be an indirect source of airway or digestive tract colonization (Sarfati et al., 1996). One typical example is pepper, which has been found to be massively contaminated by *A. flavus*, *A. fumigatus*, and *A. niger* (De Bock et al., 1989; Eccles and Scott, 1992; Vargas et al., 1989). Microbial contamination of food given to neutropenic patients can be eradicated by heating at 210°C, irradiation, or treatment in a microwave oven. However, the efficiency of the last two procedures is not proven (Emam et al., 1995), and heating cannot be applied to some types of foods, such as fruits, fruit juices, bread, and cheese.

Bouakline et al., (2000) studied the degree fungal contamination of food in hematology units. The degree and type of fungal contamination varied a lot according to the type of food (Table 1.1). The heaviest and most frequent fungal contamination occurred in pepper and tea. Herbal teas were less frequently contaminated than regular tea and did not contain *Aspergillus* species. Three of 15 samples of freeze-dried soup contained spores of *Aspergillus* and *Mucorales*. *A. fumigatus* was also found in two samples of heat-sterilized grapefruit juice.

Table 1.1 Fungal load and thermotolerant species recovered from food contaminated by fungus (Bouakline et al., 2000)

Food	No. of samples examined	% Contamination	Fungus	Score[a]
Pepper	15	100	*A. fumigatus*	+++
		100	*A. flavus*	+++
		100	*Mucorales*	+++
Regular tea	15	100	*A. fumigatus*	+++
		100	*A. niger*	+++
		33	*Mucorales*	+++
Apricot	15	66	*A. fumigatus*	+
		66	*A. niger*	+
		66	*Trichoderma* sp.	+
Peach	4[b]	50	*A. fumigatus*	+
Kiwi	8[b]	50	*A. fumigatus*	+
		50	*Trichoderma* sp.	+
Banana	15	33.3	*A. fumigatus*	++
Herbal tea	22	27.3	*Mucorales*	++
Apple	15	20	*A. fumigatus*	+
Orange	15	20	*A. fumigatus*	+
		20	*Aspergillus* sp.	+
Freze-dried soup	15	20	*A. fumigatus*	++
		20	*A. niger*	++
		20	*Mucorales*	++
Crackers	15	13.3	*Chaetomium* sp.	++
Grapefruit juice	15	13.3	*A. fumigatus*	+
Lemon	8[b]	12.5	*A. fumigatus*	+
Sweet biscuits	15	6.7	*Mucorales*	+
Soft cheese	20	100	*Geotrichum* sp.	+++
		100	*C. norvegensis*	+++

[a] +, 1 to 5 CFU; ++, 6 to 10 CFU; +++, >10 CFU.

[b] n < 15 because products were seasonal

As pepper is usually sprinkled on food just before eating, there is a major risk of producing an aerosol of *Aspergillus* spores and exposing the patient to an airborne contamination. Regular tea was also consistently contaminated by molds, as were most herbal teas (Elshafie et al., 1999; Kuminsky et al., 1996; Le Bars and Le Bars, 1988). The risk associated with tea bags is probably lower than that associated with pepper, as the tea is usually prepared in the kitchen by the addition of boiling water, which likely kills the spores, although this has not been clearly demonstrated. Similar uncertainty applies to freezedried soups, which may also contain spores of *Aspergillus* and *Mucorales*. Fruits with a downy skin may also pose a risk of contamination if they are directly handled by the patient.

A. fumigatus contamination is also a problem in honey. Martins et al., (2003) analyzed 80 samples of honey and, 71(88.8%) were contaminated with fungi; of these samples, 46 were contaminated with moulds and yeasts, and 25 samples presented only yeasts. The moulds identified were: *A. candidus* (28.7%), *A. flavus* (57.5%), *A. fumigatus* (45.0%), *A. niger* (51.3%), *Mucor sp.* (31.3%) and *Penicillium spp.* (38.8%), with levels ranging from 10^1 to 10^2 cfu/g.

Apart from the pathogenicity of the thermotolerant fungi, potential impacts on health are being discussed from both allergological and toxicological points of view. To estimate the health risks reliably, it is not sufficient to measure total spore counts of fungi, which do not give information on the potential impact of fungal metabolites present in airborne spores. Numerous toxic properties such as nephro- and hepathotoxic, tremorgenic, and carcinogenic effects have been described for mycotoxins, mainly in connection with food borne intoxication of both animals and man (Fischer et al., 1999).

1.2.1 Culture and morphological characteristics

The organism is characterized by green echinulate conidia, 2.5 to 3 μm in diameter, produced in chains basipetally from greenish phialides, 6 to 8 by 2 to 3 μm in size (Figure 1.1). A few isolates of *A. fumigatus* are pigmentless and produce white conidia (Sarfati et al.). No sexual stage is known for this species. *A. fumigatus* is a fast grower; the colony size can reach 4±1 cm within a week when grown on

Czapek-Dox agar at 25°C (Raper and Fennell, 1965). *A. fumigatus* is a thermotolerant species, with optimum growth temperature at 45°C and growth occurring at temperatures as high as 55°C and survival maintained at temperatures up to 70°C (Haines, 1995; Kwon-Chung and Bennett, 1992; Raper and Fennell, 1965; Samson and Van Reenen-Hoekstra, 1988).

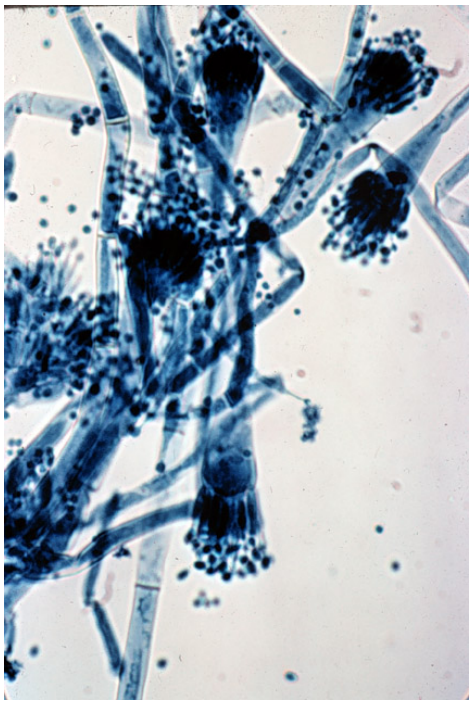


Figure 1.1 Microscopic view of *A. fumigatus* (http://www.aspergillus.man.ac.uk)

A. fumigatus is morphologically more variable (Leslie et al., 1988; Samson, 1994; Schmitt and Wolff, 1997) than was originally described by Raper and Fennell (Raper and Fennell, 1965). These variations have led to the description of several varieties of *A. fumigatus*, including *acolumnaris*, *phialiseptus*, *ellipticus*, and *sclerotiorum*, with the distinctions being based on only slight morphological differences. *A. fumigatus*, *A. brevipes*, *A. duricaulis*, *A. unilateralis*, *A. viridinutans*, together with anamorphs of species within the perfect genus *Neosartorya*, a genus in

which morphologically related species have been grouped, are classified as *Aspergillus* sect. *fumigati* (Latgé, 1999).

1.2.2 Mycotoxins

Mycotoxins comprise a group of more than 300 different chemical compounds (Betina, 1989), and pre- and post-harvest contamination of food and feed crops by mycotoxigenic fungi is a common problem throughtout the world. In domestic animals, such as dairy cattle, swine, ostrich, and poultry, mycotoxin contamination reduces growth efficiency, lowers feed conversion and reproductive rates, impairs resistance to infectious diseases, reduces vaccination efficiacy, and induces pathological damage to the liver and other organs (Coulombe, 1993).

Some important mycotoxins are aflatoxins, *Alternaria* toxins, citrinin, ochratoxins, patulin, penicillic acid, sterigmatocystin and zearalenone (Jay, 1991).

Aflatoxins are clearly the most widely studied of all mycotoxins. Knowledge of their existence dates from 1960, when more than 100,000 turkey poults died in England after eating peanut meal imported from Africa and South America. From the poisonous feed were isolated *A. flavus* and a toxin produced by this organism that was designated aflatoxin. It was later determined that *A. parasiticus* also produced aflatoxins (Jay, 1991).

Several species of *Alternaria* (including *A. citri, A. alternata, A. solani, and A. tenuissima*) produce toxic substances that have been found in apples, tomatoes, blueberries, and others. The toxins produced include alternariol monomethyl ether, altenuene, tenuazonic acid, and altertoxin-I (Jay, 1991).

Citrinin is produced by *Penicillium citrinum, P. viridicatum*, and other fungi. It has been recovered from polished rice, moldy bread, cured hams, wheat, oats, rye, and other similar products (Jay, 1991).

The ochratoxins consist of a group of at least seven structurally related secondary metabolites of which ochratoxin A is the best known and the most toxic. Ochratoxin B is dechlorinated ochratoxin A and along with ochratoxin C, it may not occur naturally. Ochratoxin A is produced by a large number of storage fungi, including *A. ochraceus, A. alliaceus, A. ostianus, A. mellus,* and other species of

aspergilli. Among penicillia that produce ochratoxin A are *P. viridicatum, P. cyclopium, P. variable,* and others. This mycotoxin has been found in corn, dried beans, cocoa beans, soybeans, oats, barley, citrus fruits, peanuts and other similar products (Jay, 1991).

Patulin (clavicin, expansin) is produced by a large number of penicillia, including *P. claviforme, P. expansum, P. patulum*; by some aspergilli (*A. clavatus, A. terreus* and others), and by *Byssochlamys nivea* and *B. fulva*. Its biological properties are similar to those of penicillic acid. Some patulin-producing fungi can produce the compound below 2 ºC. This mycotoxin has been found in moldy bread, sausage, fruits, cider (Jay, 1991).

Penicillic acid is produced by a large number of fungi, including many penicillia (*P. puberulum,* for example), as well as members of the *A. ochraceus*. It has been found in corn, beans, and other field crops (Jay, 1991).

Sterigmatocystin is produced by *A. versicolor, A. nidulans, A. rugulosus,* and others. Although not often found in natural products, they have been found in wheat, oats, and coffee beans. They act by inhibiting DNA synthesis (Jay, 1991).

There are at least five naturally occuring zearalenones, and they are produced by *Fusarium* spp., mainly *F. graminearum* and *F. tricinctum.* They have been found in corn, oats, wheat, barley, and sesame (Jay, 1991).

Thermotolerant *A. fumigatus* is the major problem causing mold in silage, and many strains can produce several mycotoxins that cause problems in dairy cattle (Dutkiewicz et al., 1989). Mycotoxins produced by *A. fumigatus* include tremorgens (Land et al., 1993), clavine alkaloids that appear to be responsible for abortions (Moreau, 1979), and gliotoxin that affects the immune system (Belkacemi et al., 1999; Yamada et al., 2000). There have been numerous syndromes in ruminants supposedly due to the ingestion of the fungi or their toxins in spoiled silage (Seglar, 1999). Cole et al. (1977) noted that the predominant fungi in molded silage from three silos examined were isolates of *A. fumigatus*.

Mycotoxins produced by *A. fumigatus* can produce various signs and symptoms, depending on the animal, the organ system involved, and the dose and type of mycotoxins ingested. The symptoms can include acute death, immunosuppression, skin lesions, and signs of hepatotoxicity, nephrotoxicity,

neurotoxicity, or genotoxicity. In addition to concerns over adverse effects of mycotoxins on animals directly, there is also public health concern over the potential for human beings to consume animal-derived food products such as meat, milk, or eggs, containing residues of those mycotoxins or their metabolites.

1.2.2.1 Gliotoxin

One highly toxic metabolite that can be produced by *A. fumigatus* is gliotoxin, which can also be produced by other mould species such as *Penicillium* (Richard et al.,1994), *Gliocladium* (Wilhite and Straney,1996) and *Candida* (Shah et al.,1995).

One of the best-studied fungal toxins is gliotoxin, first described as an antibiotic because of its wide range of antimicrobial properties. Interest in gliotoxin as an antibiotic diminished when its toxicity precluded its clinical use. Gliotoxin is lethal at relatively low concentrations and is produced by at least 10 species of fungi including *A. fumigatus*.

The properties of gliotoxin are as follows (http://www.aspergillus.man.ac.uk):

Chemical Type: Tricyclic Alkaloid

Formula: C_{13} H_{14} N_2 O_4 S_2

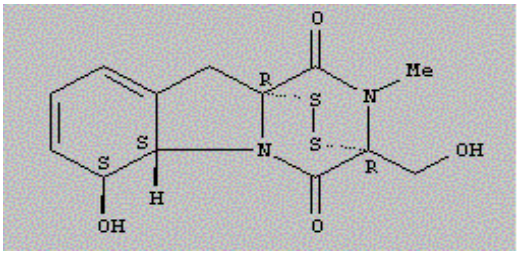
Molecular Weight: 326.4

Produced by: *A. fumigatus, A. terreus, Eurotium chevalieri, Penicillium* spp*, Candida* spp

Toxicity: Gliotoxin posseses a spectrum of biological activities including antibacterial and antiviral activities, and it is also a potent immunomodulating agent. Gliotoxin is also an inducer of apoptotic cell death in a number of cell types. Its high in vitro and in vivo immuno-suppressive potency makes it one of the putative

virulence factors of *A. fumigatus* and *Candida albicans*, and it is hypothesized that it could play a major role in the pathogenesis processes of invasive aspergillosis and candidiasis (Eichner et al., 1988; Sutton et al., 1996; Latgé, 2001; Shah et al., 1995). Its acute toxicity in mice is relatively high for a mycotoxin: LD50 is 7.8 mg/kg by intravenous injection, 32 mg/kg by intraperitoneal injection (Larin et al., 1965). Its oral LD50 are, respectively, 67 and 15 mg/kg in mice and hamster (De Clerq et al., 1978; Frame and Carlton, 1988), and 100% mortality is obtained after giving oral doses of 7.5 mg/kg to turkeys (Mc Dougall, 1969). Consequently, gliotoxin can be noxious for both animal and human health if accidentally ingested with contaminated food (Waring and Beaver, 1996), and one case of intoxication in camels has been reported so far (Gareis and Wernery, 1994).

Structure:



CA Index Name: 10H-3,10a-Epidithiopyrazino[1,2-a]indole-1,4-dione, 2,3,5a,6-tetrahydro-6-hydroxy-3-(hydroxymethyl)-2-methyl-, (3R,5aS,6S,10aR) (9CI)

Other Names: 10H-3,10a-Epidithiopyrazino[1,2-a]indole-1,4-dione, 2,3,5a,6-tetrahydro-6-hydroxy-3-(hydroxymethyl)-2-methyl-,[3R-(3α,5aβ,6β,10aα)]-(8CI); Gliotoxin (6CI, 7CI); Aspergillin; S. N. 12870

1.2.2.2 Fumigallin

The properties of fumigallin are as follows:
(http://www.aspergillus.man.ac.uk)

Chemical Type: Cyclohexane derivative

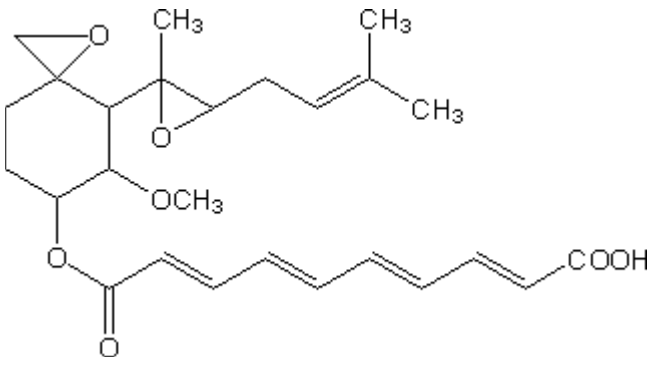
Formula: $C_{26}H_{34}O_7$

Molecular Weight: 458.55

Produced by: *A. fumigatus*

Toxicity: Originally isolated because of its antibiotic properties, especially against protozoa such as *Entamoeba histolytica*. LD50 in mice 800 mg/kg body-weight subcutaneously although as much as 2 mg/kg body-weight could be tolerated orally.

Structure:



1.2.2.3 Verruculogen

The properties of verruculogen are as follows:
(http://www.aspergillus.man.ac.uk)

Chemical Type: Indole Alkaloid

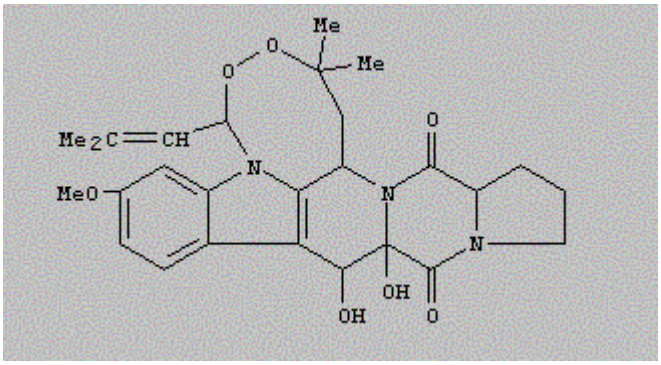
Formula: C_{27} H_{33} N_3 O_7

Molecular Weight: 511.57

Produced by: *A. fumigatus, Neosartorya fischeri*

Toxicity: Verruculogen is a tremorgen. Tremorgenic mycotoxins are lipophilic molecules that may cross the blood brain barrier and gain access to the central nervous system, clinical studies indicate that these compounds affect neurotransmitter release. Verruculogen increases spontaneous glutamate and aspartate release. *In vivo,* in guinea pig ileum preparations, verruculogen causes an increase in contractile responses due to electrical field stimulation, attributed to enhancement of acetylcholine from presynaptic nerve terminals. Verruculogen also was shown to increase presynaptic neurotransmitter release at the locust neuromuscular junction.

Structure:



CA Index Name: 5H,12H-3,4-Dioxa-5a,11a,15a triazacyclooct [lm] indeno [5,6b] fluorene-11,15 (2H,13H)-dione, 1, 10, 10a, 14, 14a, 15b hexahydro-10, 10a-dihydroxy-7methoxy-2, 2-dimethyl-5-(2-methyl-1-propenyl), (5R, 10S, 10aR, 14aS, 15bS)-(9CI)

Other Names: 5H,12H-3,4-Dioxa-5a,11a,15a-triazacyclooct[lm]indeno[5,6-b]fluorene-11,15(2H,13H)-dione, 1,10,10a,14,14a,15b-hexahydro-10,10a-dihydroxy-7-methoxy-2,2-dimethyl-5-(2-methyl-1-propenyl)-, [5R-(5a,10a,10aa,14aa,15ba)]-; NA 209A; TR 1; TR 1 toxin; Verruculogen; Verruculogen TR 1

1.2.2.4 Fumitremorgin A

The properties of fumitremorgin A are as follows:
(http://www.aspergillus.man.ac.uk)

Chemical Type: Alkaloid

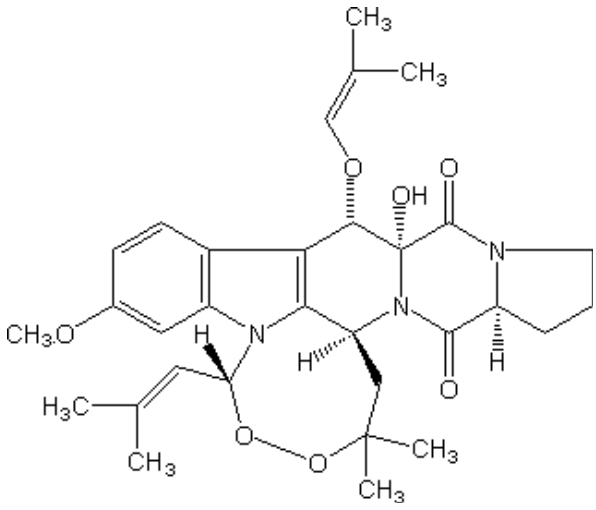
Formula: $C_{32}H_{41}N_3O_7$

Molecular Weight: 579.69

Produced by: Produced by *Penicillium brasilianum, A. fumigatus, Aspergillus caespitosus* and *N. fischeri.*

Toxicity: A tremorgenic mycotoxin. Intraperitoneal injection of 1 mg in mice caused visible tremors and a dose of 5 mg caused both sustained tremors and 70% mortality.

Structure:



1.2.2.5 Fumigaclavine

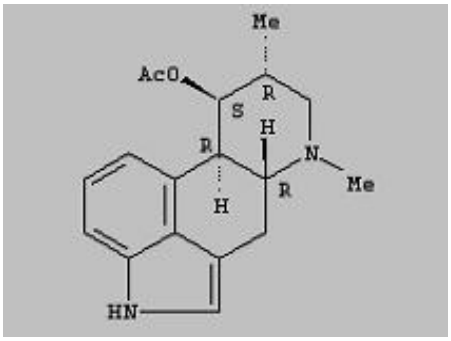
The properties of fumigaclavine are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{18} H_{22} N_2 O_2

Molecular Weight: 298.38

Produced by: *A. fumigatus*

Structure:



CA Index Name: Ergolin-9-ol, 6,8-dimethyl-, acetate (ester), (8a,9b)- (9CI)

Other Names: : Fumigaclavine A (6CI, 7CI, 8CI); Indolo[4,3 fg]quinoline, ergolin-9-ol deriv.

1.2.2.6 Helvolic Acid

The properties of helvolic acid are as follows:
(http://www.aspergillus.man.ac.uk)

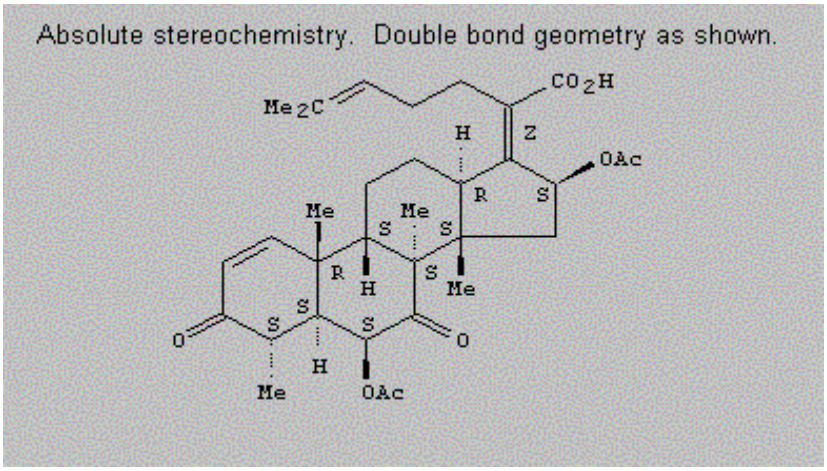
Formula: C_{33} H_{44} O_8

Produced by: *A. fumigatus, Cephalosporium caeruleus, and Emericellopsis terricola* (Chain et al., 1943; Cole and Cox, 1981)

Toxicity: Helvolic acid caused complete ciliostasis and epithelial cell disruption (Amitani et al., 1995).

Mitchell et al. (1997) have shown that helvolic acid, produced by *A. fumigatus*, inhibits the oxidative burst of macrophages.

Structure:



CA Index Name: 29-Nordammara-1,17(20),24-trien-21-oic acid, 6,16-bis(acetyloxy)-3,7-dioxo-, (4a,6b,8a,9b,13a,14b,16b,17Z)- (9CI)

Other Names: Helvolic acid (6CI, 7CI); (Z)-6b,16b-Dihydroxy-3,7-dioxo-29-nor-8a,9b,13a,14b-dammara-1,17(20),24-trien-21-oic acid diacetate; Fumigacin

1.2.2.7 Brevianamide A

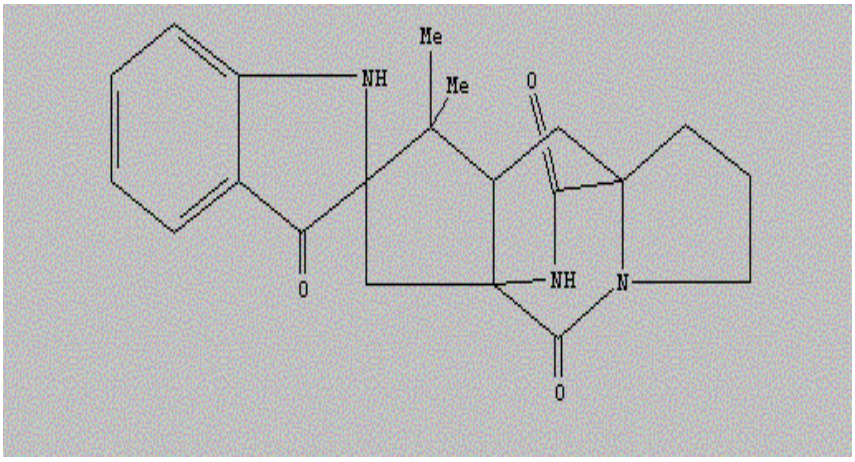
The properties of brevianamide A are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{21} H_{23} N_3 O_3

Molecular Weight: 365.2

Produced by: *A. fumigatus*

Structure:



CA Index Name: Spiro[5H,6H-5a,9a-(iminomethano)-1H-cyclopent[f]indolizine-7(8H),2'-[2H]indole]-3',5,10(1'H)-trione, 2,3,8a,9-tetrahydro-8,8-dimethyl-, (2'R,5aR,8aS,9aR)- (9CI)

Other Names: Spiro [5H, 6H-5a, 9a-(iminomethano)-1H-cyclopent [f] indolizine-7(8H),2'-indoline]-3', 5,10-trione, 2,3,8a,9-tetrahydro-8,8-dimethyl- (8CI); (+)-Brevianamide A; Brevianamid A; Brevianamide A; Spiro[5H,6H-5a,9a-(iminomethano)-1H-cyclopent[f]indolizine-7(8H),2'[2H]indole]-3',5,10(1'H)-trione, 2,3,8a,9-tetrahydro-8,8-dimethyl-, [5aR-(5aa,7b,8ab,9aa)]-

1.2.2.8 Phthioic Acid

The properties of phthioic acid are as follows:
(http://www.aspergillus.man.ac.uk)

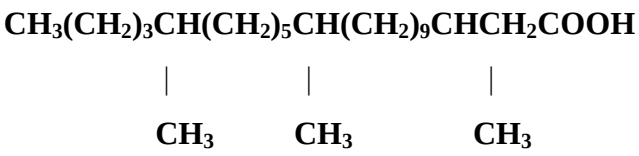
Chemical Type: Fatty Acid

Formula: C_{26} H_{52} O_2

Molecular Weight: 395.6

Produced by: *A. fumigatus*

Structure:



Other Names: : 3,13,19 TRIMETHYLTRICOSANOIC ACID

1.2.2.9 Sphingofungins

Sphingofungin A

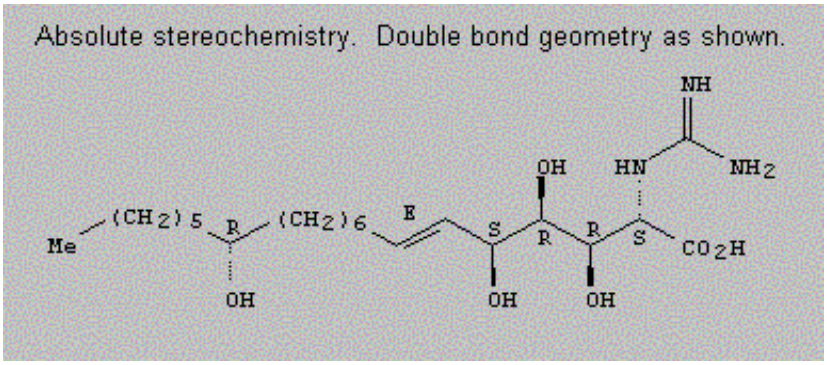
The properties of sphingofungin A are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{21} H_{41} N_3 O_6

Molecular Weight: 431.57

Produced by: *A. fumigatus*

Structure:



CA Index Name: 6-Eicosenoic acid, 2-[(aminoiminomethyl)amino]-3,4,5,14-tetrahydroxy-, (2S,3R,4R,5S,6E,14R)- (9CI)

Other Names: 6-Eicosenoic acid, 2-[(aminoiminomethyl)amino]-3,4,5,14-tetrahydroxy-, [2S-(2R*,3S*,4S*,5R*,6E,14S*)]-; Sphingofungin A

Sphingofungin B

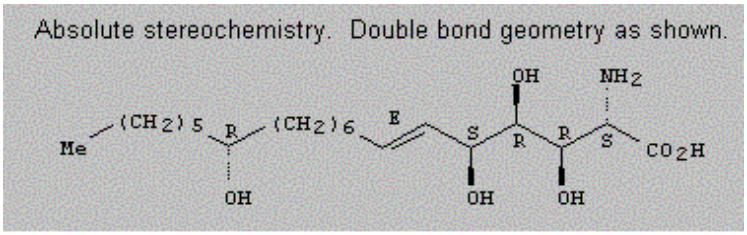
The properties of sphingofungin B are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{20} H_{39} N O_6

Molecular Weight: 389.53

Produced by: *A. fumigatus*

Structure:



CA Index Name: 6-Eicosenoic acid, 2-amino-3,4,5,14-tetrahydroxy-, (2S,3R,4R,5S,6E,14R)- (9CI)

Other Names: 6-Eicosenoic acid, 2-amino-3,4,5,14-tetrahydroxy-, [2S (2R*,3S*,4S*,5R*,6E,14S*)]-; Sphingofungin B

Sphingofungin C

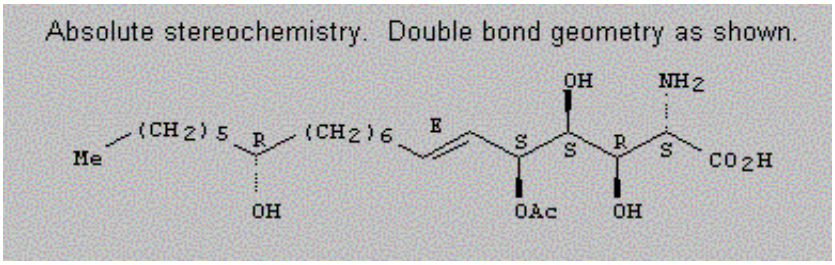
The properties of sphingofungin C are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{22} H_{41} N O_7

Molecular Weight: 431.56

Produced by: *A. fumigatus*

Structure:



CA Index Name: 6-Eicosenoic acid, 5-(acetyloxy)-2-amino-3,4,14-trihydroxy-, (2S,3R,4S,5S,6E,14R)- (9CI)

Other Names: 6-Eicosenoic acid, 5-(acetyloxy)-2-amino-3,4,14-trihydroxy-, [2S-(2R*,3S*,4S*,5R*,6E,14S*)]-; Sphingofungin C

Sphingofungin D

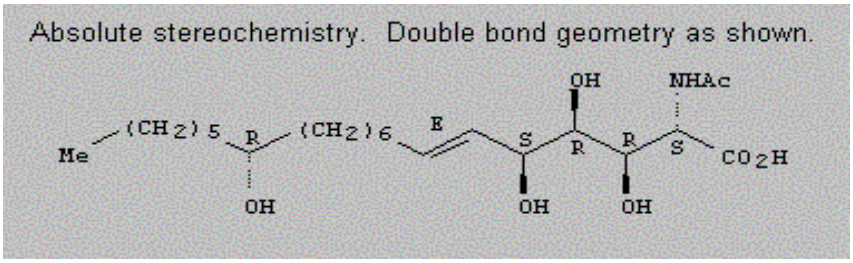
The properties of sphingofungin D are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{22} H_{41} N O_7

Molecular Weight: 431.56

Produced by: *A. fumigatus*

Structure:



CA Index Name: 6-Eicosenoic acid, 2-(acetylamino)-3,4,5,14-tetrahydroxy-, (2S,3R,4R,5S,6E,14R)- (9CI)

Other Names: 6-Eicosenoic acid, 2-(acetylamino)-3,4,5,14-tetrahydroxy-, [2S-(2R*,3S*,4S*,5R*,6E,14S*)]-; Sphingofungin D

Sphingofungin E

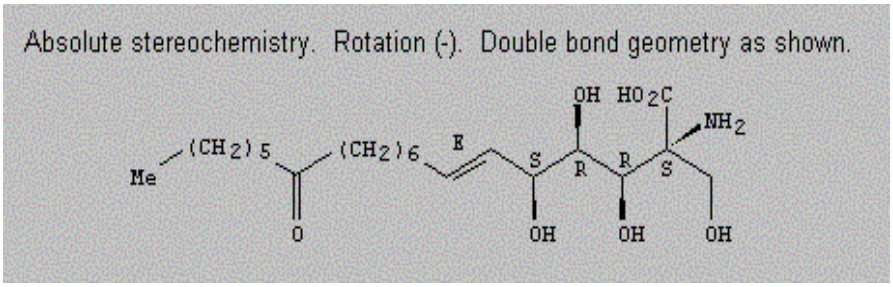
The properties of sphingofungin E are as follows:
(http://www.aspergillus.man.ac.uk)

Formula: C_{21} H_{39} N O_7

Molecular Weight: 417.54

Produced by: *A. fumigatus*

Structure:



CA Index Name: 6-Eicosenoic acid, 2-amino-3,4,5-trihydroxy-2-(hydroxymethyl)-14-oxo-, (2S,3R,4R,5S,6E)- (9CI)

Other Names: (-)-Sphingofungin E; Sphingofungin E

Sphingofungin F
(http://www.aspergillus.man.ac.uk)

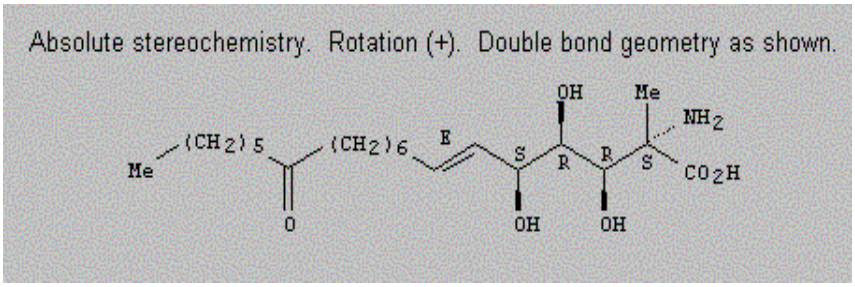
The properties of sphingofungin F are as follows:

Formula: C_{21} H_{39} N O_6

Molecular Weight: 401.54

Produced by: *A. fumigatus*

Structure:



CA Index Name: 6-Eicosenoic acid, 2-amino-3,4,5-trihydroxy-2-methyl-14-oxo-, (2S,3R,4R,5S,6E)- (9CI)

Other Names: 6-Eicosenoic acid, 2-amino-3,4,5-trihydroxy-2-methyl-14-oxo-, [2S-(2R*,3S*,4S*,5R*,6E)]-; (+)-Sphingofungin F; Sphingofungin F

1.2.3 Detection Methods of *Aspergillis fumigatus*

Identification of *A. fumigatus* is important because it is one of the most important fungal pathogens (Wang et al., 2000). *Aspergillus* spp. isolates are typically identified by microscopic demonstration of characteristic morphologic structures after growth on appropriate media. Identification may be delayed if the isolate fails to form the diagnostically appropriate structures. Furthermore, inexperience in microscopy may lead to misidentification. These problems may be obviated by using DNA-based methods for identification and species assignment of isolates (Brandt et al., 1998). These include RAPD, restriction fragment length polymorphism (RFLP) detection, and Southern hybridization with various repetitive sequence-based probes (Radford et al., 1998).

Recent approaches for the detection of *A. fumigatus* also include detection of a galactomannan antigen by ELISA (Maertens et al., 1999) and the demonstration of DNA by PCR in samples of blood and bronchoalveolar lavage liquid (BAL) (Kappe and Rimek, 1999). These rapid methods are helpful for the early diagnosis of aspergillosis in high risk patients (Maertens et al., 1999; Sklandny et al., 1999;

Verweij et al., 1996). In solid phase cytometry, with an enzymatic "viability" staining procedure, fungal hyphae can be detected non-specifically within the hour. By combining this procedure with an immunofluorescence labelling, a distinction between *Aspergillus* spp. and other clinically important fungi is possible, except for *Penicillium* spp. due to cross-reactivity.

Detection of specific fungal metabolites has been discussed as an alternative to antibody, antigen or nucleic acid-based tests (Yeo and Wong, 2002). Gliotoxin, a well-characterised fungal metabolite, has potent immunosuppressive effects and is indicative of invasive aspergillosis (Denning, 1998). Similarly, Mitchell et al. (1997) have shown that helvolic acid, produced by *A. fumigatus*, inhibits the oxidative burst of macrophages. Consequently, the appearance of these toxins may be indicative of invasive aspergillosis since neither toxin has been associated with infections caused by other clinically relevant fungi.

1.2.3.1 Detection and Quantitation of *Aspergillus fumigatus* Using Polymerase Chain Reaction

Due to the detection limitations associated with traditional culture analyses, molecular biology techniques can be utilized for the rapid and sensitive detection of target organisms in indoor environments. This technique utilizes the polymerase chain reaciton (PCR) to detect specific microorganisms by amplifying DNA sequences unique to the organism of interest. Table 1.2 shows the specimens and the probes used for the detection of *A. fumigatus* (Latgé, 1999).

PCR assays show a superior sensitivity for the detection of *A. fumigatus* in blood and organs. Real time PCR also offers a sensitive and fast tool for the quantification of *A. fumigatus* DNA, which is especially beneficial for blood specimen study.

In food analysis, *A. fumigatus* is identified by its macroscopic and microscopic appearence after lactophenol cotton blue staining and its mycotoxins are detected with ELISA (Bouakline et al., 2000). However, PCR assays can be applied to detect the mycotoxin producer directly, namely *A. fumigatus*.

Table 1.2 The specimens and the probes used for the detection of

A. fumigatus (Latgé, 1999).

Specimen	Probe
Urine	18-kDa ribotoxin
BAL fluid	rDNA
BAL fluid	33-kDa alkaline protease
BAL fluid	rRNA
BAL fluid	mtDNA
Serum	mtDNA
Serum	rRNA
Plasma	rRNA
Plasma	rRNA

1.3 Aim of the Study

This study was aimed at the development of a PCR-based specific method for the detection of *Aspergillus fumigatus* IMI 385708 by random cDNA cloning. In this respect, cDNA library, constructed earlier by Yalçındağ (B. Yalçındağ M. Sc. Thesis, 2002), was plated and plaques were chosen randomly. These phages were subjected to phagemid excision and plasmid isolation. For the characterization of inserts, restriction digestion, PCR analysis were done and after being sequenced, these insert sequences were analyzed by BLAST search of the gene bank, and by using relevant software to find coding proteins. These data were used to design three pairs of primers specific to *A. fumigatus*. A software was also designed to differentiate the nonconserved regions among a set of genes compared by multiple sequence alignment. Designed primers were used in PCR against different *Aspergillus* species including *A. fumigatus* to analyze specificity of primers to *A. fumigatus.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 cDNA Library

cDNA library of *Aspergillus fumigatus* IMI 385708 was prepared by Banu Yalçındağ (B. Yalçındağ M. Sc. Thesis, 2002).

A. fumigatus was grown in a medium containing locus bean gum and mRNA was isolated from fifth day's mycelia. Then cDNA was synthesized by ZAP Express® cDNA synthesis kit.

2.1.2 Fungal Strains

A. fumigatus was kindly provided by Prof. Dr. Peter Biely from the Slovak Academy of Sciences, Institute of Chemistry, as part of a colloborative research work.

Aspergillus nidulans, *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae* were kindly provided by Dr. Ceyda Pembeci from Food Science and Technology Research Institute, Marmara Research Center, TÜBİTAK, from the TÜBİTAK MRC Culture Collection.

2.1.3 Bacterial Strains

The RecA$^-$ *E.coli* host strain XL1-Blue MRF' was supplied with the ZAP Express cDNA synthesis kit. Because the pBK-CMV phagemid vector does not require a *supF* genotype, the amplified library grows very efficiently on the XL1-Blue MRF$'$ strain.

A nonsuppressing *E.coli* strain XLOLR was provided with the ZAP Express cDNA synthesis kit. XLOLR cells are resistant to lambda infection, preventing lambda DNA contamination after excision.

2.1.4 Chemicals and Enzymes

The list of chemicals and enzymes used and their suppliers are given in Appendix A.

2.1.5 Growth Media, Buffers and Solutions

The preparation of the growth media, buffers and solutions used are given in Appendix B.

2.2 Methods

2.2.1 Maintenance and Cultivation of the Strains

Stock cultures of *A. fumigatus* were grown on YpSs agar slants (Appendix B) at 45°C and maintained at 4°C.

For DNA and RNA isolation, *A. fumigatus* was cultivated in 200 ml YpSs broth (Appendix B) in 500 ml erlenmeyer flasks at 45°C and 155 rpm for 2 days.

Stock cultures of *A. nidulans*, *A. parasiticus*, *A. flavus*, *A. niger*, *A. oryzae* were grown on YpSs agar slants (Appendix B) at 25°C and maintained at 4°C.

For DNA isolation, *A. nidulans*, *A. parasiticus*, *A. flavus*, *A. niger*, *A. oryzae* were cultivated in 200 ml YpSs broth (Appendix B) in 500 ml erlenmeyer flasks at 25°C and 155 rpm for 2 days.

Stock cultures of *E. coli XL1 Blue MRF'* and *E. coli XLOLR* were grown overnight on LB Tetracycline agar plates (Appendix B) at 37°C and maintained at 4°C. The plates were refreshed every month.

For plating the cDNA library, 50 ml LB medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B) was inoculated with a single colony of *E. coli XL1 Blue MRF'* cells, incubated at 37ºC, for 4-6 hours or overnight at 30ºC, shaking at 155 rpm.

Recombinant *E. coli XL1 Blue MRF'* and *E. coli XLOLR* strains were grown overnight on LB Kanamycin agar plates (Appendix B) at 37°C and maintained at 4°C. The plates were refreshed every month.

For plasmid excision, 50 ml NZY broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B) was inoculated with a single colony of *E. coli* XL1 Blue MRF' cells, and incubated in shake flasks overnight at 30ºC. *E. coli* XLOLR strain was grown in 50 ml NZY broth (Appendix B) overnight in shake flasks at 30°C.

For transformation of *E. coli* XL1 Blue MRF' cells, 5 ml LB medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B) was inoculated with a single colony of *E.* coli XL1 Blue MRF' cells, incubated in shake flasks overnight at 37ºC. Then, 100 ml of LB medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B) was inoculated with 1 ml of an overnight culture of *E. coli* XL1 Blue MRF' and incubated at 37°C until the OD_{550} reached 0.4-0.5.

For plasmid purification, 3 ml LB kanamycin broth (Appendix B) was inoculated with a single colony of *E. coli XL1 Blue MRF'*, incubated at 37ºC, for 12-16 hours in shake flasks, until OD_{600} reached 1-1.5.

2.2.2 Plating the cDNA Library

The library was plated according to the instructions given in the ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene).

E. coli XL1 Blue MRF' cells were cultivated as explained in section 2.2.1. Then the cells were centrifuged at 1500 rpm for 10 min at room temperature and the supernatant was discarded. The cells were resuspended in half the original volume with sterile 10 mM $MgSO_4$ (Appendix B). After resuspension, the cells were diluted to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$. Bacteria were used immediately after dilution.

Phage particles to 5,000-10,000 pfu/plate were added into 600 µl of host cells at an OD_{600} of 0.5. Bacteria and phage mixture was incubated at 37ºC for 15 min to allow the phage to attach the cells. Then, 6.5 ml of NZY top agar (Appendix B) was added to the bacteria and phage mixture. The plating culture was quickly poured onto a 150 mm NZY agar plate (Appendix B) which was at least 2 days old and the plate was carefully swirled to distribute the cells evenly. The plate was inverted and incubated at 37ºC for about 8 hours.

2.2.3 The Polymerase Chain Reaction (PCR)

A 20 µl PCR reaction mixture contained

- Sterile double distilled water to give a final volume of 20 µl
- 10x reaction buffer to give a final concentration of 1x
- 0.2 mM dNTP mix
- 1 units of Taq DNA polymerase
- 50 pmols from each primer or 100 pmols from RAPD primer
- phage DNA or 0.25 µg genomic DNA

15 µl of mineral oil was added to prevent evaporation.

For phage DNA, amplifications were performed according to the following cycle:

94ºC 2’
94ºC 1’
55ºC 1’ } x 30 cycle
72ºC 1’
72ºC 1’

For 0.25 µg genomic DNA with RAPD primer, amplifications were performed according to the following cycle:

90ºC 5’
89ºC 1’
32ºC 1’ } x 35 cycle
72ºC 1.5’
50ºC 3’

For 0.25 µg genomic DNA, amplifications were performed according to the following cycle:

94ºC 2’
94ºC 1’
60ºC 30’’ } x 30 cycle
72ºC 1’
72ºC 1’

2.2.4 Agarose Gel Electrophoresis

To analyze PCR products, plasmids, 0.8 % (w/v) agarose gel (Appendix B) was used.

The gel was melted in a microwave oven (Vestel Goldstar) and cooled to 50-60°C. After adding ethidium bromide at a concentration of 0.5 µg/ml, the gel was

poured into mould and allowed to solidify for about 15 min. Then, it was placed in the electrophoresis tank filled with 1x TAE buffer (Appendix B). Electrophoresis was carried out at 70 V for 1 hr. Nucleic acids were visualized on UV transilluminator at 320 nm and photographed with Nikon Coolpix 4500 digital camera.

2.2.5 Plasmid Excision

Plasmid excision was made according to the instructions given in the ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene).

The plaque of interest was cored from the agar plate and transferred with a needle to a sterile microcentrifuge tube containing 500 µl of SM buffer and 20 µl chloroform. The microcentrifuge tube was vortexed to release the phage particles into the SM buffer. The mixture was incubated for 1-2 hours at room temperature or overnight at 4°C.

E. coli XL1 Blue MRF' and *E. coli* XLOLR cells were cultivated as explained in section 2.2.1. The cultures were centrifuged at 1500 rpm for 10 min and the cells were resuspended at an OD_{600} of 1.0 in 10 mM $MgSO_4$ (Appendix B). 200 µl of *E. coli* XL1 Blue MRF', 250 µl of phage stock, 1 µl of the ExAssist helper phage were combined in a Falcon tube and incubated at 37°C for 15 min. 3 ml NZY broth was added and incubated for 2.5-3 hours or overnight at 37°C with shaking. The tube was heated at 65-70°C for 20 min and centrifuged at 1500 rpm for 15 min. The supernatant was decanted into a sterile Falcon tube. This stock contains the excised pBK-CMV phagemid vector packaged as filamentous phage particles.

To plate the excised phagemids, two tubes, each containing 200 µl of freshly grown *E. coli* XLOLR cells were prepared. 10 µl and 100 µl of the phage supernatant were added in these two Falcon tubes respectively and the mixtures were incubated at 37°C for 15 min. Then, 300 µl of NZY broth was added to the tubes and incubated at 37°C for 45 min. Finally, 200 µl of the cell mixture from each Falcon tube was plated on LB kanamycin agar plates (Appendix B) and incubated overnight at 37°C.

Colonies appearing on the plate contain the pBK-CMV double-stranded phagemid vector with the cloned DNA insert. Helper phage does not grow, since

helper phage is unable to replicate in Su^- (nonsuppressing) XLOLR strain and does not contain kanamycin resistance genes. *E. coli* XLOLR cells are also resistant to lambda phage infection, thus preventing lambda phage contamination after excision.

2.2.6 Transformation of *E.coli XL1 Blue MRF'*

2.2.6.1 Preparation of Competent Cells

E. coli XL1 Blue MRF' cells were cultivated as explained in section 2.2.1. The culture was dispensed into 2 falcon tubes. The tubes were chilled on ice for 10 min and centrifuged at 6 000 rpm for 5 min at 4°C. Then, the supernatant was discarded and the cells were resuspended in a total volume of 50 ml (25 ml for each tube) ice-cold Solution A (Appendix B), kept on ice for 15 min, centrifuged at 6 000 rpm for 5 min at 4°C. Again, the supernatant was discarded, the cells were resuspended in a total volume of 7 ml (3.5 ml for each tube) ice-cold Solution A (Appendix B) and 100% sterile glycerol was added to a final concentration of 20% (700 µl for each tube). Finally, 300 µl aliquots were dispensed into eppendorf tubes and stored at -80°C.

2.2.6.2 Transformation of Competent Cells

0.5 µl plasmid was mixed with 50 µl TE buffer (Appendix B). This mixture was mixed with one batch of competent cells (300 µl) and kept on ice for 30 min. The mixture was transferred into a 42°C water bath for precisely 2 min. Then, 1 ml LB medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B) was added and kept at 37°C for 1 hr. 50-200 µl aliquots were finally spread on LB kanamycin agar plates (Appendix B) and incubated overnight at 37°C.

2.2.7 Plasmid Purification

Plasmids were purified according to the instructions given in the Plasmid Mini Kit (Qiagen®), as described below:

E. coli XL1 Blue *MRF'* cells were cultivated as explained in section 2.2.1. The cells were centrifuged at 6 000 rpm for 8 min and the supernatant was discarded. The bacterial pellet was resuspended in 0.3 ml of buffer P1 (Resuspension Buffer) (Appendix B). Then, 0.3 ml of buffer P2 (Lysis Buffer) (Appendix B) was added and mixed gently for 4-6 times, incubated at room temperature for exactly 5 min. After that, 0.3 ml of buffer P3 (Neutralization Buffer) (Appendix B) was added, gently mixed for 4-6 times and incubated on ice for 5 min. Then, the mixture was centrifuged at 13 000 rpm for 10 min. The supernatant was removed promptly and applied to the column which was equilibrated earlier by applying 1 ml QBT buffer (Equilibration Buffer) (Appendix B), allowed it to enter the resin by gravity flow. The column was washed 4 times with 1 ml QC buffer (Wash Buffer) (Appendix B). After that, plasmid DNA was eluted with 0.8 ml QF buffer (Elution Buffer) (Appendix B). If required, the size of plasmid DNA was checked at this stage by running 10 µl of the solution on a 0.8 % (w/v) agarose gel (Appendix B). Plasmid DNA was precipitated with 0.7 volumes of room temperature isopropanol. Then, the mixture was centrifuged immediately at 13000 rpm for 30 min, and the supernatant was decanted carefully. The plasmid DNA was washed with 1 ml of 70% ethanol (Appendix B), centrifuged at 13 000 rpm for 5 min. The supernatant was decanted, the pellet was air dried for 5 min, and redissolved in a 15 µl of double distilled water.

2.2.8 Restriction Enzyme Digestion

A 20 µl restriction enzyme digestion mixture contained

- Sterile double distilled water to give a final volume of 20 µl
- appropriate 10x restriction enzyme buffer to give a final concentration of 1x
- 0.1 to 5 µg DNA
- 4 to 40 units restriction enzyme

15 µl of mineral oil was added to prevent evaporation.

The reaction mixture was incubated overnight at 37ºC. If double digest was required, the restriction enzyme buffer for best activity of double digestion reactions suggested by the supplier was chosen.

2.2.9 Total RNA Isolation

Total RNA was isolated according to the instructions of the Rneasy Plant Mini Kit (Qiagen®).

A. fumigatus cells were cultivated as explained in section 2.2.1. The mycelia were filtered through Wathman 3MM filter paper, and placed in liquid nitrogen, and ground thoroughly with a mortar and pestle. 75 mg ground mycelia were placed into Rnase-free 1.5 ml microcentrifuge tube. Then, 450 µl Buffer RLC was added into the tube and vortexed vigorously. The lysate was directly pipetted onto a QIAshredder spin column placed in 2 ml collection tube, and centrifuged for 2 min at maximum speed. The supernatant was transferred carefully to a new microcentrifuge tube. 0.5 volume ethanol was added to the cleared lysate, and mixed immediately by pipetting. Then, the sample was applied to an RNeasy mini column placed in a 2 ml collection tube, and centrifuged for 15 s at ≥8 000 x g (≥10 000 rpm). 700 µl Buffer RW1 was added to the RNeasy column and centrifuged for 15 s at ≥8 000 x g (≥10000 rpm) to wash the column. The RNeasy column was transferred into a new 2 ml collection tube. 500 µl Buffer RPE was pipetted onto the RNeasy column and centrifuged for 15 s at ≥8 000 x g (≥10 000 rpm) to wash the column. Another 500 µl Buffer RPE was added to the RNeasy column and centrifuged for 2 min at ≥8 000 x g (≥10 000 rpm) to dry the RNeasy silica-gel membrane. Finally, 30-50 µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and centrifuged for 1 min at ≥8 000 x g (≥10 000 rpm) to elute.

2.2.10 Northern Blotting

2.2.10.1 Sample Preparation

2 µl isolated total RNA (up to 20 µg) was mixed with 2 µl 10x MOPS electrophoresis buffer (Appendix B), 4 µl formaldehyde, 10 µl formamide. The mixture was incubated at 55 °C for 60 minutes, and chilled on ice for 10 minutes. Then, the mixture was centrifuged for 5 seconds to deposit all of the fluid in the bottom of the microfuge tube. 2 µl of 10x formaldehyde gel-loading buffer (Appendix B), and the tube was placed on ice until electrophoresis.

2.2.10.2 Electrophoresis of RNA

Agarose/formaldehyde gel (Appendix B) was installed in a horizontal electrophoresis box. Sufficient amount of 1x MOPS electrophoresis buffer (Appendix B) was added to cover the gel to a depth of approximately 1 mm. The RNA sample and a marker (GeneRuler™ 100 bp DNA Ladder Plus) were loaded into the wells and the gel was run at 75 V for 2 hours. After electrophoresis, a photograph of the gel was taken together with a ruler.

2.2.10.3 Northern Transfer

After electrophoresis in an agarose/formaldehyde gel (Appendix B), the gel was equilibrated in 20x SSC (DEPC-treated) (Appendix B) for 2 x 15 minutes.

For the preparation of the northern transfer apparatus, a tray was filled with 10X SSC as the blotting buffer. The platform was covered with three sheets of Whatman 3MM filter paper saturated with 10X SSC. The gel was placed onto the filter papers avoiding air bubbles between gel and filter papers and was surrounded with cling film to prevent the blotting buffer being absorbed directly by the paper towels above. The nylon membrane (Roche) was cut to exact size of the gel and placed on top of the gel, carefully again avoiding air bubbles. Three sheets of Whatman 3MM filter paper wetted with 10X SSC were placed on top of the

membrane. Plenty of paper towels were put on top of the filter papers. Lastly, a glass plate was placed on top of the towel and approximately 1 kg of weight was set on the very top. The transfer was allowed to proceed overnight at 4 °C. After blotting, the apparatus was dismantled and the membrane was removed.

The membrane was placed on a sheet of Whatman 3MM filter paper and the transferred RNA was fixed by baking at 100°C for 30 minutes.

2.2.10.4 Preparation of Probe DNA

For the preparation of the probe to be used in hybridization, amplification with PCR, isolation and purification of the fragment and labeling were done. To prepare the probe, the insert in the plasmid was amplified with the primers that are specific to the vector arms. To obtain the intended concentration at the end of isolation, 10 reaction tubes, each 50 µl, were made ready for PCR. PCR was performed as described in section 2.2.3. Following amplification, the desired PCR fragment was directly recovered from the reaction mixture using a DNA Extraction Kit (Fermentas).

For recovering PCR products from residual reaction compounds, first the mineral oil was removed. The remaining reaction mixture was collected to 1.5 ml microcentrifuge tubes. 3 volumes of binding solution was added to 1 volume of sample. The resuspended silica powder suspension was added as 2 µl per 1µg of DNA. The mixture was incubated at 55°C for 5 minutes and was mixed by vortexing every 2 minutes to keep silica powder in suspension. The silica powder / DNA complex was then centrifuged at 13 000 rpm for 5 seconds and the supernatant was discarded. 500 µl ice cold extraction wash buffer (Appendix B) was added and vortexed. The tube was centrifuged at 13 000 rpm for 5 seconds and supernatant was discarded. This washing procedure was repeated three times. During each washing the pellet was resuspended completely.

After the supernatant from the last wash had been removed, the tube was centrifuged again and the remaining liquid was removed. The pellet was air-dried for 20 min and the pellet was resuspended in an aliquot of double distilled water and

incubated at 55°C for 5 minutes. The tube was centrifuged at 13 000 rpm for 1 min and the supernatant was transferred into a new tube.

The concentration of resulting DNA was determined by running the DNA on an agarose gel, against a marker of known concentration, and comparing the intensity of the band with the marker band, nearest in size.

2.2.10.5 Probe Labeling

15 µl isolated PCR fragment (20 to 100 µg/µl) was heat-denatured by boiling in a water bath for 10 minutes and immediately chilled on ice for 30 seconds. On ice, 2 µl hexanucleotide mixture (10X), 2 µl dNTP labeling mixture (10X), 1 µl Klenow enzyme were added respectively. The reaction was incubated at 37°C overnight, preferably 20 hours. Then the reaction was terminated by adding 2 µl 200 mM EDTA, pH = 8.0 (Appendix B).

2.2.10.6 Hybridization

Membranes were placed in a roller bottle or hybridization bag and prehybridized at 68°C at least 30 minutes using enough prehybridization solution (DIG Easy Hyb, ready-to-use hybridization solution, Roche).

The DIG-labeled probe was heat-denatured by boiling in a water bath for 10 minutes and immediately chilled on ice for 30 seconds. The probe was added to 20 ml hybridization solution (DIG Easy Hyb, ready-to-use hybridization solution, Roche) at a concentration of 20 ng/ml. The prehybridization solution was discarded and hybridization solution was added. The membranes were hybridized to the probe at 68°C overnight.

Membranes were washed twice with low stringency wash buffer (Appendix B) for 5 minutes at room temperature., then twice with high stringency wash buffer (Appendix B) at 68°C for 15 minutes.

2.2.10.7 Detection

Membrane were washed with 100 ml washing buffer (Appendix B) for 5 minutes, and then blocked with 80 ml 1X blocking solution (Appendix B) for 30 minutes. After removing the blocking solution, 80 ml antibody solution (Appendix B) was added. Membranes were washed twice with 100 ml washing buffer (Appendix B) to remove unbound antibody for 15 minutes. In continuation, the membrane was equilibrated with 80 ml detection buffer (Appendix B) for 2 minutes. All incubations were performed at room temperature with shaking and great care was taken not to dry the membrane between the steps.

Finally, the membrane was placed on a sheet of plastic transparency film, DNA side facing up. For each 100 cm^2 of membrane, 500 µl CSPD diluted with detection buffer (Appendix B), (1/100 dilution of 25 mM stock) was added. The damp membrane was covered with a second sheet of transparency film. Excess liquid was dripped off from the membrane and the transparency was heat sealed. The sealed bag, containing the membrane was exposed to X-ray film (XBM Blue Sensitive, RETINA) in the Hypercasette™ (Amersham) for at least 30 minutes, at most 3 hours at 37°C.

2.2.11 Genomic DNA Isolation

Fungal strains were cultivated as explained in section 2.2.1. The mycelia were filtered by Wathman 3MM filter paper, and dried at 45°C for 1 hours then, placed in liquid nitrogen, and ground thoroughly with a mortar and pestle. Powdered mycelia were transferred to a 50 ml falcon tube. Then 25 ml TTE buffer (Appendix B) was added and centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded. 15 ml of lysis buffer (Appendix B) was added to the pellet and mixed by inversion. It was incubated at 37°C for 15 minutes. After the addition of equal volume of phenol:chloroform:isoamylalcohol (25:24:1), the mixture was centrifuged at 5500 rpm for 8 minutes, upper phase was transferred to a new tube. Phenol:chloroform:isoamylalcohol (25:24:1) extraction was repeated until the interphase was clear. The upper phase from the last extraction was transferred to a

Sorvall tube. 0.1 volume sodium acetate (3 M, pH = 5.2) (Appendix B), and 2-2.5 volume cold absolute ethanol was added to the collected upper phase. The mixture was left overnight at -20°C. Then the tubes were centrifuged at 12 000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 1 ml 70 % ethanol (Appendix B). The tubes were centrifuged at 6000 rpm for 3 minutes and the supernatant was discarded. After being allowed to air-dry for 20 minutes, the pellet was dissolved in 100 µl sterile double distilled water at 4°C overnight. To the dissolved DNA solution, 10 µl, 10 mg/ml DNase free RNase (Appendix B) was added and incubated overnight at 37°C. The concentration of resulting DNA was determined by running on an agarose gel (Appendix B) with a marker of known concentration (λ.DNA/*Eco*RI+*Hin*dIII Marker) and comparing the intensity of the band with the marker's bands.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Experimental Strategy

The experimental strategy for the development of a PCR-based specific method for the detection of *Aspergillus fumigatus* is shown in Figure 3.1.

As explained in section 2.1.1, cDNA library of *A. fumigatus* was constructed earlier on λZAP Express vector from mRNA purified from days cultures of the fungus grown on locust bean gum (0.2%) (B. Yalçındağ, M. Sc. Thesis, 2002). To perform random cDNA cloning, cDNA library of *A. fumigatus* was plated and phages were selected randomly. Insert sizes of random clones were determined by PCR. Then, recombinant plasmids were excised and transformed into *E. coli* XL1 Blue MRF'. Purified plasmids were sequenced and random clones were characterized by comparison of cDNAs with genomic DNA of *A. fumigatus*, amino acid sequence determination and multiple sequence alignment. Then, specific primers for *A. fumigatus* genomic DNA were designed and tried against *A. fumigatus* and closely related *Aspergillus* species' genomic DNAs by PCR.

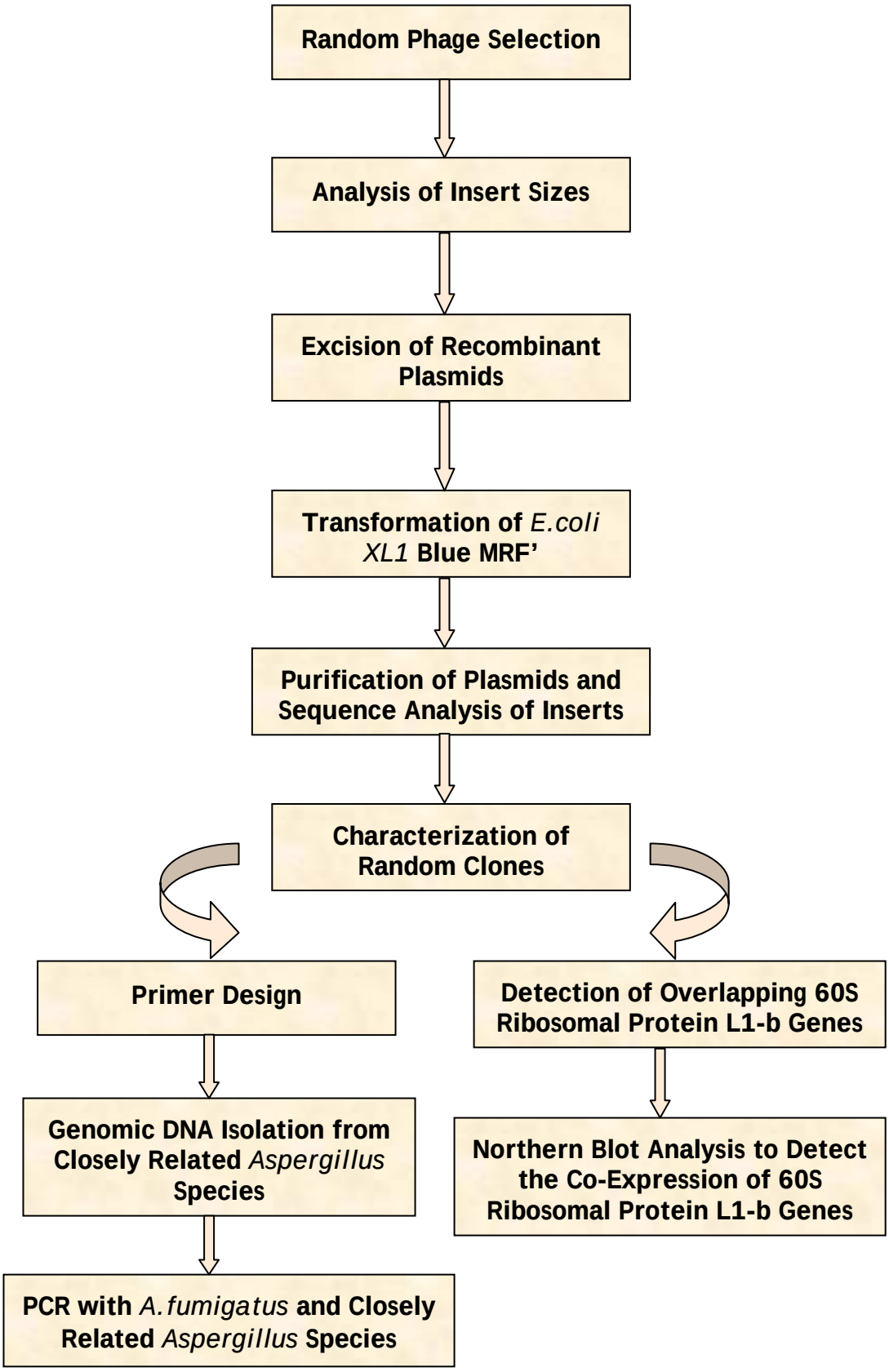


Figure 3.1 Flow chart of the experimental strategy

3.2 Plating the cDNA Library and Random Phage Selection

cDNA library was diluted to 1/20 with SM buffer and plated onto a 150 mm plate according to the procedure given in Section 2.2.2. At the end of the incubation period, 200 separate, non-touching plaques were formed.

3.3 Analysis of the Insert Size of Random Clones by PCR

14 plaques were randomly chosen from the plate. These distinct plaques were cored from the agar plate and transferred with a needle to PCR tubes, containing all the other reaction components. Also the same phages were transferred to sterile microcentrifuge tubes containing 500 µl of SM buffer and 20 µl chloroform for further studies, and stored.

Amplification studies were performed with the primers 315 and 316 (Appendix C), which are specific to the pBK-CMV phagemid vector arms (Appendix D), against phage DNAs using the PCR conditions outlined in Section 2.2.3 with an annealing temperature of 55°C (Figure 3.2).

According to the PCR results, amplification was not observed in phage DNAs 8 and 16. Phage DNAs 9 and 19 gave *c.*1375 bp bands; 13,17,18,20,21,23 and 24 gave approximately 1000-1100 bp bands; 15 and 22 gave 950 bp bands; and 14 gave a 600 bp band.

Because of the small insert size of phage DNA 14, this phage was eliminated.

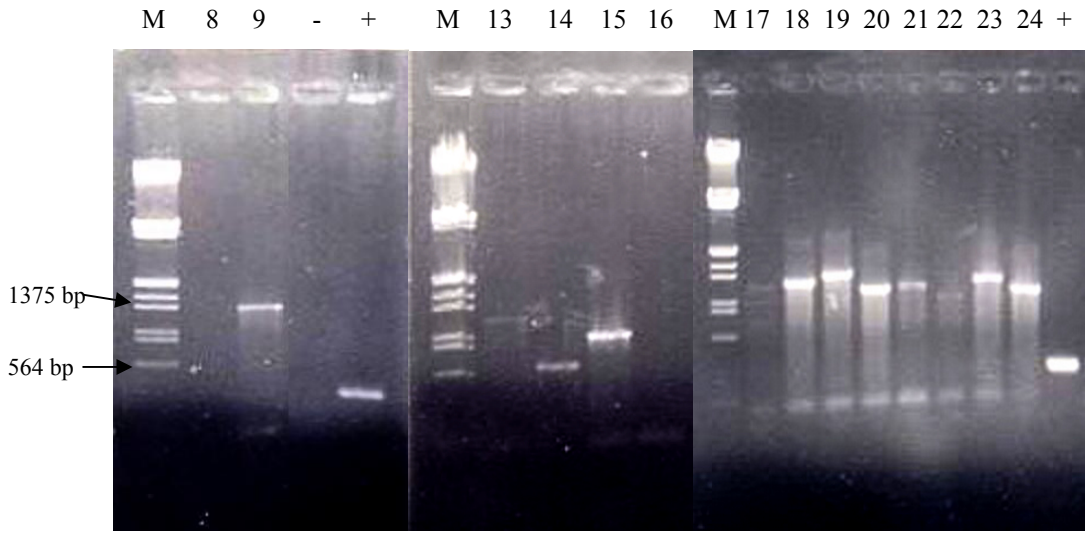


Figure 3.2 Amplification of phage DNAs with the primers 315 and 316. **M**, λ DNA/*Eco*RI+*Hin*dIII Marker; **+**, positive control; **-**, negative control; the numbers denote for the number of phages

3.4 Excision of Recombinant Plasmids

Plasmid excision of 11 phages were performed according to the procedure given in section 2.2.6. After the incubation period of the LB-kanamycin agar plates, growth of the colonies were detected.

In this procedure, individual lambda phage or an amplified library are allowed to infect *E. coli* cells which are co-infected with filamentous helper phage. Inside the cell, trans-acting proteins from the helper phage recognize initiator (I) and terminator (T) domains within the ZAP Express® vector arms. Both of these signals are recognized by the helper phage gene II protein and a new DNA strand is synthesized, displacing the existing strand. The displaced strand is circularized and packaged as a filamentous phage by the helper phage proteins, and secreted from the cell. pBK plasmids are recovered by infecting an F' strain and growing in the presence of kanamycin (Figure 3.3).

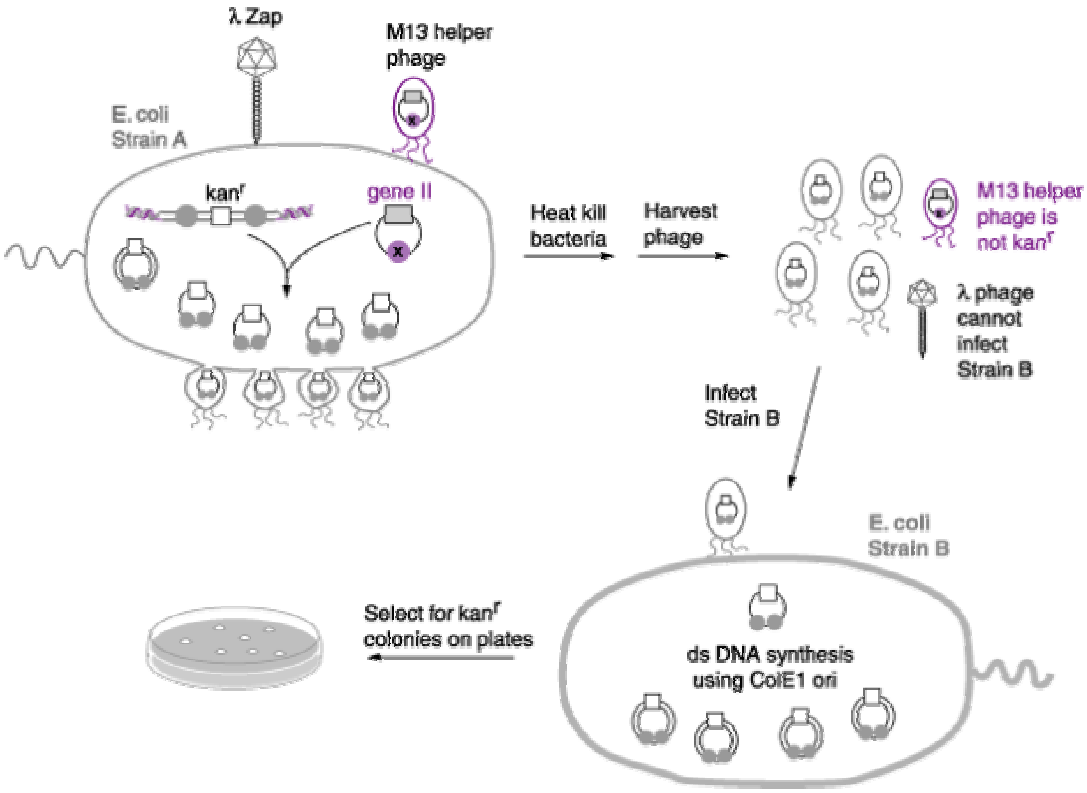


Figure 3.3 Plasmid excision

3.5 Transformation of *E.coli* XL1 Blue MRF'

Firstly, *E.coli* XL1 Blue MRF' competent cells were prepared and then, 11 excised plasmids were transformed into *E.coli* XL1 Blue MRF' according to the procedure given in section 2.2.7. The plates were incubated overnight at 37ºC and colonies were formed.

3.6 Purification and Analysis of Plasmids

The plasmids were purified according to the procedure given in section 2.2.8 and visualized by agarose gel electrophoresis (Figure 3.4).

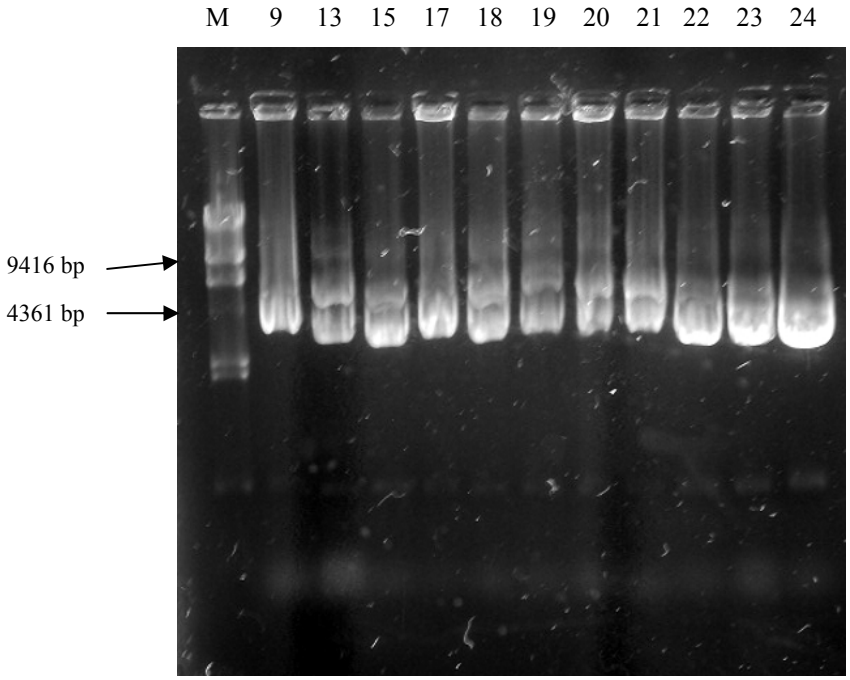


Figure 3.4 Plasmid purification. **M**, λ DNA/*Hin*dIII Marker;
The numbers denote for the purified plasmid numbers.

Eight purified plasmids were randomly chosen for further sequence analysis. In order to observe the insert and determine its size, these plasmids were digested with *Xho*I and *EcoR*I enzymes according to the procedure given in section. The digested plasmids were shown in Figure 3.5.

According to Figure 3.5, the insert sizes of the plasmids ranged from 650 bp to 1100 bp. Insert sizes found in section 3.3 by PCR are different from the restricted enzyme digested plasmids' insert sizes. This may be caused by picking multiple plaques during the random plaque selection step. In this step, plaque of interest was cored and picked with needle, and then analyzed by PCR. However, on the needle, there were probably more than one type of plaque. The smaller plasmids are likely to be selected over the larger ones during the excision/transformation steps.

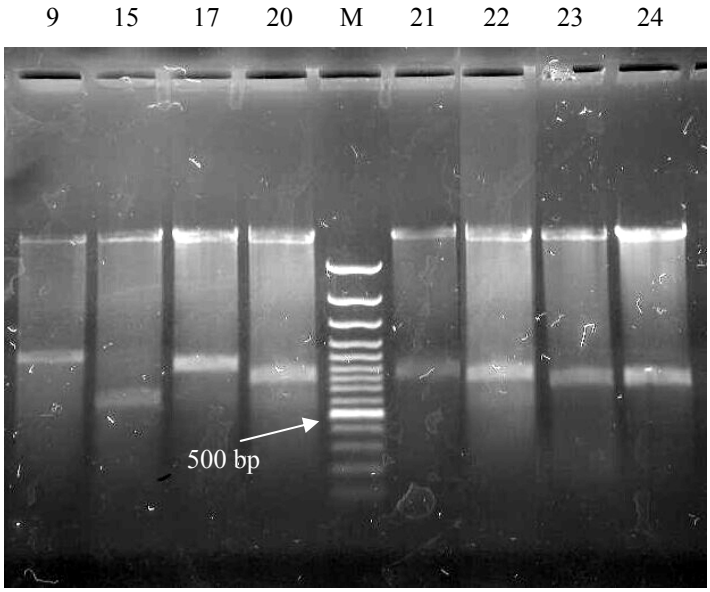


Figure 3.5 M, GeneRuler™ 100 bp DNA Ladder Plus;
The numbers denote for *Xho*I and *EcoR*I enzyme digested plasmid numbers.

3.7 Characterization of Random Clones

Plasmids were first sequenced with the reverse primer 315 (Appendix C). Then, plasmids 15 and 23 were sequenced further with the forward primer 316 (Appendix C). The resulting sequences obtained from these plasmids are shown in Figures F.1, F.2, F.3, F.4, F.5, F.6, F.7 and F.8 in Appendix F.

95 % of the preliminary genome sequence data of *A. fumigatus* was released in July 28, 2003 (http://www.tigr.org/tdb/e2k1/afu1/) while these studies were being conducted. The Sanger Institute and its collaborators, Dr. David Denning and Dr. Andrew Brass at the University of Manchester, have been funded to carry out a pilot genomic analysis of *A. fumigatus*. The group has selected a clinical isolate, Af293 as the strain to be sequenced. This involves preparing a bacterial artificial chromosome (BAC) library of *A. fumigatus*, to fingerprint 3000 BAC clones generating a physical map and then to sequence 10 physically linked BAC clones. They are also funded to carry out a whole genome shotgun of *A. fumigatus* in collaboration with The Institute of Genome Research (TIGR).

The finishing of the *A. fumigatus* Af293 genome sequence is continuing at TIGR and the Sanger Institute. TIGR currently has 8 finished contigs/scaffolds and one additional scaffold broken into 2 contigs with an ~6.5 kb gap. These 10 contigs account for 14.8 Mb of sequence. TIGR has identified 8 telomeric areas in this data. In addition, TIGR has finished the 32 kb mitochondrial genome and a contig containing rDNA repeats which will be released shortly when they are cleared by the TIGR closure group.

The Sanger Institute has released 9 contigs corresponding to 5 scaffolds, with two scaffolds consisting of three contigs each. The total amount of sequence in these 9 contigs is 13.2 Mb.

Contigs from both TIGR and Sanger, 28 Mb of sequence data, are available from TIGR web site for download and searching.

The sequences of the inserts were searched by using BLASTN 2.0 in *A. fumigatus* genome. Then, intron and exon analyses of the sequences were done by comparison of the cDNA insert with *A. fumigatus* genome sequence. Once the sequences were available, it was also possible to perform a BLAST search to find the identity of the randomly cloned genes. Thus, cDNA sequences were searched with BLASTX 2.2.9 which is the protein database search program that finds homology between the translated query and available protein sequences.

3.7.1 Characterization of Clone 9

3.7.1.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, partial DNA sequence of the sense strand of cDNA on clone 9 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome and a homologous region was found in the minus strand of contig 59. The gene on plasmid 9 was characterized accordingly, as shown in Figure 3.6.

Query submitted to BLAST: insert of the plasmid 9

Result: >a_fumigatus|chr_0|TIGR.5237|59

Length = 2,797,657

Minus Strand HSPs:

Identities = 225/226 (99%), Positives = 225/226 (99%), Strand = Minus / Plus

```
Q:      495 TCTGCTCATCAAAATCATGATGTCTATTTCTACTGACTTGCAAATCGGATCGATGAACTA 436
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S:  2009139 TCTGCTCATCAAAATCATGATGTCTATTTCTACTGACTTGCAAATCGGATCGATGAACTA 2009198

Q:      435 GATGCATACTTTTGATAGTTGGAATCACAGTGCGCGCCTTGAACGCCTCCCAGGAATATA 376
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S:  2009199 GATGCATACTTTTGATAGTTGGAATCACAGTGCGCGCCTTGAACGCCTCCCAGGAATATA 2009258

Q:      375 GACTGCTGAACTAAAAGATAATACCAACGTGAATGCGGACTTCTGGAACGGAACTGGTAT 316
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
S:  2009259 GACTGCTGAACTAAAAGATAATACCAACGAGAATGCGGACTTCTGGAACGGAACTGGTAT 2009318

Q:      315 CAAGGTGCGGAGGGCCGCTTAAGGACAAGATTAGACCAGGAGGACT 270
            |||||||||||||||||||||||||||||||||||||||||||||
S:  2009319 CAAGGTGCGGAGGGCCGCTTAAGGACAAGATTAGACCAGGAGGACT 2009364
```

Identities = 190/200 (95%), Positives = 190/200 (95%), Strand = Minus / Plus

```
Q:      269 GCATTGACCGTGCCATCGTTCTCAGGAGTATTGGTGATCTGCGCAGATTTACCCCATACG 210
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
S:  2009425 GCATTGACCGTGCCATCGTTCTCAGGAGTATTGGTGATCTGCGCATATTTACCCCATACG 2009484

Q:      209 ACCTTTCCGCTTGTCGTGACGAGACC-AATTCGGAGACGTTGACTTCGATGATGGTTCCC 151
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
S:  2009485 ACCTTTCCGCTTGTCGTGACGAGACCCAATTCGGAGACGTTGACTTCGATGATGGTTCCC 2009544

Q:      150 TTGGTCAGGAN-CCCAGTTGGGTGTA-AGAGGGT-CTGNGGGT-CT-CTTGAC-CCCAGG 97
            |||||||||  ||||||||||||| ||||||| ||| |||| || |||||| ||||||
S:  2009545 TTGGTCAGGACTCCCAGTTGGGTGTAGAGAGGGTTCTGCGGGTTCTTCTTGACGCCCAGG 2009604

Q:       96 ATGGGCAGTTGCACGGTGAC 77
            ||||||||||||||||||||
S:  2009605 ATGGGCAGTTGCACGGTGAC 2009624
```

Figure 3.6 Comparison of cDNA sequence on clone 9 with genomic DNA sequence of *A. fumigatus*. "Q" denotes for Query and "S" denotes for Subject.

As can be seen from Figure 3.6, these sequences were obtained by TIGR. Two parts of sequences were found, one of them has 99% identity with query and the other one has 95% identity with query. 100% identity was expected but it may be caused from the sequencing errors and genomic sequence differences between the strains IMI 385708 (Query) and Af293 (Subject). Also, these two sequences shows that there is one or more intron sequences.

3.7.1.2 Determination of Amino Acid Sequence

Intron and exon analyses of the sequences were done by comparison of the cDNA insert with *A. fumigatus* genome sequence. As shown in Figure 3.7, two intron sequences were found.

GATGCGCAAGCGCATCAAGGCACAGGAGGAAAAGAACGTCAAGTCCTCCGCCCCCGA
CGAGCCGTCCAAGACCCCTCTGCCCCAGTACCTGCTCGACCGTTCGCAGGCGACCAA
CGCCAAGGCTCTGTCCAGTGCTATCAAGGATAAGCGTGCTGAGAAGGCGGCCAAGTT
CGCGGTGCCCCTGCCCAAGGTGAAGGGTATCAGTGAGGAGGAGATGTTCAAGGTCGT
CAACACGGGCAAGAAGACGCACAAGAAGTCGTGGAAGCGGATGATCACCAAGCCCAC
ATTTGTCGGTAGCGACTTCACCCGGCGACCGGTCAAGTACGAGCGTTTCATCCGGCC
GATGGGTCTGCGTTACAAGAAGGCCAATGTCACGCAGTATGCTGCCTTCCTTCCCTC
TAAGCTTTTTGCGTTGCAACCCAACTGACAATTTATCAGCCCGGAGCTCGGCGTCAC
CGTGCAACTGCCCATCCTGGGCGTCAAGAAGAACCCGCAGAACCCTCTCTACACCCA
ACTGGGAGTCCTGACCAAGGGAACCATCATCGAAGTCAACGTCTCCGAATTGGGTCT
CGTCACGACAAGCGGAAAGGTCGTATGGGGTAAATATGCGCAGATCACCAATACTCC
TGAGAACGATGGCACGGTCAATGCGTATGTGACCTTTCACCAATGTATCTCCCAAGA
CAGAGTCATGCTAATCTTCGTGCGCAGAGTCCTCCTGGTCTAATCTTGTCCTTAAGC
GGCCCTCCGCACCTTGATACCAGTTCCGTTCCAGAAGTCCGCATTCTCGTTGGTATT
ATCTTTTAGTTCAGCAGTCTATATTCCTGGGAGGCGTTCAAGGCGCGCACTGTGATT
CCAACTATCAAAAGTATGCATCTAGTTCATCGATCCGATTTGCAAGTCAGTAGAAAT
AGACATCATGATTTTGATGAGCAGATCTTCACGTCGGCCGTCGATTACCTTGTATAC
ATACATATACGTCGTTCACCAAGCTACGGTATATATTCAGTTAACATACGTATCCTC

TAATTTTAAATCCATATTTATGTTGCTTTACTGTCCATATTCCAGACATCTTGGTAT
ATGGTTATGTCGCAAGCATACAGTAAA

Figure 3.7 Intron and exon sequences of *A. fumigatus* genome for plasmid 9; yellow highlighted red sequences denote exon sequences and blue highlighted black sequences denote intron sequences.

The sequencing step is very important to find intron and exon sequences. Because misreading and nonreading sequences create a problem to determine the sequences.

An open reading frame of the partial cDNA sequence from clone 9 was found by a program called WinGene 2.31. Accordingly, cDNA on plasmid 9 encodes a protein with 101 amino acid long partial peptide sequence shown below in Figure 3.8.

L H P A T G Q V R A F H P A D G S A L Q E G Q C H A P E L
G V T V Q L P I L G V K K N P Q N P L Y T Q L G V L T K G
T I I E V N V S E L G L V T T S G K V V W G K Y A Q I T N T
P E N D G T V N A V L L V -

Figure 3.8 Partial amino acid sequence of protein encoded cDNA on clone 9

3.7.1.3 Multiple Sequence Alignment

cDNA sequence of clone 9 was searched with BLASTX 2.2.9 which is the protein database search program that finds homology between the translated query and other proteins. The result of the homology search is given as the type of protein, accession number, amino acid length and identities in Appendix H. Multiple sequence alignment of the results is shown in Figure 3.9.

```
M.musculus  MPQNEYIELHRKRYGYRLDYHEKKRKKEGWEAHERSKKAKKMIGLKAKLYHKQHHAEKIQ 60
H.sapiens   MPQNEYIELHRKRYGYRLDYHEKKRKKESREAHERSKKAKKMIGLKAKLYHKQRHAEKIQ 60
S.pompe     MPQNEYIEESIRKHGRRFDHEERKRKKAAREAHDASLYAQKTRGIKAKLYQEKRRKEKIQ 60
A.nidulans  MPQNEYIERWTKQHGKRLDHDERVRKREARQSHQQSKDAQNLRGLRAKLYQQKRHAEKIQ 60
N.crassa    MPQNEYIERAQKLHGKRLDTEERARKKAAREGHKQSENAQNLRGLRAKLFAKERHAQKIQ 60
A.fumigatus ------------------------------------------------------------

M.musculus  MKKTIKMHEKRNTKQKDDEKTPQRAVSAYLLDREGQSRAKVLSNMIKQKRKEKVGKWEVP 120
H.sapiens   MKKTIKMHEKRNTKQKNDEKTPQGAVPAYLLDREGQSRAKVLSNMIKQKRKEKAGKWEVP 120
S.pompe     MKKTIKQHEERNATQRGSDAQTQGAVPTYLLDREQESQAKMLSSAVKQKRKEKAAKYSVP 120
A.nidulans  MRKRIKAQEEKNVKSSAPSEPSKTPLPQYLLDRSEATNAKALSSAIKDKRAEKAAKFAVP 120
N.crassa    MRKAIKQHEERNVKGAPEEKDPSNPVPAYLLDRSNPTSAKALSSQIKSKRAEKAARFSVP 120
A.fumigatus ----------------------------------------LHPATGQVR---------- 9
                                                     *     . *

M.musculus  LPKVRAQGETEVLKVIRTGKR-KKKAWKRTVTKVCSVGDGFTRKPPKYERFIRPMGLRFK 179
H.sapiens   LPKVRAQGETEVLKVIRTGKR-KKKAWKRMVTKVCFVGDGFTRKPPKYERFIRPMGLRFK 179
S.pompe     LPQVRGVAEEEMFKVIRTGKS-KKNSWKRMITKATFVGDGFTRRPVKYERFIRPMALRQK 179
A.nidulans  LPKVKGISEEEMFKVVNTGKKTHKKSWKRMITKPTFVGNDFTRRPVKYERFIRPMGLRYK 180
N.crassa    IPKVKGISEEELFKVVKTGKKVHKKGWKRVVTKPTFVGPDFTRRPVKYERFIRPMGLRYK 180
A.fumigatus ------------------------------------------------AFHPADGSALQ 20
                                                               *  . .   :

M.musculus  KAHVTH--------------------PELKATFCLPILGVKKNPSSPLYTTLGVITKGT 218
H.sapiens   KAHVTH--------------------PELKATFCLPILGVKKNPSSPLYTTLGVITKGT 218
S.pompe     KANVTH--------------------KELGVTMQLPIIGVKKNPQSPTYTQLGVLTKGT 218
A.nidulans  KANVTQYVLSLATIGVYLGFTDKFSCSPEMAVTVQLPILSVKKNPQNPLYTQLGVLTKGT 240
N.crassa    KANVTH--------------------PTLNVTVQLPILGVKKNPSNPLYTQLGVLSKGT 219
A.fumigatus EGQCHA--------------------PELGVTVQLPILGVKKNPQNPLYTQLGVLTKGT 59
            :.:                       : .*. ***:.*****..* ** ***::***

M.musculus  VIEVNVSELGLVTQGGKVIWGKYAQVTNNPENDGCINAVLLV 260
H.sapiens   VIEVNVSELGLVTQGGKVIWGKYAQVTNNPENDGCINAVLLV 260
S.pompe     VIEVNVSELGLVTSGGKVVWGKYAQITNNPELDGCVNALLLT 260
A.nidulans  VIEVNVSELGIVTAGGKVAWGKYAQITNTPENDGCVNAVLLV 282
N.crassa    IIEVNVSDLGMVTASGKIAWGRYAQITNNPENDGCLNAVLLV 261
A.fumigatus IIEVNVSELGLVTTSGKVVWGKYAQITNTPENDGTVNAVLLV 101
            :******:**:** .**: **:***:**.** ** :**:**.
```

Figure 3.9 Multiple sequence alignment of amino acids from *A.fumigatus* and some important homolog species. *Mus musculus,* and *Homo sapiens* amino acid sequence code for Transforming Growth Factor (TGF) beta-inducible nuclear protein 1; *Schizosaccharomyces pombe, Aspergillus nidulans*, and *Neurospora crassa* amino acid sequence code for hypothetical protein.

As can be seen from Figure 3.9 and Appendix H, clone 9 has a very high homology (%78) with *Mus musculus* and *Homo sapiens*' TGF beta-inducible nuclear protein 1. According to these results, clone 9 can be TGF beta-inducible nuclear protein 1.

TGF-beta is the prototype of a large family of signalling molecules with more than 40 members. TGF-beta plays an essential role in a wide array of cellular processes, including early embryonic development, cell growth, differentiation, motility, and apoptosis (http://breast-cancer-research.com/content/5/6/R187).

3.7.2 Characterization of Clone 15

3.7.2.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, DNA sequence of the sense strand of cDNA clone 15 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome and a homologous region was found in the minus strand of contig 70. The gene on plasmid 15 was characterized accordingly, as shown in Figure 3.10.

Query submitted to BLAST: insert of the plasmid 15
Result: >a_fumigatus|chr_0|Sanger.Af0121f02.p1c|70
Length = 2,671,085

Minus Strand HSPs:
Identities = 361/368 (98%), Positives = 361/368 (98%), Strand = Minus / Plus

```
Q:    491 TTTAGGTACCAGTCGTGGACATCACATTTCCTATCTATGGCATATACAATCCCACATCTG 432
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 832707 TTTAGGTACCAGTCGTGGACATCACATTTCCTATCTATGGCATATACAATCCCACATCTG 832766

Q:    431 AGCGCCTAAAAACCCCTGTCACAAAGCTAATTGCGCAGAGAATCTAGTAGAGACGCTTGG 372
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 832767 AGCGCCTAAAAACCCCTGTCACAAAGCTAATTGCGCAGAGAATCTAGTAGAGACGCTTGG 832826

Q:    371 GGGGAGACATGGTAGCCTTGAGGACAAGGCTGCCAACGTTCTGCCAACCCTTCTTCAGCA 312
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 832827 GGGGAGACATGGTAGCCTTGAGGACAAGGCTGCCAACGTTCTGCCAACCCTTCTTCAGCA 832886

Q:    311 GGGAGACGAGGTAGTTGATGGCCAGCATGACGTTGGCGACGAGCTCTTCCTTGGTCATGC 252
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 832887 GGGAGACGAGGTAGTTGATGGCCAGCATGACGTTGGCGACGAGCTCTTCCTTGGTCATGC 832946

Q:    251 CGACGTTGCCAACGGCAACACCGAGGCAGAGAACCTTCTTGAGCTGGAACT-GATGGTAG 193
          |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
S: 832947 CGACGTTGCCAACGGCAACACCGAGGCAGAGAACCTTCTTGAGCTGGAACTTGATGGTAG 833006
```

```
Q:    192 ACTTGATCTCGGTGACCTTGTTGGC-ATGTCCTC-CTGTGANA-ACGGGGGTAGGAAATT 136
          ||||||||||||||||||||||||| |||||||| |||||| | |||||||||||| ||||
S: 833007 ACTTGATCTCGGTGACCTTGTTGGCCATGTCCTCGCTGTGAGAGACGGGGGTAGGGAATT 833066

Q:    135 TACCANCT 128
          ||||| ||
S: 833067 TACCAGCT 833074
```

Identities = 195/320 (60%), Positives = 195/320 (60%), Strand = Minus / Plus

```
Q:    356 CCTTGAGGACAAGG-CTGCCAACGTTCTGCCAAC----CCTTCTTCAGCAGGGAGAC-GA 303
          | |||  ||| ||  || ||   ||  |||| ||    ||  |  || ||  ||| | ||
S: 832917 CGTTGGCGACGAGCTCTTCCTTGGTCATGCCGACGTTGCCAACGGCAACACCGAGGCAGA 832976

Q:    302 GGTAGTTGATGGCCAGCATGACGTTGGC-GACGAGCTCTTCCTTGGTCATGCCGACGTTG 244
          |    ||  ||  | | |    | |||  |||  | ||| |  ||  | ||  | |  ||
S: 832977 GAACCTTCTTGAGCTGGAACTTGATGGTAGACTTGATCT-CGGTGACCTTGTTGGCCATG 833035

Q:    243 CCAACG--GC-A-ACACCGAGGCAGAGAACCTTCTTGAGCTGGAACTGATGGTAG-ACTT 189
           |  ||  |  | | || | || || |||  | |   |||||   | || | ||| | ||
S: 833036 TCCTCGCTGTGAGAGACGGGGGTAGGGAATTTACC--AGCTGCGCC-GA-GTTAGCATTT 833091

Q:    188 GATCTCGGTGACCTTGTTGGCATGTCCTCCTGTGANAACGGGGGTAGGAAATTTACCANC 129
          | |   ||  |    |   ||  | | | ||| |  ||| |    || | |  ||||  |
S: 833092 GCTAGAGGCTATGCCGCGCGCTGGGCGTTCTGCGGGAACCGA---AG-ATACGTACC--C 833145

Q:    128 TTGGACAGACCGGGACC-AGGAGACGAGGAATCTGCT-GAT-AGGGTGTCG-AAGCA--G 75
          ||||||||||||||||| |||||||||||||||||| ||| ||||||||| |||||  |
S: 833146 TTGGACAGACCGGGACCCAGGAGACGAGGAATCTGCTTGATGAGGGTGTCGGAAGCAAGG 833205

Q:     74 AA-GC-TCGT-CTTGCGAGC 58
          || || |||| |||||||||
S: 833206 AAAGCATCGTACTTGCGAGC 833225
```

Figure 3.10 Comparison of cDNA sequence on clone 15 with genomic DNA sequence of *A. fumigatus*. "Q" denotes for Query and "S" denotes for Subject.

As can be seen from Figure 3.10, this contig was sequenced by the Sanger Institute. Two part of sequences were found, one of them has 98% identity with query and the other one has 60% identity with query. 100% identity was expected but it may be caused from the sequencing errors and genomic sequence differences between the strains IMI 385708 (Query) and Af293 (Subject). Also, these two sequences shows that there is one or more intron sequences.

3.7.2.2 Determination of Amino Acid Sequence

Intron and exon analyses of the sequences were done by comparison of the cDNA insert with *A. fumigatus* genome sequence. As shown in Figure 3.11, two intron sequences were found.

GGAGTGCGCGAGAATGTCGAGCAGCTGCTCAACTACTCTCAGAATGAGAAGAAGAGA
AACTTCCTCGAGACCGTCGAGCTTCAGATCGGTCTGAAGAACTACGACCCCCAGCGT
GACAAGCGTTTCTCTGGCACCATCAAGCTGCCCACCGTTCCCCGCCCCAACATGACC
ATCTGGTACGTCAACTTTGCCGACCGACCGATCCTTCGGTGGAATGTCTTGATGCGC
ATCATCGGGAGAAGGATGAGGTAACGCACAAGTTATGGGACTAAGGGTTGCTGACGG
TGTTGTCTGTAGTGTTCTTGGTGACCAGCACGATCTCGACCGTGCCAAGCACCACGG
CATCGATGCCATGTCCGCTGATGACCTGAAGAAGCTCAACAAGAACAAGAAGCTCAT
CAAGAAGCTGGCTCGCAAGTACGATGCTTTCCTTGCTTCCGACACCCTCATCAAGCA
GATTCCTCGTCTCCTGGGTCCCGGTCTGTCCAAGGGTACGTATCTTCGGTTCCCGCA
GAACGCCCAGCGCGCGGCATAGCCTCTAGCAAATGCTAACTCGGCGCAGCTGGTAAA
TTCCCTACCCCCGTCTCTCACAGCGAGGACATGGCCAACAAGGTCACCGAGATCAAG
TCTACCATCAAGTTCCAGCTCAAGAAGGTTCTCTGCCTCGGTGTTGCCGTTGGCAAC
GTCGGCATGACCAAGGAAGAGCTCGTCGCCAACGTCATGCTGGCCATCAACTACCTC
GTCTCCCTGCTGAAGAAGGGTTGGCAGAACGTTGGCAGCCTTGTCCTCAAGGCTACC
ATGTCTCCCCCCAAGCGTCTCTACTAGATTCTCTGCGCAATTAGCTTTGTGACAGGG
GTTTTTAGGCGCTCAGATGTGGGATTGTATATGCCATAGATAGGAAATGTGATGTCC
ACGACTGGTACCTAAATCTTTTTTTCGGGAATTCCGTAGTGTGTGCTGGCATACTGC
ACATGTTCCAATCTTCATCTATTCTAGGATATACGTCCGCTGCATAGAGAACATAAA
CAGTACCGTCCGTAAAGATAAATATACAAGTGGGATAAAGTAGGGTAAAAAGTTACT

Figure 3.11 Intron and exon sequences of *A. fumigatus* genome for plasmid 15; yellow highlighted red sequences denote exon sequences and blue highlighted black sequences denote intron sequences.

An open reading frame of the partial cDNA sequence from clone 15 was found by a program called WinGene 2.31. Accordingly, cDNA on plasmid 15 encodes a protein with 161 amino acid long partial peptide sequence shown below in Figure 3.12.

T V P R P N M T I C V L G D Q H D L D R A K H H G I D A M
S A D D L K K L N K N K K L I K K L A R K Y D A F L A S D
T L I K Q I P R L L G P G L S K A G K F P T P V S H S E D M
A N K V T E I K S T I K F Q L K K V L C L G V A V G N V G M
T K E E L V A N V M L A I N Y L V S L L K K G W Q N V G S
L V L K A T M S P P K R L Y -

Figure 3.12 The amino acid sequence of cDNA on clone 15

3.7.2.3 Multiple Sequence Alignment

cDNA sequence of clone 15 was searched with BLASTX 2.2.9. Multiple sequence alignment of the results is shown in Figure 3.13.

```
A.fumigatus  ----------------------------------------------------TVPR 4
N.crassa     MSKISVAAVRQHVTDLLEYSNETKKRNFLETVELQIGLKNYDPQRDKRFSGTIRLPSIPR 60
S.pompe      MSKVSVASVRSNVEQILKGS-EEKKRNFTETVELQIGLKNYDPQRDKRFSGTIKLPNVPR 59
S.cerevisiae MSKITSSQVREHVKELLKYSNETKKRNFLETVELQVGLKNYDPQRDKRFSGSLKLPNCPR 60
C.albicans   MSKITSSGVREYVHKLLEYSTETKKRNFLETVELQVGLKNYDPQRDKRFSGTLKLPQVPR 60
                                                                  **

A.fumigatus  PNMTICVLGDQHDLDRAKHHGIDAMSADDLKKLNKNKKLIKKLARKYDAFLASDTLIKQI 64
N.crassa     PNMSICILGDQHDIDRAKHGGVDAMSVDDLKKLNKNKKLIKKLARKYDAFVASEALIKQI 120
S.pompe      PNMAICILGDAHDLDRAKHGGVDAMSVDDLKKLNKNKKLVKKLAKKYDAFIASEVLIKQI 119
S.cerevisiae PNMSICIFGDAFDVDRAKSCGVDAMSVDDLKKLNKNKKLIKKLSKKYNAFIASEVLIKQV 120
C.albicans   PNMTTCIFGDAFDFDRAKSLGVDAMSVDDLKKLNKNKKLIKKLAKKYNAFIASEVLIKQI 120
             ***: *::** .*.****  *:****.***********:***::**:**:**:.****:

A.fumigatus  PRLLGPGLSKAGKFPTPVSHSEDMANKVTEIKSTIKFQLKKVLCLGVAVG---MTKEELV 121
N.crassa     PRLLGPGLSKAGKFPTPVSHSDDLTGKLNEVKSTIKFQLKKVLCMGVAVGNVGMTQEQLV 180
S.pompe      PRLLGPGLSKAGKFPSPVSHADDLYGKITEVKSTIKFQLKKVLCLGVAVGHVEMSEEQLI 179
S.cerevisiae PRLLGPQLSKAGKFPTPVSHNDDLYGKVTDVRSTIKFQLKKVLCLAVAVGNVEMEEDVLV 180
C.albicans   PRLLGRTLSKAGQFPTPVSHNDDLYSKVTDVKSTIKFQLKKVLCLAVAVGNVDMEEDVLV 180
             *****  *****:**:**** :*: .*:.:::************:.****   * :: *:
```

```
A.fumigatus  ANVMLAINYLVSLLKKGWQNVGSLVLKATMSPPKRLY 158
N.crassa     GNIMLAINYLVSLLKKGWQNVGSLTIKATMSPPKRLY 217
S.pompe      ANIMLAVNFLVSLLKKGWQNIGSLVVKSTMGKPHRLY 216
S.cerevisiae NQILMSVNFFVSLLKKNWQNVGSLVVKSSMGPAFRLY 217
C.albicans   NQIMMAANFLVSLLKKNWQNVGSLVIKSTMGPSFRIY 217
              ::::: *::******.***:***.:*::*. . *:*
```

Figure 3.13 Multiple sequence alignment of amino acids from *A.fumigatus* and some important homolog species. *Schizosaccharomyces pombe, Saccharomyces cerevisiae*, and *Candida albicans* amino acid sequence code for 60S ribosomal protein L10a; *Neurospora crassa* amino acid sequence codes for hypothetical protein.

As can be seen from Appendix H, clone 15 codes for a putative 60S ribosomal protein L1-b of *Aspergillus fumigatus*.

3.7.3 Characterization of Clone 17

3.7.3.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, partial DNA sequence of the sense strand of cDNA clone 17 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome and a homologous region was found in the minus strand of contig 58. The gene on plasmid 17 was characterized accordingly, as shown in Figure 3.14.

Query submitted to BLAST: insert of the plasmid 17
Result: >a_fumigatus|chr_0|TIGR.5231|58
Length = 2,455,402

Minus Strand HSPs:
Identities = 227/231 (98%), Positives = 227/231 (98%), Strand = Minus / Plus

```
Q:    473 TTGATAACGGATGAACATGTTATAAGCATGTATATGCAATACAGTACATTGATCGCTCAT 414
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 888073 TTGATAACGGATGAACATGTTATAAGCATGTATATGCAATACAGTACATTGATCGCTCAT 888132
```

```
Q:    413 TCTTACACCGATNTGCCAAACCTCATATGCAACAATTGCTGTTTTGANACTTCATATCGA 354
          |||||||||||| ||||||||||||||||||||||||||||||||| ||||||||||||
S: 888133 TCTTACACCGATATGCCAAACCTCATATGCAACAATTGCTGTTTTGAGACTTCATATCGA 888192

Q:    353 AGGAGGAGAAGCACCAAGAACTCATAAAACGCGACCGAGGAACGAACAAAACATNTAGAA 294
          |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
S: 888193 AGGAGGAGAAGCACCAAGAACTCATAAAACGCGACCGAGGAACGAACAAAACATATAGAA 888252

Q:    293 AAAGTAGAAATCGAAATACGGAGAACAAAGAGTCCGTCAAAAACACGCCTC 243
          |||||||||||||||||||||||||||||||||||||||||||||||||| |
S: 888253 AAAGTAGAAATCGAAATACGGAGAACAAAGAGTCCGTCAAAAACACGCCGC 888303
```

Identities = 150/167 (89%), Positives = 150/167 (89%), Strand = Minus / Plus

```
Q:    250 CACGCCTCTCAGGACCTTCTCCATCTCCTCCTCCGTCAGNGTCTCATACTCGATCAGTGC 191
          | |||||||||||||||||||||||||||||| |||||| ||||||||||||||||||||
S: 888495 CTCGCCTCTCAGGACCTTCTCCATCTCCTCCTTCGTCAGCGTCTCATACTCGATCAGTGC 888554

Q:    190 CTTGGTCAGAAGCTC-AGTTCGTGTCTCCGTTCAGTCANAATCTTGGTGGCACGCATCCG 132
          |||||||||||| || |||||||||||||||||||||| ||||||||||||||||||||
S: 888555 CTTGGTCAGAAGTTCCAGTTCGTGTCTCCGTTCAGTCAGAATCTTGGTGGCACGCATCCG 888614

Q:    131 -GCTTC-TC-AC-AGTCGTCG-ACT-CGGATTC-ATTCTGGTTGGTT 92
           ||||| || || |||||||| ||| ||||||| ||| |  ||| ||
S: 888615 CGCTTCCTCGACCAGTCGTCGCACTTCGGATTCGATT-TC-TTGTTT 888659
```

Figure 3.14 Comparison of cDNA sequence on clone 17 with genomic DNA sequence of *A. fumigatus*. "Q" denotes for Query and "S" denotes for Subject.

As can be seen from Figure 3.14, this contig was sequenced by TIGR. Two part of sequences were found, one of them has 98% identity with query and the other one has 89% identity with query. 100% identity was expected but it may be caused from the sequencing errors and genomic sequence differences between the strains IMI 385708 (Query) and Af293 (Subject). Also, these two sequences shows that there is one or more intron sequences.

3.7.3.2 Determination of Amino Acid Sequence

Intron and exon analyses of the sequences were done by comparison of the cDNA insert with *A. fumigatus* genome sequence. As shown in Figure 3.15, one intron sequence was found.

GACAAGGTCACAAGTGGTATTTCGGCGGTAAGTTCTATTCTCAGACTTCAAAAGTCT
GTTCACGATCACTAATATTCTGCAGGATATTCAACAAGCCACTGAAACCGCGTTTAC
ACTGATCACCAGATTCGGATACTCCAAGAAACTTGGTAACGTCGACCTCTCGACCAA
CTATGATAGTCTATCATCTGAAACCAAACAAGAAATCGAATCCGAAGTGCGACGACT
GGTCGAGGAAGCGCGGATGCGTGCCACCAAGATTCTGACTGAACGGAGACACGAACT
GGAACTTCTGACCAAGGCACTGATCGAGTATGAGACGCTGACGAAGGAGGAGATGGA
GAAGGTCCTGAGAGGCGAGAAACTAGACAAGATGGAGTCGGTACCATCGGCTCCGCT
CAAGCTGCCTGAGGCTTTGCAGACCGCAAAATTGAATCATCCATCCGCCCAGGCCGA
GGAACCGTCGGCCGCAGCCGAATGATAGGTTTCTCCCTCACGACTTTGTACTCTCCA
TATTTCTTCCATCACTCACCTCCTCTCTGCCGAAATCAGGCGGCGTGTTTTTGACGG
ACTCTTTGTTCTCCGTATTTCGATTTCTACTTTTTCTATATGTTTTGTTCGTTCCTC
GGTCGCGTTTTATGAGTTCTTGGTGCTTCTCCTCCTTCGATATGAAGTCTCAAAACA
GCAATTGTTGCATATGAGGTTTGGCATATCGGTGTAAGAATGAGCGATCAATGTACT
GTATTGCATATACATGCTTATAACATGTTCATCCGTTATCAATGAAGTTGTAAAGTT
GGGATTTTGCACCTGTGCTTGTAAGAAGGCCATATTGAAATCAGGTTGCTATTGATG
CAGCTTTCATTTAAAACCTACACCACAACCAAGGCACAACAACGATGCTGCGGAAAT
TCCTTTTTGATTGATATGTATACAGAGTTATCCGTCGAAACACGGTTTATATAAGTG
TAGTGT

Figure 3.15 Intron and exon sequences of *A. fumigatus* genome for plasmid 17; yellow highlighted red sequences denote exon sequences and blue highlighted black sequences denote intron sequences.

An open reading frame of the partial cDNA sequence from clone 17 was found by a program called WinGene 2.31. Accordingly, cDNA on plasmid 17 encodes a protein with 101 amino acid long partial peptide sequence shown below in Figure 3.16.

K Q E I E S E V R R L V E E A R M R A T K I L T E R R H E L
E L L T K A L I E Y E T L T K E E M E K V L R G V F L T D S
L F S V F R F L L F L Y V L F V P R S R F M S S W C F S S F
D M K S Q N S N C C I -

Figure 3.16 Partial amino acid sequence of protein encoded by cDNA on clone 17

3.7.3.3 Multiple Sequence Alignment

cDNA sequence of clone 17 was searched with BLASTX 2.2.9. Multiple sequence alignment of the results is shown in Figure 3.17.

As can be seen from Figure 3.17 and Appendix H, clone 17 has approximately 40% homology with protease genes at the N-terminus. However, C-terminus of the protein had no homology with the protease genes. Accordingly, it was not possible to suggest that clone 17 codes for a protease.

```
S.pompe       ------------------------------------------------------------
A.tumefaciens ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    MASQLPILKPNVSASGSWPSMSKFLKSPLHRTGQSFATIGSAIQDSRRRLHTETFARKST 60
A.fumigatus   ------------------------------------------------------------


S.pompe       ----------------------------------------MSRVLHPIFLFGKTSFLYS 19
A.tumefaciens ------------------------------------------------------------
N.crassa      --------------------MSSRQLAMANPLFRRSFSALMSRPLGTVNTLRSMSTHQP 39
A.nidulans    PSEVVAFGVYSAPVHRLPRSLLSSKRNPLSSSWTASDSGDLESSIFTPSSFAGRHCHPLA 120
A.fumigatus   ------------------------------------------------------------


S.pompe       GCSKFGGR----LFNNSIVHGWLRTRSYALASGLHPLRKQKLAHFEDLANANMSDPYMQA 75
A.tumefaciens ------------MN-------PNFRNFALWAVIALLLIALFSMFQTSPTQTGSREIPYS 40
N.crassa      GRIPSFFR----SPVHSSLGFTLQVRSFGNGGLSHNLLAAREAAANQFPTSAGAQYAFYQ 95
A.nidulans    SKTYSTLAPPLNRTRNVLIPSTLQQQRFIFGGPSHSLLAQKEKTANNNPSSANAQNAFYQ 180
A.fumigatus   ------------------------------------------------------------


S.pompe       KLYKELADNFPEAIISRYETQGVARNSACDRYYQEALRKKSWSRSLSNNISLSQSSSSPA 135
A.tumefaciens QFIRDVDS-------GRVRDVTVTGNRVLGTYTENGTAFQTYSPVIDD--SLMERLQSKN 91
N.crassa      ALLKANMP---AIIIERYQSGRFATNEQVDQIYQQALAMSTGQPYTPANNTVDNNGYHPS 152
A.nidulans    ALLRANMP---AIVIERYRSGHFSSNAVSEQIYLKALER-VGGGVSAPAANLNQGLRSDQ 236
A.fumigatus   ------------------------------------------------------------
```

```
S.pompe       TSSFSDPKAFSAGVPKFTSDTSSTVSSTPSLNHSLQNSMPPSTPTPPPVWAPTIVSSALG 195
A.tumefaciens VTIVARP----------ESDGSSGFLS----------------------------YLG 111
N.crassa      GFTASQIHAAGTAAAAQHTGGNMAMVKP---------------------------IAA 183
A.nidulans    IQAVGQAVAAQ------NQGGQIGISSK---------------------------QSGT 262
A.fumigatus   ------------------------------------------------------------


S.pompe       TSSKTPVYVVVDEPRFTKFFRIFKFIAGLSVASYFVLLGMSIFAETSGLNNIMTNTTEQE 255
A.tumefaciens T--LLPMFLILG----VWLFFMRQMQGGSRGAMGFGKSKAKLLTEAHGR----------- 154
N.crassa      GAKTGPLHIVVDESFGSSALRWVKFLMWFTLFTYLSMVVITMVFEGLSSIKRPGGKLEAS 243
A.nidulans    GAKEAPLYVVVEESLGSAVFRWVKFIVLFCAFAYASMIVLSIVLETTGVLKNIKG-PHSN 321
A.fumigatus   ------------------------------------------------------------


S.pompe       PMEERAINVRFSDVQGVDEAKEELEEIVDFLRDPTHFTRLGGKLPRGVLLTGPPGTGKTM 315
A.tumefaciens --------VTFDDVAGVDEAKQDLEEIVEFLRDPQKFQRLGGKIPRGVLLVGPPGTGKTL 206
N.crassa      EVKPENQKARFADVHGCDEAKEELQELIDFLRNPEKYSTLGGKLPKGVLLVGPPGTGKTL 303
A.nidulans    EAQPEHQTVRFSDVHGCDEAKDELQELVEFLLNPERFSSLGGKLPKGVLLVGPPGTGKTL 381
A.fumigatus   ------------------------------------------------------------


S.pompe       LARAVAGEANVPFFFMSGSQFDEMYVGVGAKRVRELFAAARKQAPSIIFIDELDAIGQKR 375
A.tumefaciens LARSVAGEANVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKNAPCIIFIDEIDAVGRHR 266
N.crassa      LARAVAGEAGVPFFNMSGSEFEEVYVGVGAKRVRDLFAAAKAKAPSIVFIDELDAIGGRR 363
A.nidulans    LARAVAGEAGVPFFYMSGSEFDEVYVGVGAKRVRELFNQARSKSPAIIFIDELDAIGAKR 441
A.fumigatus   ------------------------------------------------------------


S.pompe       NA---RDAAHMRQTLNQLLVDLDGFSKNEDLAHPVVFIGATNFPESLDPALTRPGRFDRH 432
A.tumefaciens GAGLGGGNDEREQTLNQLLVEMDGFEANEG----IILIAATNRPDVLDPALLRPGRFDRQ 322
N.crassa      NS---RDATYVRQTLNQLLTELDGFEQNSG----VIIIGATNFPESLDKALTRPGRFDRN 416
A.nidulans    NE---RDAAYVKQTLNQLLTELDGFSQSTG----VIILAATNYPELLDKALTRPGRFDRK 494
A.fumigatus   ------------------------------------------------------------


S.pompe       IHVPLPDVRGRLAILLQHTRHVPLGKDVDLSIIARGTSGFAGADLANLINQAAVYASKNL 492
A.tumefaciens VVVPNPDIVGRERILKVHVRNVPLAPNVDLKILARGTPGFSGADLMNLVNEAALMAARRN 382
N.crassa      VVVSLPDVRGRMAILQHHAKRIKAAADVNLEAIASRTSGLSGAELENIVNQAAIHASKLK 476
A.nidulans    VVVDLPDVRGRMDILKHHMKNVQISTDVDVAVIARGTSGFSGADLENLVNQAAIFASRNK 554
A.fumigatus   ------------------------------------------------------------


S.pompe       STAVSMRDLEWSKDRILMGAERKSAFITPENKLMTAYHEGGHALVALFTKN-AMRPYKAT 551
A.tumefaciens KRVVTMQEFEDAKDKIMMGAERRSSAMTEAEKKLTAYHEAGHAITALKVAV-ADPLHKAT 441
N.crassa      AQAVTQKDFEWAKDKVIMGAEKRSMVITAKEKEMTAYHEAGHALVGYYAKDSASSLYKVT 536
A.nidulans    QSKVTPKDFDYAKDKIIMGAEARSRIIQDKDKLLTAYHEAGHALVAYFSPS-SMPLYKIT 613
A.fumigatus   ------------------------------------------------------------


S.pompe       IMPRGSSLGMTISLPDMDKDSWTREEYLAMLDVTMGGRAAEELLYGKDKITSGAHNDIDK 611
A.tumefaciens IIPRGRALGMVMQLPEGDRYSMSYKWMVSRLVIMMGGRVAEELTFGKENITSGASSDIEQ 501
N.crassa      ILPRGQTLGHTAYLPEMDKHSFTVRDYLGMIDRAMGGKVAEEIVYGNELVTSGVSADLDM 596
A.nidulans    IMPRGMSLGSTHFLPEMDMVSKNYVQYLSDIDVSMGGKVAEELIYGEDKVTSGISADLAS 673
A.fumigatus   ------------------------------------------------------------


S.pompe       ATQVARRMVTEFGMSDRIGPVSLEAEMD-----------NLSPATRALVESEIKSLLEA 659
A.tumefaciens ATKLARAMVTQWGFSDALGQVAYGENQQEVFLGHSVSQSKNVSEATAQTIDTEVRRLIDE 561
N.crassa      ATRTAWQMVAQLGMSEKLGPVEYLRKYN-----------QLSSETRAMVESEVKRVLDE 644
A.nidulans    ATRTAFTLVTRFGYSKKLGNVDLYANYD-----------SLSSETKQEIEAEVRRLVEE 721
A.fumigatus   ----------------------------------------KQEIESEVRRLVEE 14
                                                        :::*:: :::
```

```
S.pompe       SYERSLSLLKSHKKELDALATALVDYEFLTAEEMNRVVKGD---RDLLRNKLS------- 709
A.tumefaciens AYTEARRILTDNHDGFVAIAEGLLEYETLTGEEIKALLRGEKPARDLGDDSPGSRGSAVP 621
N.crassa      SYERARNLLTSKRNELDYLAKALVEYETLDKKEVERVIRGEK-LKDRISVPPGPMAIPKP 703
A.nidulans    ARQRATNILTERRHELELLTKALIKYETLTKEEMEKVLRGES-LDRLVVPADAPMKLPEP 780
A.fumigatus   ARMRATKILTERRHELELLTKALIEYETLTKEEMEKVLRGVFLTDSLFSVFRFLLFLYVL 74
              :  .:  :*...:. :  :: .*:.** *  :*:: :::*

S.pompe       ----------------------------------
A.tumefaciens KAGAKK-----DGPSEAKGDGEAEGGMEPQPH--- 648
N.crassa      SDTLEPGLPLPPLPGDVPPPGDSGPGPAPPPPVPA 738
A.nidulans    IS----------ATNLSPNQGVEESGNRASAE--- 802
A.fumigatus   FVPRSRFMSSWCFSSFDMKSQNSNCCI-------- 101
```

Figure 3.17 Multiple sequence alignment of amino acids from *A.fumigatus* and some important homolog species. *Neurospora crassa, Agrobacterium tumefaciens, Schizosaccharomyces pombe,* and *Aspergillus nidulans* amino acid sequences code for intermembrane space AAA protease IAP-1, metalloprotease, putative metallopeptidase, and hypothetical protein, respectively.

3.7.4 Characterization of Clone 20

3.7.4.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, partial DNA sequence of the sense strand of cDNA clone 20 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome. However, a homologous region could not be found. It may be caused from the non-sequenced contigs of the *A. fumigatus* genome. Therefore, intron and exon analyses of the insert could not be done.

An open reading frame of the partial cDNA sequence from clone 20 was found by a program called WinGene 2.31. Accordingly, cDNA on plasmid 20 encodes a protein with 64 amino acid long partial peptide sequence shown below in Figure 3.18.

V S R R P H T Q X D L L S S E W N R P Y S R E A A A Y P L P Y L V E K K F W P S V T R V D D A Y G D Q N L F C T C G P V E E T D -

Figure 3.18 Partial amino acid sequence of protein encoded by cDNA on clone 20

3.7.4.2 Multiple Sequence Alignment

cDNA sequence of clone 20 was searched with BLASTX 2.2.9. Multiple sequence alignment of the results is shown in Figure 3.19.

```
A.fumigatus   ------------------------------------------------------------
S.pompe       -----------MFRACSKLQYHGVNTS---LSRHLFLAKRNLSISSACLEAKNSQK---- 42
N.crassa      MTAPGRVGRLGHIVGPSTMAFRGAMPARLPLRASTTISCPQPLVAASCLRLASSESSQRG 60
A.nidulans    MAAS------LCLRGSRQLALRSQLRS---IHPSITRSRLLPTYRAVHSSSQQSHSARRP 51
S.cerevisiae  ----------MLRTRVTALLCRATVRS---STNYVSLARTRSFHSQSILLKTAATDITST 47
H.sapiens     -----------MQSCARAWGLR--------LGRGVGGGRRLAGGSGPCWAPRSRDSSSGG 41

A.fumigatus   ------------------------------------------------------------
S.pompe       ----------------FPALDTFEPRHIGPSKTDQQYQLESLG--YKDFDSFLKDVIPD 83
N.crassa      KLMKIIPSTAWVQYPELFPVREDFASRHIGPDNSSIQEMLGVLDPPVESLDQFVQEVIPA 120
A.nidulans    IYTSSVADHGVPHPRDVFQPVDTFPRRHIGPSPEAAEEMLAVLDPPVKSLDEFVKQVLPA 111
S.cerevisiae  QYSRIFN----PDLKNIDRPLDTFARRHLGPSPSDVKKMLKTMG--YSDLNAFIEELVPP 101
H.sapiens     GDSAAAG--ASRLLERLLPRHDDFARRHIGPGDKDQREMLQTLG--LASIDELIEKTVPA 97

A.fumigatus   ------------------------------------------------------------
S.pompe       SVRTPESQLMAFGSVNPNEKNPPVN--YSESEFTTLANNVANQNKL-IKSFIGMGYYNVK 140
N.crassa      DILSKRELFPQTRVRFHATKKYPTRQGHQEWEIMKIAESMASSNRHSVKAQIGAGYYGTL 180
A.nidulans    DILSKKDLAVTAPSADNGLPRSSVHGGLGETDMLKLLDKYREQIDVSGKTYIGAGYYPTI 171
S.cerevisiae  NILKRRPLKLEAPSK-----------GFCEQEMLQHLEKIANKNHYKVKNFIGKGYYGTI 150
H.sapiens     NIRLKRPLKMEDPVC--------------ENEILATLHAISS-KNQIWRSYIGMGYYNCS 142

A.fumigatus   ------------------------------------------------------------
S.pompe       LPAAIQRNVLENPEWYTQYTPYQAEISQGRLESMMNYQTMIADLTGLSISNASLLDEGTA 200
N.crassa      TPEVIKRNVLESPAWYTSYTPYQPEISQGRLESLLNFQTMVTDLTGLPIANASLLDEGTA 240
A.nidulans    VPPVILRNILENPAWYTSYTPYQPEISQGRLESLLNFQTLTADLTGLPFANASVLDEATA 231
S.cerevisiae  LPPVIQRNLLESPEWYTSYTPYQPEISQGRLEALLNFQTVVSDLTGLPVANASLLDEGTA 210
H.sapiens     VPQTILRNLLENSGWITQYTPYQPEVSQGRLESLLNYQTMVCDITGLDMANASLLDEGTA 202

A.fumigatus   ------------------------------------------------------------
S.pompe       AGEAMVM-----LMANDKKKRKTFLVDKNIYPNTLSVLRTRASGFGIKIELDNITP---- 251
N.crassa      AAEAMTMSLNALPASRAKRPAKTYVLSNRLHPQTRAVLRGRAEGFGVNIITLDFHDPEFP 300
A.nidulans    AAEAMTMSLATQPLAKQKKAGKTYVVSHLCHPQTVAVMRSRAEGFGINLVIGDILADDFK 291
S.cerevisiae  AGEAMLLS-----FNISRKKKLKYVIDKKLHQQTKSVLHTRAKPFNIEIIEVDCSDIKKA 265
H.sapiens     AAEALQLC-------YRHNKRRKFLVDPRCHPQTIAVVQTRAKYTGV------LTELKLP 249
```

```
A.fumigatus   ------------------------------------------------------------
S.pompe       ELITKSAKHVFGIFVQYPAADGSIFD---YGHLAATARSFNMHVVAATDLLALTILKSPG 308
N.crassa      SKLEELGDDLVGVMVQYPDTTGQVLD---HRQLADLVHKQGALLSVATDLLALTMLTPPG 357
A.nidulans    -IVKDQGDNLIGVLAQYPDTEGGIYD---FQGLSDAIHTAGGTFSVATDLLALTVLKAPG 347
S.cerevisiae  VDVLKNPD-VSGCLVQYPATDGSILPPDSMKQLSDALHSHKSLLSVASDLMALTLLKPPA 324
H.sapiens     CEMDFSGKDVSGVLFQYPDTEGKVED---FTELVERAHQSGSLACCATDLLALCILRPPG 306

A.fumigatus   ------------------------------------------------------------
S.pompe       EWGADVAVGSTQRFGLPMGYGGPHAGFFACSEEFKRKIPGRLIGLSKDRLENPAYRLALQ 368
N.crassa      EWGADIAFGNSQRFGVPLGFGGPHAAFFAVQEKHKRKMPGRLIGVSKDRLGGRALRLSLQ 417
A.nidulans    EFGADIAFGNAQRFGVPMGYGGPHAAFFACADKYKRKVPGRVVGVSKDRLGNRALRLALQ 407
S.cerevisiae  HYGADIVLGSSQRFGVPMGYGGPHAAFFAVIDKLNRKIPGRIVGISKDRLGKTALRLALQ 384
H.sapiens     EFGVDIALGSSQRFGVPLGYGGPHAAFFAVRESLVRMMPGRMVGVTRDATGKEVYRLALQ 366

A.fumigatus   ------------------------------------------------------------
S.pompe       TREQHIRREKATSNICTAQALLANMSAFYAIYHGPNGLQEIANRIYASTSFLKSALESSG 428
N.crassa      TREQHIRREKATSNVCTAQALLANISSFYAVYHGPEGLRAIAERCNLGARVLESAAKFCG 477
A.nidulans    TREQHIRREKATSNICTAQALLANMSAMYAVYHGPSGLKTIAQRIMSMTAALRERLAALG 467
S.cerevisiae  TREQHIKRDKATSNICTAQALLANVASSYCVYHGPKGLQNISRRIFSLTSILANAIENDS 444
H.sapiens     TREQHIRRDKATSNICTAQALLANMAAMFRIYHGSHGLEHIARRVHNATLILSEGLKRAG 426

A.fumigatus   ------------------------------------------------------------
S.pompe       YKIVNKS------HFFDTLTIEVESAD---KVLAKAL-DHGYNLRKVDDSHVGLSLDETV 478
N.crassa      LQLYSPNNSCS-AVPFDTLVINQDHIG--KVLVYAAR-ERGINIRFISTDSAGISVDETT 533
A.nidulans    YNVPAKSNVSDGAAVFDTITIEFSNSEEADAIIAAAR-QNSIFLRRVSATKVGISLDETA 526
S.cerevisiae  CPHELIN-----KTWFDTLTIKLGNGISSEQLLDKALKEFNINLFAVDTTTISLALDETT 499
H.sapiens     HQLQHDL-------FFDTLKIHCGCSVK--EVLGRAA-QRQINFRLFEDGTLGISLDETV 476

A.fumigatus   ------------------------------------------------------------
S.pompe       CDKDIQALFSIFNINKSVDQYYMEIATSEPNGNSAS---------TVDNLSICSLPENF 528
N.crassa      TENDLISLIGAFQDAARSLKVTGRDEALDANPQVIFEHFLKHHAEQIKQSGPLGHLPEPL 593
A.nidulans    GREELKAILQVFSAHAKAEAALDQELGLAS------------------------IPASL 561
S.cerevisiae  TKADVENLLKVFDIENSSQFLSEDYS-------------------------NSFPREF 532
H.sapiens     NEKDLDDLLWIFGCESSAELVAESMGEECR----------------------GIPGSVF 513

A.fumigatus   ------------------------------------------------------------
S.pompe       RRTTLYLQHPVFNRYHSETELMRYIHHLQSKDLSLAHAMTPLGSCTMKLNAVTEMMPITN 588
N.crassa      RRTSSYLTHPVFNTHHSETELLRYIHHLQSKDLSLVHSMIPLGSCTMKLNASAEMALITL 653
A.nidulans    ERTSAYLTHPVFNTHHSETEMLRYIRHLESKDLSLAHSMIPLGSCTMKLNATTEMIPVSW 621
S.cerevisiae  QRTDEILRNEVFHMHHSETAMLRYLHRLQSRDLSLANSMIPLGSCTMKLNSTVEMMPITW 592
H.sapiens     KRTSPFLTHQVFNSYHSETNIVRYMKKLENKDISLVHSMIPLGSCTMKLNSSSELAPITW 573

A.fumigatus   ------------------------------------------------------------
S.pompe       PLFANIHPYVPEEQAKGYRHVIEDLQLMLTTITGFDAACFQPNSGAAGEYTGLSVIRAYQ 648
N.crassa      PGFSNLHPFVPPDQSEGYSRLTKVLESQLIDITGMDACSLQPNSGAQGEFAGLRVIRKYL 713
A.nidulans    PEFSQMHPFLPADVAKGYTQMIDDLEQQLADITGMAEVTVQPNSGAQGEFAGLRVIKKYQ 681
S.cerevisiae  PQFSNIHPFQPSNQVQGYKELITSLEKDLCSITGFDGISLQPNSGAQGEYTGLRVIRSYL 652
H.sapiens     KEFANIHPFVPLDQAQGYQQLFRELEKDLCELTGYDQVCFQPNSGAQGEYAGLATIRAYL 633

A.fumigatus   ------------------------------------------------------------
S.pompe       RSIGQGHRNICLIPVSAHGTNPASAAMAGFTVIPVKCLNN-GYLDMQDLKEKASKHADKL 707
N.crassa      QSRAQSQRDICLIPVSAHGTNPASASMAGMRVVPIKCDTKTGNLDLADLEAKCKQYENEL 773
A.nidulans    EATGSSKRNICLIPVSAHGTNPASAAMAGMKVVTIKCDTKTGNLDLDDLKAKCEKHKDEL 741
S.cerevisiae  ESKGENHRNVCLIPVSAHGTNPASAAMAGLKVVPVNCLQD-GSLDLVDLKNKAEQHSKEL 711
H.sapiens     NQKGEGHRTVCLIPKSAHGTNPASAHMAGMKIQPVEVDKY-GNIDAVHLKAMVDKHKENL 692
```

```
A.fumigatus   ------------------------------------------------------------
S.pompe       AAFMVTYPSTFGIFEPDVKEALEVIHEHGGQVYFDGANMNAMVGLCKAGDIGADVCHLNL 767
N.crassa      AAMMITYPSTFGVFEPAIKKVCQIVHAHGGQVYMDGANMNAQVGLCSPGEIGADVCHLNL 833
A.nidulans    AAIMITYPSTFGVYEPGVKKACDLVHQYGGQVYMDGANMNAQIGLCSPGEIGADVCHLNL 801
S.cerevisiae  AAVMITYPSTYGLFEPGIQHAIDIVHSFGGQVYLDGANMNAQVGLTSPGDLGADVCHLNL 771
H.sapiens     AAIMITYPSTNGVFEENISDVCDLIHQHGGQVYLDGANMNAQVGICRPGDFGSDVSHLNL 752

A.fumigatus   ------------------------------------------------------------
S.pompe       HKTFCIPHGGGGPGVGPICVKKHLADFLPSHP----VVSCGGKNGITSVSSSPFGSAGIL 823
N.crassa      HKTFCIPHGGGGPGVGPICVKEHLAGFLPTT------KTMSNTELNLPVSSASYGSASIL 887
A.nidulans    HKTFCIPHGGGGPGVGPIGVAEHLRPYLPSHPNSEYLQSKRTEKSSPPISAAPWGSASIL 861
S.cerevisiae  HKTFSIPHGGGGPAGAPICVKSHLIPHLPKHDVVDMITGIGGSKSIDSVSSAPYGNALVL 831
H.sapiens     HKTFCIPHGGGGPGMGPIGVKKHLAPFLPNHPVIS-LKRNEDACPVGTVSAAPWGSSSIL 811

A.fumigatus   ------------------------------------------------------------
S.pompe       PISWAYMRMMGLAGLRDASKAALLNANYMAKRLSSHYKLVYTNKN---NLCAHEFILDAR 880
N.crassa      PISWAYNALMGGAGLKKATQVTLLNANYLLSRLKEHYPILYTNEH---GRCAHEFIIDAR 944
A.nidulans    PITFNYINMMGSKGLTHATKITLLNANYILSRLKDHYPILYTNDN---GRCAHEFILDVR 918
S.cerevisiae  PISYAYIKMMGNEGLPFSSVIAMLNSNYMMTRLKDHYKILFVNEMSTLKHCAHEFIVDLR 891
H.sapiens     PISWAYIKMMGGKGLKQATETAILNANYMAKRLETHYRILFRGAR---GYVGHEFILDTR 868

A.fumigatus   ------------------------------------------------------VSRR- 4
S.pompe       EFKATAGVDATDIAKRLQDYSFHAPTLSWPIANTLMIEPTESESMYEMDRFCDALISIRQ 940
N.crassa      PFEKTSGIQAIDIAKRLQDYGFHAPTMSWPVANTLMIEPTESESKEELDRFVDALIAIRE 1004
A.nidulans    KFKDTCGIEAIDIAKRLQDYGFHAPTMSWPVANTLMIEPTESENKAELDRFCDALISIRK 978
S.cerevisiae  EYKAKG-VEAIDVAKRLQDYGFHAPTLAFPVPGTLMIEPTESENLEELDRFCDAMISIKE 950
H.sapiens     PFKKSANIEAVDVAKRLQDYGFHAPTMSWPVAGTLMVEPTESEDKAELDRFCDAMISIRQ 928
                                                                  :: :

A.fumigatus   -------------------PHTQXDLLSS------EWNRP-YSREAAAYPLPYLV-EKK 36
S.pompe       EIREIEEGLQPKDNNLLVNAPHPQKDIASE------KWDRP-YTRERAVYPVPLLK-ERK 992
N.crassa      EIREVEEGKQPREGNVLKMSPHPISDIIGGDGEAGNKWDRP-YSREKAAYPLPWLR-EKK 1062
A.nidulans    EIAAVESGEQPRDGNVLRMAPHTQRDLLAT------EWDRP-YTREQAAYPLPYLL-EKK 1030
S.cerevisiae  EINALVAGQP--KGQILKNAPHSLEDLITS-----SNWDTRGYTREEAAYPLPFLR-YNK 1002
H.sapiens     EIADIEEGRIDPRVNPLKMSPHSLTCVTSS------HWDRP-YSREVAAFPLPFMKPENK 981
                           **.   :         .*:   *:** *.:*:* :   .*

A.fumigatus   FWPSVTRVDD------------------------AYGDQNLFCTCGPVEETD------- 64
S.pompe       FWPSVARLDD------------------------AYGDKNLFCTCSPVV---------- 1017
N.crassa      FWPSVARVND------------------------TYGDLNLFCTCPPVEDTTGGNQSSI 1097
A.nidulans    FWPSVTRVDDDAIGGSRRLPGTSRSQCGVRHPRCSTLSDIKASTFSGSLESHRSAARSRL 1090
S.cerevisiae  FWPTVARLDD------------------------TYGDMNLICTCPSVEEIANETE--- 1034
H.sapiens     FWPTIARIDD------------------------IYGDQHLVCTCPPMEVYESPFSEQK 1016
              ***:::*::*                          .* :    . .:

A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      QEQ----------------------------------------------------------- 1100
A.nidulans    GPSPDLCQDMESKITLFYGTFVDLPRTRSGEKHELAIRHGAIWVSSATGRIQGFDWSIAN 1150
S.cerevisiae  ------------------------------------------------------------
H.sapiens     RASS---------------------------------------------------------- 1020

A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    EAELQSLLRKKGWTGVPIIRALEQENEFFFPGFIGLFGSSTLLDWLETYTFPLESSMSNL 1210
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------
```

```
A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    DKARTAYNAVISRTLANGTTCASYYATIHVPATNLLASLCHTRGQRALIGRVCMDNPAFC 1270
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------


A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    PDYYRDESAEASIELTKETIAHIHSLPDSDKESERLVKPIITPRFAPTCSTSALTSLGQL 1330
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------


A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    AASHTPPLHIQTHISENPNEVSLVQSLFPEHPSYAAVYDACSLLTHRTILAHAVHLTQPE 1390
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------


A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    KELIASRNAKISHCPASNSALGSGLAPVRDLIDNGITVGLGTDVSGGYSPSILEAVRQAC 1450
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------


A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    LVSRLLRHSTASTSSSGNSTQNETEGREVLSVEEALYLATRGGAAVIDMPNELGGFEVGM 1510
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------


A.fumigatus   ------------------------------------------------------------
S.pompe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.nidulans    FWDVQLIRLGATVQETPQTGSHSDSRSVVDIFGWESWAEKVHKWVWTGNDRNVRRVWVGG 1570
S.cerevisiae  ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------


A.fumigatus   ----------------------------------------------------
S.pompe       ----------------------------------------------------
N.crassa      ----------------------------------------------------
A.nidulans    AVVHDLDDGSCVGEETMLGSWFGKSLQRDWTRWAVASVGVAILGFAIGRRSLGSR 1625
S.cerevisiae  ----------------------------------------------------
H.sapiens     ----------------------------------------------------
```

Figure 3.19 Multiple sequence alignment of amino acids from *A.fumigatus* and some important homolog species. *Schizosaccharomyces pombe, Neurospora crassa,* and *Aspergillus nidulans, Saccharomyces cerevisiae,* and *Homo sapiens* amino acid sequences code for putative glycine dehydrogenase, hypothetical protein, hypothetical protein, putative glycine dehydrogenase, glycine dehydrogenase, respectively.

As can be seen from Figure 3.19 and Appendix H, clone 20 has a very high homology (around %60) with glycine dehydrogenase genes. According to these results, clone 20 is likely to code for glycine dehydrogenase. Interestingly, its corresponding genomic could not be found on the *A. fumigatus* genome. It may be possible that this region is still not sequenced.

3.7.5 Characterization of Clone 21

3.7.5.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, partial DNA sequence of the sense strand of cDNA clone 21 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome. However, a homologous region could not be found. Therefore, intron and exon analyses of the insert could not be done.

cDNA sequence of clone 21 was searched with BLASTX 2.2.9, however, no significant similarity was found.

3.7.6 Characterization of Clone 22

3.7.6.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, partial DNA sequence of the sense strand of cDNA clone 22 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome and a homologous region was found in the plus strand of contig 72. The gene on plasmid 22 was characterized accordingly, as shown in Figure 3.20.

As can be seen from Figure 3.20, this contig was sequenced by the Sanger Institute. One part of sequences was found with 89% identity. 100% identity was expected but it may be caused from the sequencing errors and genomic sequence differences between the strains IMI 385708 (Query) and Af293 (Subject). Also, these one sequence data shows that there is no intron sequences.

Query submitted to BLAST: insert of the plasmid 22

Result: >a_fumigatus|chr_0|Sanger.Af0346e02.q1ca|72

Length = 2,962,292

Plus Strand HSPs:

Identities = 409/455 (89%), Positives = 409/455 (89%), Strand = Plus / Plus

```
Q:     27 AGTCCACCC-ACAAACTCCGACCAGGCCTTACATATATCGACAACAGGTTACGAC-TGCC 84
          | | | ||| ||||   ||  | |   | | |||  | | ||| | |  |||||| || |
S: 106770 AATACTCCCGACAAGGGCCT-CTACATCAT-CATCGA-CAACAGC-GTCTACGACGTGAC 106825

Q:     85 CA-GTT-ATCGACGA-CATCCCGGCGGCGCCAAGATTNTNA-GCGT-TGGCGGGNA-GGA 138
          || ||| |||||||| ||||||||||||||||||||||| | | |||| ||||||| | |||
S: 106826 CAAGTTCATCGACGAGCATCCCGGCGGCGCCAAGATTCTCAAGCGTGTGGCGGGCAAGGA 106885

Q:    139 TGCCTCGAAGCAGTTTTG-AAGGTGCGTTNTACCTTACCAACGNGCATATGNACAGAGGG 197
          |||||||||||||||||| |||||||||| |||||||||||| ||||||| ||||||||
S: 106886 TGCCTCGAAGCAGTTTTGGAAGGTGCGTTCTACCTTACCAACGCGCATATGAACAGAGGG 106945

Q:    198 CTGANCTGATGTTGATGGTTGTAGTACCNCNATGAGGGCGTGTTGAAGAAGTATTCGCCC 257
          |||| |||||||||||||||||||||| | ||||||||||||||||||||||||||||||
S: 106946 CTGAGCTGATGTTGATGGTTGTAGTACCACAATGAGGGCGTGTTGAAGAAGTATTCGCCC 107005

Q:    258 NAGTTGAAGATTGGGGAGGTGAAAGAGGCTGCGAANCTGTGATTTATATATTGATATGGA 317
           |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
S: 107006 AAGTTGAAGATTGGGGAGGTGAAAGAGGCTGCGAAGCTGTGATTTATATATTGATATGGA 107065

Q:    318 CTTGGAATTGCNCTGGACTGGACTGGGATCGGGATTGATNTACGGAGCATAATATCCTGA 377
          |||||||||||| |||||||||||||||||||||||||| ||||||||||||||||||||
S: 107066 CTTGGAATTGCACTGGACTGGACTGGGATCGGGATTGATCTACGGAGCATAATATCCTGA 107125

Q:    378 TGGAAGAGAAGGTGTATCAGGCGGATGACCTTGATGGTTGTGTCCCNCGATAGAGATATA 437
          |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
S: 107126 TGGAAGAGAAGGTGTATCAGGCGGATGACCTTGATGGTTGTGTCCCGCGATAGAGATATA 107185

Q:    438 TATTATATATGCATATTGACTGACTTTTCAAGACG 472
          |||||||||||||||||||||||||||||||||||
S: 107186 TATTATATATGCATATTGACTGACTTTTCAAGACG 107220
```

Figure 3.20 Comparison of cDNA sequence on clone 22 with genomic DNA sequence of *A. fumigatus*. "Q" denotes for Query and "S" denotes for Subject.

3.7.6.2 Determination of Amino Acid Sequence

Intron and exon analyses of the sequences were done by comparison of the cDNA insert with *A. fumigatus* genome sequence. As shown in Figure 3.21, no intron sequence was found.

CAGTCAAACCACAAGATCTCCACATCGACATCACCAAATCCTCAACATTCTCCTCCA
GGAATTACCACGATAACAAGAGAGATAGAACTTCAAAGAACGCTGAGAACGAAATGG
CCCAATCCTTCACCCCTGCCGAGGTCGCAGCCCAC**AATACTCCCGACAAGGGCCTCT**
ACATCATCATCGACAACAGCGTCTACGACGTGACCAAGTTCATCGACGAGCATCCCG
GCGGCGCCAAGATTCTCAAGCGTGTGGCGGGCAAGGATGCCTCGAAGCAGTTTTGGA
AGGTGCGTTCTACCTTACCAACGCGCATATGAACAGAGGGCTGAGCTGATGTTGATG
GTTGTAGTACCACAATGAGGGCGTGTTGAAGAAGTATTCGCCCAAGTTGAAGATTGG
GGAGGTGAAAGAGGCTGCGAAGCTGTGATTTATATATTGATATGGACTTGGAATTGC
ACTGGACTGGACTGGGATCGGGATTGATCTACGGAGCATAATATCCTGATGGAAGAG
AAGGTGTATCAGGCGGATGACCTTGATGGTTGTGTCCCGCGATAGAGATATATATTA
TATATGCATATTGACTGACTTTTCAAGACGACCCAATGCGGAAATACAAGCGAAGCG
TCAAGTGGTATCTCTTGCAACCTACAATAATGAACAATAATACACAACCAAATTTCA
TTTCGCCCAGTTAGAATCCTCTGAACGGATCGGGCT

Figure 3.21 Exon sequence of *A. fumigatus* genome for plasmid 22; yellow highlighted red sequences denote exon sequences.

An open reading frame of the partial cDNA sequence from clone 22 was found by a program called WinGene 2.31. Accordingly, cDNA on plasmid 22 encodes a protein with 55 amino acid long partial peptide sequence shown below in Figure 3.22.

N T P D K G L Y I I I D N S V Y D V T K F I D E H P G G A K I
L K R V A G K D A S K Q F W K V R S T L P T R I -

Figure 3.22 Partial amino acid sequence of protein encoded by cDNA on clone 22

3.7.6.3 Multiple Sequence Alignment

cDNA sequence of clone 22 was searched with BLASTX 2.2.9. Multiple sequence alignment of the results is shown in Figure 3.23.

```
A.fumigatus ---------------NTP--DKGLYIIIDNSVYDVTKFIDEHPGGAKILKRVAGKDASKQ 43
A.nidulans  --MSKTFTPAEVAKHNKP--DQGLYIIVDNSVYDVTNFVDEHPGGAKILKRVAGKDASKQ 56
N.crassa    --MSQTFTKSQVAEHKD---DKSMYIIIDDGVYDITNFLDDHPGGAKILKRMAGKDATKS 55
S.pompe     -MAEKTITVEEVLKHNT---RDDLYIVVKDKVYDISKFLDAHPGGEEVLVDLAGRDASGP 56
C.elegans   MSELRVISLDEVSKHNWEDADQSCWIVISGKVYDVTKFLNEHPGGEEVITQLAGKDATVG 60
                         :     .. :*::.. ***:::*:: **** :::  :**:**:

A.fumigatus FWK-----VRSTLPTRI------------------------------------------ 55
A.nidulans  FWKYHNDGVLKKYAPKLKIG--------EVKEGAKL----------------------- 84
N.crassa    FWKYHGKSVLEKYGTKLKVG--------TLAEAAKL----------------------- 83
S.pompe     FEDVGHSEDAQELLEKFYIGNLL-----RTEDGPQLPTTGAAAGGSGYDSSQPVKPAMWL 111
C.elegans   FLDVGHSKDAIEMANEYLIGQLPESDVPKVETAAAKPSKNEKSSSLLNDFTEIMTSPTWT 120
            * .            .

A.fumigatus ---------------------
A.nidulans  ---------------------
N.crassa    ---------------------
S.pompe     -FVLVMVVAYFAFRKYVLK-- 129
C.elegans   NFLIPTTMGLVIYAVYKCMFN 141
```

Figure 3.23 Multiple sequence alignment of amino acids from *A.fumigatus* and some important homolog species. *Aspergillus nidulans, Neurospora crassa, Schizosaccharomyces pombe, Caenorhabditis elegans* amino acid sequences code for hypothetical protein, putative cytochrome b5, probable cytochrome b5, cytochrome b5, respectively.

As can be seen from Figure 3.23 and Appendix H, clone 22 has approximately 50% homology to cytochrome b5 genes at the N-terminus. However, the C-terminus of the translated peptide had no homology to the others. According to these results, it cannot be clearly suggested whether clone 22 is a cytochrome b5 gene or not.

3.7.7 Characterization of Clone 23/24

The sequences of clones 23 and 24 were found as the same.

3.7.7.1 Comparison of cDNA and Genomic DNA of *Aspergillus fumigatus*

As indicated before, partial DNA sequence of the sense strand of cDNA clone 23/24 was determined and the insert sequence was searched by BLASTN 2.0 in *A. fumigatus* genome and a homologous region was found in the minus strand of contig 59. The gene on plasmid 23/24 was characterized accordingly, as shown in Figure 3.24.

Query submitted to BLAST: insert of the plasmid 23/24
Result: >a_fumigatus|chr_0|TIGR.5237|59
Length = 2,797,657

Minus Strand HSPs:
Identities = 447/468 (95%), Positives = 447/468 (95%), Strand = Minus / Plus

```
Q:      496 TACGAATTTNGGCATCGGGTCGTTGATTCAATTGCGTGGCTTATATACAGCCTAACCAAC 437
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
S: 1777205 TACGAATTTTGGCATCGGGTCGTTGATTCAATTGCGTGGCTTATATACAGCCTAACCAAC 1777264

Q:      436 ACCTATAAACGCCTTTACTTCGCCAGCATAAACCAATACTTCCGCTAAGAATAGAAACCT 377
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 1777265 ACCTATAAACGCCTTTACTTCGCCAGCATAAACCAATACTTCCGCTAAGAATAGAAACCT 1777324

Q:      376 GCAAATCCCAATATCAAGAACCCAGCCATATATCGGAACGAATGCAAACAGTGAATGCCC 317
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 1777325 GCAAATCCCAATATCAAGAACCCAGCCATATATCGGAACGAATGCAAACAGTGAATGCCC 1777384


Q:      316 TTAAAATTCCATGCTTTCCGATTTTACCTCATCCGGATCTTCATTTCCTCGTTTGCCGAC 257
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 1777385 TTAAAATTCCATGCTTTCCGATTTTACCTCATCCGGATCTTCATTTCCTCGTTTGCCGAC 1777444

Q:      256 GCGTTCCTCTTTGTTTAGAGAAGCGATGAACGAACGGAAGTAGTCAATATATGGCTTAAG 197
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 1777445 GCGTTCCTCTTTGTTTAGAGAAGCGATGAACGAACGGAAGTAGTCAATATATGGCTTAAG 1777504

Q:      196 GCTGGTCTTCTCGTAATCTTGCAATTTGCGACTTCCCTGGGAGTCGCTAGGTGTGTGGCT 137
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S: 1777505 GCTGGTCTTCTCGTAATCTTGCAATTTGCGACTTCCCTGGGAGTCGCTAGGTGTGTGGCT 1777564
```

```
Q:     136 T-CATCTTGACCAGCTTCGGCGCCTGCATTAC-GCTA-AGTGC-CAGCATCC-AGGCATC 82
           | |||||||||||||||||||||||||||||| |||| ||||| |||||||| |||||||
S: 1777565 TTCATCTTGACCAGCTTCGGCGCCTGCATTACCGCTAGAGTGCTCAGCATCCCAGGCATC 1777624

Q:      81 -ATCTCCGC-AGTAAT-GAGTGATGTTGGGACGGAAAGAGATCAATCG 37
            |||||||| |||||| ||||||   | ||   | || |||  | |||
S: 1777625 TATCTCCGCCAGTAATTGAGTGACTGTTGGGACGGAA-AGAGGA-TCG 1777670
```

Figure 3.24 Comparison of cDNA sequence on clone 23/24 with genomic DNA sequence of *A. fumigatus*. "Q" denotes for Query and "S" denotes for Subject.

As can be seen from Figure 3.24, this contig was sequenced by TIGR. One part of sequences was found with 95% identity. 100% identity was expected but it may be caused from the sequencing errors and genomic sequence differences between the strains IMI 385708 (Query) and Af293 (Subject). Also, these one sequence data shows that there is no intron sequences.

3.7.7.2 Determination of Amino Acid Sequence

Intron and exon analyses of the sequences were done by comparison of the cDNA insert with *A. fumigatus* genome sequence. As shown in Figure 3.25, no intron sequence was found.

CGTTACATCCGCATATGTCCCGTAGTCTTGAAATTCTCAAACCCTTCTTCGCCCAAA
CTACACTTATCGACCAGGATACATTCGCAAGCTCAGAACAAGCAGAGCGCCTCCTCT
CATTGCTGCCGGACAAGACACTGAATGATGCACTGCGCAAGAAATGGGATTCGTCGC
CTGGCCGGTCCAGTACGAACAAATGGGCGGACATCGACGCGCTTGCAAAGACTGGCA
AGAGCAGCACTCTTAATCCTACTACTCTGAGAGACGCGAAACAGGATATCGTACTCG
AATATACATACCCGCGACTCGACGCTGAAGTCAGCAAGAAAATGATCCATTTACTCA
AGAGCCCTTTTGTCATCCATCCCGGTACTGGGCGTGTTTGTGTTCCCATTGACGCCA
AAAAAGCGGACGGATTCGATCCTCTTTCCGTCCCAACAGTCACTCAATTACTGGCGG
AGATAGATGCCTGGGATGCTGAGCACTCTAGCGGTAATGCAGGCGCCGAAGCTGGTC
AAGATGAAAGCCACACACCTAGCGACTCCCAGGGAAGTCGCAAATTGCAAGATTACG
AGAAGACCAGCCTTAAGCCATATATTGACTACTTCCGTTCGTTCATCGCTTCTCTAA

ACAAAGAGGAACGCGTCGGCAAACGAGGAAATGAAGATCCGGATGAGGTAAAATCGG
AAAGCATGGAATTTTAAGGGCATTCACTGTTTGCATTCGTTCCGATATATGGCTGGG
TTCTTGATATTGGGATTTGCAGGTTTCTATTCTTAGCGGAAGTATTGGTTTATGCTG
GCGAAGTAAAGGCGTTTATAGGTGTTGGTTAGGCTGTATATAAGCCACGCAATTGAA
TCAACGACCCGATGCCAAAATTCGTACTTTATTGGTCACTATTCGCTACTATGGTAA
ACTCGGCGGTAATTTTGATGACGGCTTGAATGAGAGGTTTGACATTCTCAATTAGAT
GTGAACTACGTACAACACATGATCCTGAATTCGTTTCTAATGATACAATGGGGGAAT
AGCGGATAAGTAGTTATGATTTTT

Figure 3.25 Exon sequence of *A. fumigatus* genome for plasmid 23/24; yellow highlighted red sequences denote exon sequences

An open reading frame of the partial cDNA sequence from clone 23/24 was found by a program called WinGene 2.31. Accordingly, cDNA on plasmid 23/24 encodes a protein with 179 amino acid long partial peptide sequence shown below in Figure 3.26.

D S S P G R S S T N K W A D I D A L A K T G K S S T L N P T
T L R D A K Q D I V L E Y T Y P R L D A E V S K K M I H L L
K S P F V I H P G T G R V C V P I D A K K A D G F D P L S V
P T V T Q L L A E I D A W D A E H S S G N A G A E A G Q D
E S H T P S D S Q G S R K L Q D Y E K T S L K P Y I D Y F R
S F I A S L N K E E R V G K R G N E D P D E V K S E S M E F
-

Figure 3.26 Partial amino acid sequence of protein encoded by cDNA on clone 23/24

3.7.7.3 Multiple Sequence Alignment

cDNA sequence of clone 23/24 was searched with BLASTX 2.2.9. Multiple sequence alignment of the results is shown in Figure 3.27.

```
A.fumigatus   ------------------------------------------------------------
A.nidulans    MPHWVSSDGSSTS----------HNDVEALPDAPPLTDVSNGAEKDVAAAG--------- 41
N.crassa      MPHSESPPESENSPIPMANVDQEQEDAVAESTTAQVPEAQQPADEDVDMADSNAVLSAPT 60
S.pompe       ------------------------------------------------------------
H.sapiens     ------------------------------------------------------------
S.cerevisiae  ------------------------------------------------------------

A.fumigatus   ------------------------------------------------------------
A.nidulans    ----------------------------------KTTAGVKIEDIFDDDEDEETEFPA 65
N.crassa      NGTTHISESKAESQAETPNNQDTPVPEAATGAPSESKPATGVKLEELFDEMDSDDDEFPT 120
S.pompe       ---------------------------------------MTVQIDELDDKDLDEIIANGT 21
H.sapiens     ---------------------------------------MTVQIDELDDKDLDEIIANGT 21
S.cerevisiae  ------------------------------------------------------------M 1

A.fumigatus   ------------------------------------------------------------
A.nidulans    SSAPAETRVGS-------AEASAPVPVQVDTETMLQFYQRLFPFRYLFQWLNHGIVPSPD 118
N.crassa      TKAAKREPASSPDLLSSQSDMEIDLHEASDPEVMRTFYQRLFPWRYLFQWLNHSPTPTND 180
S.pompe       LDGAKQG--------------------AVDSETMIQYYRHLFPWKYLFQWLNHGPVVTND 61
H.sapiens     LDGAKQG--------------------AVDSETMIQYYRHLFPWKYLFQWLNHGPVVTND 61
S.cerevisiae  TNSVKTN--------------------GPSSSDMEYYYKSLYPFKHIFNWLNHSPKPSRD 41

A.fumigatus   ------------------------------------------------------------
A.nidulans    FGNREFALTLQNDAYLRYQSYPTADLFRKDILKMNPSRFEIGPVYNRNPRDRKTL-GSGQ 177
N.crassa      FGHREFAFTLQNDAYLRYQSFTTSDLLRKDVLRLMPSRFEIGPVYTANPRDRKTLRNSSA 240
S.pompe       FAHREFAFTLPNDAYIRYLSFSNWEELKKEALNLCPSRFEVGPVYSANPRDRKTIRKST- 120
H.sapiens     FAHREFAFTLPNDAYIRYLSFSNWEELKKEALNLCPSRFEVGPVYSANPRDRKTIRKST- 120
S.cerevisiae  MINREFAMAFRSGAYKRYNSFNSVQDFKAQIEKANPDRFEIGAIYNKPPRERDTLLKSE- 100

A.fumigatus   ------------------------------------------------------------
A.nidulans    LKPLAKELVFDIDLTDYDDIRTCCTKANICAKCWAFVTMAIKVVDTALRQDFGFQHILWV 237
N.crassa      FRPLSKELCFDIDLTDYDDIRTCCDKANICQKCWQFITMAIKVVDTALREDFGFKHIMWV 300
S.pompe       FHPLKKELVFDIDMTDYDDVRTCCSKTNICEKCWPFITIAVQVLDICFHEDFGFKHILWV 180
H.sapiens     FHPLKKELVFDIDMTDYDDVRTCCSKTNICEKCWPFITIAVQVLDICFHEDFGFKHILWV 180
S.cerevisiae  LKALEKELVFDIDMDDYDAFRTCCSGAQVCSKCWKFISLAMKITNTALREDFGYKDFIWV 160

A.fumigatus   ------------------------------------------------------------
A.nidulans    YSGRRGAHAWVCDSRARNLSDDRRRGIAGYLDLVRGGTNSGKRVN--LKRPLHPHMTRSL 295
N.crassa      YSGRRGAHAWVCDKSARALDDQKRRAIAGYLEVIRGGAQSGKKVN--LRRPLHPHLVRSL 358
S.pompe       YSGRRGIHAWICDEIACSLDDRSRRMIASYLQVVVGNPQGGVRLINNLKRPLHPHLTRSL 240
H.sapiens     YSGRRGIHAWICDEIACSLDDRSRRMIASYLQVVVGNPQGGVRLINNLKRPLHPHLTRSL 240
S.cerevisiae  FSGRRGAHCWVSDKRARALTDVQRRNVLDYVNVIR-DRNTDKRLA--LKRPYHPHLARSL 217

A.fumigatus   ---------------------------------------------DSSPGRSSTNKWA 13
A.nidulans    EILKPYFVQTTLVDQDTFASPEQEQRLLSLLP---DKGLNDSLRRKWESAPDRSSTNKWA 352
N.crassa      DILKQHFQSDVLEAQDPWRTEDQQEKLLALLPSD-QKQLVSALRNKWASSPDRPSTLKWA 417
S.pompe       NILKSAFVKIVLEDQDPWASKEGAENLLKLLP---DKDLASALRKKWEVDPERSSKNKWS 297
H.sapiens     NILKSAFVKIVLEDQDPWASKEGAENLLKLLP---DKDLASALRKKWEVDPERSSKNKWS 297
S.cerevisiae  EQLKPFFVSIMLEEQNPWE--DDQHAIQTLLPALYDKQLIDSLKKYWLDNPRRSSKEKWN 275
                                                          * *.*. **

A.fumigatus   DIDALAKT---GKSSTLNPTTLRDAKQDIVLEYTYPRLDAEVSKKMIHLLKSPFVIHPGT 70
A.nidulans    DIDALAKA---GKSSTLNPATLREAKQDIVLEYTYPRLDSEVSKKMIHLLKSPFVIHPGT 409
N.crassa      DIDSVAKT---STAKNFDTKSLLDAKQDIVLEYTYPRLDIEVSKKLNHLLKSPFVVHPGT 474
S.pompe       DIDTVLAS---GSIASISPSVIAIAKQDIVLTYLYPRLDVEVSRHLNHLLKSPFCVHPGT 354
H.sapiens     DIDTVLAS---GSIASISPSVIAIAKQDIVLTYLYPRLDVEVSRHLNHLLKSPFCVHPGT 354
S.cerevisiae  DIDQIATSLFKGPKQDSHIIKLRECKEDLVLMTLYPKLDVEVTKQTIHLLKAPFCIHPAT 335
              *** :  :   .        :  .*:*:**   **:** **:::  ****:** :**.*
```

```
A.fumigatus   GRVCVPIDAKK-ADGFDPLSVPTVTQLLAEIDAWDAEH-SSGNAGAEAGQDESHTPSDSQ 128
A.nidulans    GRVCVPIDIRN-VEKFDPLSVPTVSQLLSEIDSWDSDHPSSGAAETAEGEGSAPNASDAG 468
N.crassa      GRVCVPIDVKRGLESFDPLGVPTVQSLIREIDEWKKPEPKEGRQEQE------------- 521
S.pompe       SRVCVPIDIER-MDSFNPLKVPTVNDLLQELD--KNSQNDNGHGPTMETNTTENQKDNAR 411
H.sapiens     SRVCVPIDIER-MDSFNPLKVPTVNDLLQELD--KNSQNDNGHGPTMETNTTENQKDNAR 411
S.cerevisiae  GNVCVPI-----DESFAPEKAPKLIDLQTEME-------------------------- 362
              ..*****.     : * *  .*.: .*  *::  .    ...       .      ..:

A.fumigatus   GSRKLQDYEKTSLKPYIDYFRSFIASLNKEER--VGKR-GNEDPDE-VKSESMEF 179
A.nidulans    GTRKLQDYEKTSLKPYIDYFRSFIAGLNKEER--NGKRERHEDSTPGVKSESMDF 521
N.crassa      VEKHVVDWEKTSLKGYIEFFRSFVIGLMKDEREVKVKREREEEGGGGGGGESMEF 576
S.pompe       GQSNKGHGFSTSLNPYTLYFKSFSSQLFKETVG--NKR----------KHENLEF 454
H.sapiens     GQSNKGHGFSTSLNPYTLYFKSFSSQLFKETVG--NKR----------KHENLEF 454
S.cerevisiae  ---KNNDVSLTALQPFINQFQAYVSSLLKNELG-SVKREREDDD----EPASLDF 409
                 :  .   *:*: :   *:::   * *:      **   ..        .::*
```

Figure 3.27 Multiple sequence alignment of amino acids from *A.fumigatus* and some important homolog species. *Aspergillus nidulans, Neurospora crassa, Schizosaccharomyces pombe, Homo sapiens*, and *Saccharomyces cerevisiae* amino acid sequences code for hypothetical protein, hypothetical protein, probable DNA primase small subunit, DNA primase small subunit, and p48 polypeptide of DNA primase, respectively.

As can be seen from Figure 3.27 and Appendix H, clone 23/24 has an approximately 45% homology with DNA primase small subunit. According to these results, clone 23/24 is likely to correspond to the DNA primase small subunit gene.

3.8 Primer Design

For the specific detection of *A. fumigatus*, 3 pairs of primers were designed using the sequences of the putative 60S ribosomal protein gene (clone 15), the gene of DNA primase small subunit gene (clone 23/24) and the antigenic cell wall galactomannoprotein gene (Yuen et al., 2001). The latter information was obtained from the literature and was included into the study due to the species-specific characteristics of cell wall protein.

3.8.1 Primer Design for 60S Ribosomal Protein Gene

The sequences of 60S ribosomal protein coding genes from organisms of *A. fumigatus, Neurospora crassa, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans* and *Encephalitozoon cuniculi* were compared using the multiple sequence alignment tool ClustalW 1.82 (http://www.ebi.ac.uk/clustalw/). The results of the multiple sequence alignment are shown in Figure 3.28. Regions for the design of primers were selected among non-conserved regions of sufficient length, proper GC content (~50%), melting temperature, hairpin loop structure, homo-dimer and hetero-dimer analysis. These criteria were checked with OligoAnalyzer 3.0 software (http://www.idtdna.com/program/gateway/gateway.asp).

```
C.albicans    TACCT---CAAGTTCCGAGACCAAACATGACCACCTGTATTTTTGGTGATGCTTTTGACT 220
S.cerevisiae  TGCCA---AACTGTCCAAGACCAAACATGTCCATCTGTATCTTCGGTGATGCTTTCGATG 220
S.pombe       TGCCC---AATGTTCCTCGTCCCAACATGGCCATTTGCATTCTCGGTGATGCCCATGATT 217
N.crassa      TGCCC---AGCATTCCCCGCCCCAACATGAGCATCTGCATTCTCGGTGACCAGCACGATA 219
A.fumigatus   ---CC---ACCGTTCCCCGCCCCAACATGACCATCTGTGTTCTTGGTGACCAGCACGATC 54
E.cuniculi    TGCCCTATAGGGTGCGGAGCCTCGACAAGACGATTGTGATCGCTGACGAGGCGCATGTCA 238
                 *          *   * *   *** *   *      *    *  **       *

C.albicans    TTGATAGAGCCAAGTCTTTGGGTGTTGATGC-TATGTCCGTTGATGACTTGAAAAAATTG 279
S.cerevisiae  TTGACAGAGCTAAGTCTTGCGGTGTTGACGC-TATGTCCGTCGATGACTTGAAGAAGTTG 279
S.pombe       TGGATCGTGCCAAGCACGGTGGTGTCGATGC-TATGTCCGTCGATGACTTGAAAAAGCTT 276
N.crassa      TCGACCGTGCCAAGCACGGCGGTGTTGACGC-CATGTCCGTCGACGATCTCAAGAAGCTC 278
A.fumigatus   TCGACCGTGCCAAGCACCACGGCATCGATGC-CATGTCCGCTGATGACCTGAAGAAGCTC 113
E.cuniculi    AAGTATGTATCGA----TGCGAATCTGCCGTACGTGCCTATCGACGA----AATAAGC-- 288
                *   *     *       *     *  *    ** *    ** **    ** **

C.albicans    AACAAAAACAAGAAATTGATTAAAAAATTGGCTAAGAAATACAACGCTTTCATTGCTTCT 339
S.cerevisiae  AACAAGAACAAGAAGTTAATCAAGAAGTTGTCTAAGAAGTACAACGCTTTCATTGCTTCC 339
S.pombe       AACAAGAACAAGAAGCTTGTCAAGAAGTTGGCCAAGAAGTATGATGCTTTCATTGCTTCT 336
N.crassa      AACAAGAACAAGAAGCTCATCAAGAAGCTTGCTCGCAAGTACGATGCCTTCGTCGCCTCC 338
A.fumigatus   AACAAGAACAAGAAGCTCATCAAGAAGCTGGCTCGCAAGTACGATGCTTTCCTTGCTTCC 173
E.cuniculi    GGGGACGATAAGAAGGATATAAGAGAGTCTGTTCTAAAGAAGAATAAGTTCTTTATCCTC 348
                  *  * *****     * *   *          **  *  *    *** *

C.albicans    GAAGTTTTGATCAAACAAATTCCAAGATTATTGGGTCGAACTTTATCTAAAGCTGGCCAG 399
S.cerevisiae  GAAGTTTTGATCAAGCAAGTTCCAAGACTATTGGGTCCTCAATTGTCCAAGGCTGGTAAG 399
S.pombe       GAAGTCCTCATCAAGCAAATTCCTCGTCTGTTGGGTCCCGGTCTTTCCAAGGCTGGTAAG 396
N.crassa      GAGGCCCTTATCAAGCAGATCCCCCGTCTGCTCGGTCCCGGTCTTTCCAAGGCTGGCAAG 398
A.fumigatus   GACACCCTCATCAAGCAGATTCCTCGTCTCCTGGGTCCCGGTCTGTCCAAGGCTGGTAAA 233
E.cuniculi    TGCCCTGGATATAACAAGATTTACCAGCTCAAGAAC-----ATTCTTAGATGCGGAAAGA 403
                        **  *  *        *             * *   * ** *

C.albicans    TTCCCAACTCCAGTTTCTCACAATGATGATTTATACAGTAAAGTTACTGATGTTAAATCC 459
S.cerevisiae  TTCCCAACCCCAGTTTCTCACAACGATGACTTGTACGGTAAGGTCACTGATGTCAGATCT 459
S.pombe       TTCCCTTCCCCTGTCTCCCATGCTGACGACCTTTACGGTAAAATCACCGAGGTTAAGTCC 456
N.crassa      TTCCCCACCCCCGTCTCCCACTCCGACGACCTTACCGGCAAGCTCAACGAGGTCAAGTCT 458
A.fumigatus   TTCCCTACCCCCGTCTCTCACAGCGAGGACATGGCCAACAAGGTCACCGAGATCAAGTCT 293
E.cuniculi    CACCCCATATTC-TTAGGAACGGCGACGACATCAACGCTGTTTTCGAGACGGGAAAGAAA 462
                ***        *     *    ** **  *   *       *          *
```

```
C.albicans    ACTATCAAATTCCAATTGAAAAAAGTCTTGTGTTTGGCCG---TTGCTGTTGGTAACGTT 516
S.cerevisiae  ACCATCAAGTTCCAATTGAAGAAGGTCTTGTGTTTGGCTG---TTGCCGTTGGTAACGTT 516
S.pombe       ACTATCAAGTTCCAATTGAAGAAGGTCCTTTGCCTTGGTG---TTGCTGTCGGCCATGTC 513
N.crassa      ACCATCAAGTTCCAGCTCAAGAAGGTTCTCTGCATGGGTG---TCGCCGTCGGCAACGTT 515
A.fumigatus   ACCATCAAGTTCCAGCTCAAGAAGGTTCTCTGCCTCGGTG---TTGCCGTTGGCAACGTC 350
E.cuniculi    TCCTGCAAGCTCCGCATCCAGGACGACTTTTCCGTTACTTCGTTTACTGTAGGCCACACC 522
               *   ***  ***   *  *  * *   * *   *        *  * ** **  *

C.albicans    GATATGGAAGAAGATGTCTTGGTTAACCAAATCATGATGGCTGCTAACTTCTTGGTTTCT 576
S.cerevisiae  GAAATGGAAGAAGACGTTTTGGTTAACCAAATCTTGATGTCTGTTAACTTCTTTGTTTCT 576
S.pombe       GAGATGTCTGAGGAGCAATTGATTGCTAACATCATGCTTGCCGTCAACTTCTTGGTTTCT 573
N.crassa      GGCATGACCCAGGAGCAGCTTGTTGGTAACATCATGTTGGCCATCAACTACCTCGTCTCC 575
A.fumigatus   GGCATGACCAAGGAAGAGCTCGTCGCCAACGTCATGCTGGCCATCAACTACCTCGTCTCC 410
E.cuniculi    GGGATGGATTCCGAGCACATATACGAGAACATCAAGGTGGGGATGGGACTGCTTGTCTCC 582
              *  ***      **     *        *  **  * *              * ** **

C.albicans    TTGTTGAAAAAGAACTGGCAAAATGTTGGTTCCTTGGTTATTAAATCTACCATGGGTCCA 636
S.cerevisiae  TTGTTGAAGAAGAACTGGCAAAATGTTGGTTCCTTGGTTGTTAAGTCCTCCATGGGTCCA 636
S.pombe       TTGTTGAAGAAGGGCTGGCAAAACATTGGCTCCTTGGTCGTCAAATCTACCATGGGTAAG 633
N.crassa      CTCCTCAAGAAGGGCTGGCAGAACGTTGGTAGCCTTACCATCAAGGCTACCATGTCTCCC 635
A.fumigatus   CTGCTGAAGAAGGGTTGGCAGAACGTTGGCAGCCTTGTCCTCAAGGCTACCATGTCTCCC 470
E.cuniculi    TATCTAAAGAATGGGTCCCAGAACCTGAAAGGTGTGATGATCAAGACTGACCAGTCGCCT 642
                  * ** **    *  ** **  *        *     * **  *   *  *

C.albicans    TCATTTAGAATCTACTAA------------------------------------------ 654
S.cerevisiae  GCTTTCAGATTGTACTAA------------------------------------------ 654
S.pombe       CCTCACAGACTTTACTAA------------------------------------------ 651
N.crassa      CCCAAGCGCCTCTACTAA------------------------------------------ 653
A.fumigatus   CCCAAGCGTCTCTACTAGATTCTCTGCGCAATTAGCTTTGTGACAGGGGTTTTTAGGCGC 530
E.cuniculi    CCGGTGACTCTCTATTAA------------------------------------------ 660
               *        * ** **

C.albicans    ------------------------------------------------------------
S.cerevisiae  ------------------------------------------------------------
S.pombe       ------------------------------------------------------------
N.crassa      ------------------------------------------------------------
A.fumigatus   TCAGATGTGGGATTGTATATGCCATAGATAGGAAATGTGATGTCCACGACTGGTACCTAA 590
E.cuniculi    ------------------------------------------------------------


C.albicans    -
S.cerevisiae  -
S.pombe       -
N.crassa      -
A.fumigatus   A 591
E.cuniculi    -
```

Figure 3.28 CLUSTAL W (1.82) multiple sequence alignment results; (*) indicates the conserved bases and yellow highlighted red sequences denote primer sequences

First ~220 bases were excluded from the sequence shown in Figure 3.28. Because they did not show any homology with the 60S ribosomal protein L1-b of *A. fumigatus*.

Forward Primer Ribopro1:

Sequence: 5'- AGC GAG GAC ATG GCC AAC -3'

Length: 18 bp

GC content: 61.1 %

Melting Temperature: 58.6 °C

Reverse Primer Ribopro2:

Sequence: 5'- GCG ACG AGC TCT TCC TTG -3'

Length: 18 bp

GC content: 61.1 %

Melting Temperature: 56.2 °C

3.8.2 Primer Design for DNA Primase Gene

The sequences of DNA primase small subunit coding genes from organisms of *A. fumigatus, Aspergillus nidulans, Trichoderma reesei, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were compared using the multiple sequence alignment tool ClustalW 1.82(http://www.ebi.ac.uk/clustalw/). The results of the multiple sequence alignment were shown in Figure 3.29. Primers were designed as described in section 3.8.1.

```
A.fumigatus   --------GGGATTCGTCGCCTGGCCGGTCCAGTACGAACAAATGGGCGGACATCGACGC 52
A.nidulans    CCGGAAATGGGAATCAGCCCCCGACCGCTCTAGCACAAACAAGTGGGCTGACATTGATGC 1067
S.pombe       TAAAAAATGGGAGGTTGATCCTGAACGATCTAGCAAAAATAAATGGTCCGACATTGACAC 902
S.cerevisiae  GAAGTATTGGCTGGACAATCCAAGGAGGTCAAGCAAAGAGAAGTGGAATGATATAGATCA 836
T.reesei      TAAAAGATAAGTAG--GTTTTTGGGCCCTTTTTTTTGGG-GGGCGCGCGGGGATGCCTCT 662
                                       *               *    *  **

A.fumigatus   GCTTGCAAAGACTGGC---AAGAGCAGCA------CTCTTAATCCTACTACTCTGAGAGA 103
A.nidulans    TCTTGCAAAGGCCGGT---AAAAGCTCTA------CTCTTAACCCCGCTACCCTACGCGA 1118
S.pombe       TGTCTTAGCATCTGGTTCTATTGCCTCTA--TATCTCCTTCA-GTAATT-------GCGA 952
S.cerevisiae  GATAGCTACATCGCTCTTCAAAGGCCCCAAGCAAGACTCTCACATAATTAAGTTACGTGA 896
T.reesei      TTTTATCATAAAAAGTG--GATTACTCTG----TACTACAAAAACTGCGTTTTTTTTCAA 716
                *                     *               *                *
```

```
A.fumigatus   -CGCGAAACAGGATATCGTACTCGAATATACATACCCGCGACTCGACGCTGAAGTCAGCA 162
A.nidulans    -GGCCAAACAAGATATCGTTCTCGAGTACACATACCCACGTCTCGATTCCGAGGTCAGCA 1177
S.pombe       TTGCCAAGCAGGACATTGTTTTAACCTATTTGTATCCAAGATTGGATGTTGAGGTTTCTA 1012
S.cerevisiae  ATGT-AAGGAAGATCTCGTATTGATGACTCTTTATCCGAAGCTGGATGTGGAAGTTACAA 955
T.reesei      TAGGTAGGGGTGAAATCCCCCTGGTGGTGACACA---AAGGT---ATTCCGTGGTTTTTG 770
                *  *     **  *     *           *          *    *  **

A.fumigatus   AGAAAATGATCCATTTACTCAAGAGCCCTTTTGTCATCCATCCCGGTACTGGGCGTGTTT 222
A.nidulans    AGAAGATGATCCACTTGCTCAAGAGCCCATTCGTCATTCACCCGGGCACCGGGCGAGTTT 1237
S.pombe       GACATTTAAACCATTTGCTAAAATCTCCATTTTGCGTCCACCCCGGAACTAGTCGTGTTT 1072
S.cerevisiae  AGCAAACAATTCATTTGTTAAAGGCCCCTTTTTGTATTCATCCTGCTACGGGGAATGTCT 1015
T.reesei      ----------CCGTTTTTATGAGGGG-----TGAGAGCTATTGTGGGGTAGGGTGTGTCC 815
                       *  **     *                 *    *      *    **

A.fumigatus   GTGTTCCCATTGACGCCAAAAAAAGCGGACGGATTCGATCCTCTTTCCGTCCCAACAGTCA 282
A.nidulans    GTGTTCCTATTGACATTCGTAATGTCGAGAAGTTCGATCCTCTCTCCGTACCTACTGTCT 1297
S.pombe       GTGTTCCCATAGATATAGAGAGGATGGATTCTTTTAATCCTTTGAAAGTACCCACTGTGA 1132
S.cerevisiae  GTGTGCCTATTGAT-----------GAATCCTTTGCACCTGAAAAAGCACCTAAGCTAA 1063
T.reesei      -TATTTTAATATATGGC------------------------------------------ 831
               * *    **  *

A.fumigatus   CTCAATTACTGGCGGAGATAGATGCCTGGGATGCTGAGCACTCTAGCGGTAATGCAGGCG 342
A.nidulans    CTCAATTGCTCTCAGAGATAGACTCCTGGGACTCAGACCATCCTAGTAGTGGCGC---CG 1354
S.pombe       ATGATCTTTTGCAAGAGTTGGATAAAAATTCCCAAAATGATAACGGCCATGGTCCAACAA 1192
S.cerevisiae  TTGATCTTCAAACAGAAATGGAGAAAAAT------AATGATGTT--TCATTAAC-AGCTT 1114
T.reesei      ------------------------------------------------------------

A.fumigatus   CCGAAGCTGGTCAAGATGAAAGCCACACACCTAGCGACTCCCA------GGGAAGTCGCA 396
A.nidulans    CGGAGACTGCAGAAGGCGAAGGGAGCGCTCCTAACGCCTCTGACGCTGGAGGCACCCGTA 1414
S.pombe       TGGAAACTAATACAACAGAAAACCAAAAAGATAATGCTAGGGG--------ACAATCAAA 1244
S.cerevisiae  TACAACCTTTTATCAATCAGTTCCAAGCAT---ATGTGAGTTC--------TCTTTTGAA 1163
T.reesei      ------------------------------------------------------------

A.fumigatus   AATTGCAAGATTACGAGAAGACCAGCCTTAAGCCATATATTGACTACTTCCGTTCGTTCA 456
A.nidulans    AATTACAGGACTATGAGAAGACAAGCCTGAAACCGTACATTGACTACTTCCGTTCGTTCA 1474
S.pombe       CAAGG-GGCATGGCTTTTCAACTTCTCTCAATCCTTATACGCTGTACTTTAAATCGTTCA 1303
S.cerevisiae  AAATG-AACTGGG---TTCAG-------------TGAAAAGAGAACGTGAAGATGATGA 1205
T.reesei      ------------------------------------------------------------

A.fumigatus   TCGCTTCTCTAAACAAAGAGGAACGCGTCGGCAAACGAGGAAAT---GAAGATCCGGAT- 512
A.nidulans    TTGCGGGCCTTAACAAGGAGGAGCGCAATGGGAAGCGAGAGCGTCACGAGGATAGTACTC 1534
S.pombe       GTAGCCAGCTTTTTAAAGAAACAGTAGGAAATAAAAGAAAACAT---GAGAATTTGGAAT 1360
S.cerevisiae  TGAACCGGCTTCTTTAGATTTCTGA---------------------------------- 1230
T.reesei      ------------------------------------------------------------

A.fumigatus   --GAGGTAAAATCGGAAAGCATGGAATTTTAAGGGCATTCACTGTTTGCATTCGTTCCGA 570
A.nidulans    CAGGAGTTAAATCTGAGAGTATGGACTTTTGA---------------------------- 1566
S.pombe       TTTAA------------------------------------------------------- 1365
S.cerevisiae  ------------------------------------------------------------
T.reesei      ------------------------------------------------------------

A.fumigatus   TATATGGCTGGGTTCTTGATATTGGGATTTGCAGGTTTCTATTCTTAGCGGAAGTATTGG 630
A.nidulans    ------------------------------------------------------------
S.pombe       ------------------------------------------------------------
S.cerevisiae  ------------------------------------------------------------
T.reesei      ------------------------------------------------------------

A.fumigatus   TTTATGCTGGCGAAGTAAAGGCGTTTATAGGTGTTGGTTAGGCTGTATATAAGCCACGCA 690
A.nidulans    ------------------------------------------------------------
S.pombe       ------------------------------------------------------------
S.cerevisiae  ------------------------------------------------------------
T.reesei      ------------------------------------------------------------
```

```
A.fumigatus   ATTGAATCAACGACCCGATGCCAAAATTCGTA 722
A.nidulans    --------------------------------
S.pombe       --------------------------------
S.cerevisiae  --------------------------------
T.reesei      --------------------------------
```

Figure 3.29 CLUSTAL W (1.82) multiple sequence alignment results; (*) indicates the conserved bases and yellow highlighted red sequences denote primer sequences.

First non-homologous bases were excluded from the sequence shown in Figure 3.29.

Forward Primer Prms1:

Sequence: 5'- GCC AAA AAA GCG GAC GG -3'
Length: 17 bp
GC content: 58.8 %
Melting Temperature: 55.5 °C

Reverse Primer Prms2:

Sequence: 5'- CTC ATC CGG ATC TTC ATT TC -3'
Length: 20 bp
GC content: 45.0 %
Melting Temperature: 51.4 °C

3.8.3 Primer Design for Antigenic Cell Wall Galactomannoprotein Gene

Yuen et al. (2001) cloned the *AFMP1* gene, which encodes the first antigenic cell wall galactomannoprotein in *A. fumigatus. AFMP1* codes for a protein, Afmp1p, of 284 amino acid residues, with a few sequence features that are present in Mp1p, the antigenic cell wall mannoprotein in *Penicillium marneffei*. Regions for the design of primers were selected among non-conserved regions of sufficient length, proper

GC content (~50%), melting temperature, hairpin loop structure, homo-dimer and hetero-dimer analysis. These criteria were checked with OligoAnalyzer 3.0 software. (http://www.idtdna.com/program/gateway/gateway.asp)

For this purpose the sequences of Afmp1p protein coding genes from organisms of *A. fumigatus, Aspergillus nidulans, Aspergillus flavus* and *Penicillium marneffei* were compared using the multiple sequence alignment tool ClustalW 1.82 (http://www.ebi.ac.uk/clustalw/). The results of the multiple sequence alignment were shown in Figure 3.30.

```
A.nidulans    ---------------------------------------ATGAAGTTCACCGGCTTGTTC 21
A.flavus      ---------------------------------------ATGAGATTCTCCGCTATCTTC 21
A.fumigatus   ---------------------------------------ATGCGTTTCTCTGCCCTCCTC 21
P.marneffei   TCCAAACAGCTCTCCGATCAGGTCGCCCAAGCCCTCCAGAAAGGTATCCAAGCCTTCTCC 540
                                                     *      **   *   *   *

A.nidulans    ACCCTGGCTCTGGCCACCACCGCCCTG------GCCACCCCG---GCCAAGCG--CCAGT 70
A.flavus      ACCCTGGGTCTCGCCGGCACCGCCCTG------GCCACCCCCCTCGTTGAGCGTGCTGGT 75
A.fumigatus   GTCACTCTCGGCCTCACCGGTGCCCTG------GCCACGCCCACCCTGGTCTCTCGTGAG 75
P.marneffei   ATTAGCGCTCGCCAGGCCACCAAGGTAAAGCGTGAGGCCACCAAGGTCCAGCGTGATATT 600
                          *       *       *   *  *

A.nidulans    -CCTCTCCCGCTGATATCATCGACACCATCTCCTCCAAGGTCGATGCCCTCGGCTCTGCC 129
A.flavus      TCCTCCCCCACCGACATCATCTCCGGCATCAGCGACAAGACCGATGCTCTCGACTCCGCC 135
A.fumigatus   GCCCCTGCCGTCGGTGTCATCTCCGACATCTCGGCCCAGACCTCTGCTCTGGCCTCCGCC 135
P.marneffei   TCTGCTTTCAAGAAGGTCATCCAGAATATTAGCTTGGCTGTGAACAAGTTTAATGTTGAT 660
               *  *   *       *****      **                    *       *

A.nidulans    GTCAGCTCCTACAGCGGCGGCGACCCCTCTGATGTCCAGTCCGCCTCGGACAACCTCGTC 189
A.flavus      ATCAAGGCTTACAACGGTGGTGACCCCTCCAAGGTTGAGTCCGCCTCCGCTGACTTGATC 195
A.fumigatus   GTCTCCTCCTACAACGGTGGTGACCCCTCCGCCGTCAAGTCCGCCTCTGAGAAGCTTGTC 195
P.marneffei   ATTGAGCGTTACGTGGGCGGTGATGCTTCTCATCTTCTCGCTGACGGTAATGTACTTATC 720
               *       ***   ** ** **  * **     *     * * *          *  **

A.nidulans    AGCACCATCCGATCTGCCGTCGAGGAGGTCAACGCCGGCCCCGATCTCTCCAACTCGGAC 249
A.flavus      TCGACCATCACCAAGGGCACTGATGCCATCAAGAGCGGTGATGATATCAGCACCACCGAT 255
A.fumigatus   AGCACCATCAACTCCGGTGTCGACACCGTCAAGAGCGGCCCTGCCCTCAGCACCGCTGAT 255
P.marneffei   AAAGCTACTCTGGACGGCGTTCAGTCCCTCCAAAATGAGCCTCCGCTTAGCTCCATGGAA 780
                  * *        *      *     ** *    *         *   *  *   **

A.nidulans    GCCCTGGCTCTCACCAGCCCCATCCAGGACCTGACCGATGACGTCGAGGGCGTCATCGAC 309
A.flavus      GCTCTTGCTCTGCCTGAGCCCGTCCAGGCTTTGACCAAGAAGGTCGAGCAGGCTATCGAT 315
A.fumigatus   GCTCTGGCTCTGACCTCTCCCGTCCAGGACCTGACCAAGCAGGTCGAGGGCGTCATCGAC 315
P.marneffei   GCCCTTGCCCTTGTTGGCCCTGTTCAGGATTTAAGCAATCAAATCCTACTAGCTATTCAG 840
              ** ** ** **       **  * ****   * * * *  *  **      *  **  *

A.nidulans    CAGCTCATCTCCAAGAAGGACCAGTTCGTTGAGGCTGGTGCTGGCGGCGATGTCAAGGCC 369
A.flavus      GACATTATCGCCAAGAAGGACAAGTTCGTCGAGGCTGGCGCTGGCGGCAAGGTCAAGGAC 375
A.fumigatus   GACCTCATCTCCAAGAAGGACAAGTTCGTCGCCGCCAACGCCGGTGGCACTGTCTACGAG 375
P.marneffei   AATCTTATTGATAAGAAGGAACCTCTTGTTCAGGCTGGTTTTGGTGGTAAAGTCGAGAAC 900
               *  * **    ********     * **    **       ** **    *** *

A.nidulans    GCCCTCAGCGAGCAGTACGACGCCGCCTCCAGCCTCGCTGAGGCCCTGAGCGCCAAGGTC 429
A.flavus      TCCTTGAACCAGCAGAAGTCCGCTGCCGATGGTCTCGCCTCTGCCATCACCTCCAAGGTC 435
A.fumigatus   GACCTCAAGGCCCAGTACACCGCTGCCGACAGCCTGGCCAAGGCCATCTCCGCCAAGGTC 435
P.marneffei   AATCTTAGGCAACAGGAGGAGGCTGCCCAAAAACTCAGCGAATTGGTCTCCACAAAGGTC 960
                  * *     *** *    ** ***      **          *   * * ******
```

```
A.nidulans   CCCAGCGCTCTCGAGGACATCGCCGCCGAGCTCTCTGCCGGAATCACCAACGCTATCCAG 489
A.flavus     CCTGAGTCTCTCAAGGAGATTGCCCAGAGCCTCTCCGCTGGTATCAGCACCGCTATCCAG 495
A.fumigatus  CCTGAGTCCCTCTCCGACATCGCCGCTCAGCTGTCCGCTGGCATTACCGCCGCCATTCAG 495
P.marneffei  CCCCACGAACTCGCCGACATTTCCCGACAGCTCTCCGATGGTATCGCTGCTGGCATCAAG 1020
             **       ***   ** **  **      ** ** *  ** **       *  **  **

A.nidulans   AAGGGTGTTGACGCCTACGCTGACGTTGAGGACGGTGGCAGC------TCCAGCTCTACC 543
A.flavus     AAGGGTGTCGATGCGTACAAGGACGTTTCCGACTCCGCCCCC------TCTTCCAGCGCT 549
A.fumigatus  AAGGGTATCGACGCCTACAAGGACGCCGCCAGCTCTACCGGCACTGCTTCCTCTTCTGCC 555
P.marneffei  AAAGGTATTGATGCCTTCGCCGGCACTGGCCCCGCCCCCACTACCAGTAGTACCCCCGAA 1080
             ** *** * ** ** * *   * *        *     *

A.nidulans   TCTGCTACCGGTACTGC-----------------TACTGCCACCTCCACCGGCTCCGAG 585
A.flavus     GGCTCCTCCGCGAGCGC-----------------CACTGCCACCGGCAGCGCTTCTGAG 591
A.fumigatus  CCTGCCACTGAGACCGCGACCGCCACCGAGACCTCCACTGCCACTGGCACCGTCACCGAG 615
P.marneffei  GCCTCTACTGCTCCTGCTCCCTCCACTCCTCCTCAGACGCCTGAAGACACTCTTGTTCCT 1140
                 *  * *     **                    **  *      **

A.nidulans   ACCTCCAGCGCCACCGATGC---TCCTACCTCCACCTCCTC------CACCCCCGTCATC 636
A.flavus     ACCGGCAGCGCCTCTACTACCGGTTCTGCCTCCGCCACCTC------CAGCTCCGTGATC 645
A.fumigatus  ACGGCCACTTCCACCCCTGTCATCCCCACCGGTACCGCGTCTGGCAGCGCCTCCGCTACC 675
P.marneffei  GCCACATCTACTCCTGCTCCTGGTCCCGCTCCCACTGCTCCTGA---TTCTTCCATGGTC 1197
              *       *  *   *       *  *     *  *  *           **     *

A.nidulans   C------CCACTACCACCGGCACTG-----------------------AGTCCAGCACC 666
A.flavus     C------CCACCTCCTCCGGTGCTGCCAGCTCCTCTGCTGCCCCCTCCGGCTCCAGCACC 699
A.fumigatus  C------CCTCCACCACCGCTACCCCCACCACGGGCGGTCTCGGCTCCGGCTCCGGCTCC 729
P.marneffei  TGGCCTACCTCTACCACTGCCTCTCCCGATGTGCAGCCTACCATCACCAGCTCTGGCACT 1257
                    ** *  ** * *   *                                **  ** *

A.nidulans   CCCACCAGCTCT---CCCACCGGCA---------CCCCTGCTCCCCCCGAGTTCACTGGT 714
A.flavus     CCCACTGGCTCCGGCTCTGCCTCCG---------CCACCTCTCCTCCCTTGGCCACCGGT 750
A.fumigatus  AGCACTGGTACTGCCACTGCCTCCA---------CCAGCACCAACCTCCTCTCCACTGGC 780
P.marneffei  TCGGTTCCTGCCGCGCCAACTGGCGGTAATTCTTCGCCCGCCGTCCCTGCTTTCACTGGT 1317
                      *     *  *   *          *     *    *       *** **

A.nidulans   GCTGCTTCCAAGGAGCGCTTCTCCCTCG---TCGGCGGTGCTCTTGCTGCCGTCGCCGTC 771
A.flavus     GCTGCCAACAAGGCCACCATCGGCTACT---CCCTTGGTGCCGTCGCCATGGCCGCCATT 807
A.fumigatus  GCCGCCAGCAAGGAGCACTTCAGCTACTCCCTCGGCGGTGCCGTCGTCGCGGCCGCCATC 840
P.marneffei  GCTGCCAGCGCTAACCAGGTCAGCGGCG---CGGTTGGTCTTGCTGCCGGTCTCCTTGCT 1374
             ** **   *          **  *  *         ***      *       *

A.nidulans   GCCGTTGCCATCTAA 786
A.flavus     GCCGTCGCTGTCTAA 822
A.fumigatus  GCCGTCGCTCTCTAA 855
P.marneffei  GTCCTTGCCTTTTAA 1389
             * * * **  * ***
```

Figure 3.30 CLUSTAL W (1.82) multiple sequence alignment results; (*) indicates the conserved bases and yellow highlighted red sequences denote primer sequences

First ~500 bases of *P. marneffei* were excluded from the sequence shown in Figure 3.30. Because it did not show any homology with *Aspergillus* species' antigenic cell wall galactomannoprotein gene and may codes for a different domain.

Forward Primer Afmp1:

Sequence: 5'- CCC TGG TCT CTC GTG AGG -3'
Length: 18 bp
GC content: 66.7 %
Melting Temperature: 56.9 °C

Reverse Primer Afmp2:

Sequence: 5'- GAG GTC TCG GTG GCG GTC -3'
Length: 18 bp
GC content: 72.2 %
Melting Temperature: 60.5 °C

3.9 Isolation of Genomic DNAs from Closely Related *Aspergillus* Species

The genomic DNAs of *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus oryzae* were isolated according to the procedure given in section 2.2.14. The isolated DNAs are shown in Figure 3.31.

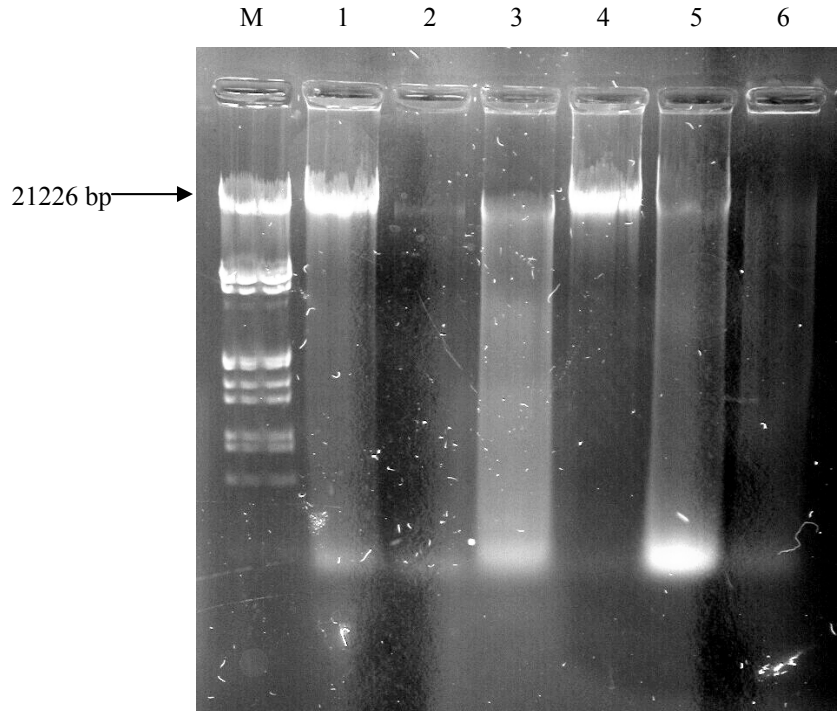


Figure 3.31 M; λ DNA/*Eco*RI+*Hin*dIII Marker; Genomic DNAs of **1**, *A. fumigatus*; **2**, *A. nidulans*; **3**, *A. flavus*; **4**, *A. niger*; **5**, *A. parasiticus*; **6**, *A. oryzae*

3.10 PCR with *A. fumigatus* and Detection Primers

Amplification studies were performed with the primers Afmp1-2, Prms1-2 and Ribporo1-2 against *A. fumigatus* genomic DNA using the PCR conditions, outlined in Section 2.2.3, with an annealing temperature of 60°C (Figure 3.32).

Single bands with expected lengths were obtained with PCR. In the negative control, a band of smaller size is observed. This band is likely to be produced by primer-dimer formation.

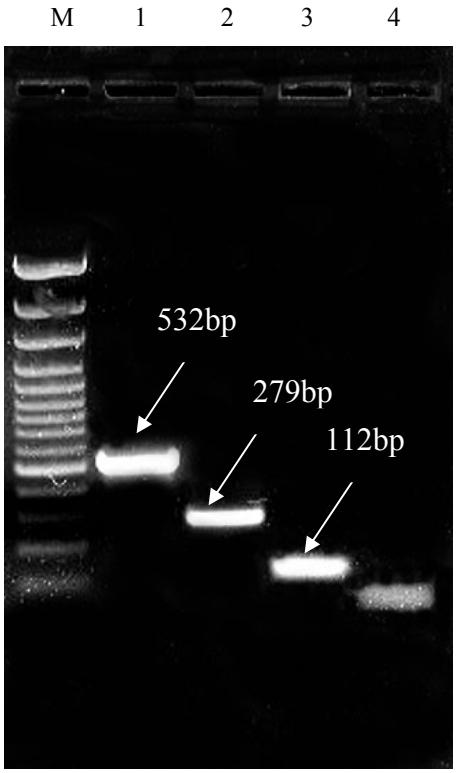


Figure 3.32 Amplification of *A. fumigatus* genomic DNA with detection primers. **M**, GeneRuler™ 100 bp DNA Ladder Plus; **1**, Amplification with primers Afmp1-2; **2**, Amplification with primers Prms1-2; **3**, Amplification with primers Ribopro 1-2; **4**, negative control

3.11 RAPD-PCR with Closely Related *Aspergillus* Species

If the detection method developed in this study is indeed specific to *A. fumigatus*; *A. nidulans, A. flavus, A. niger, A. parasiticus* and *A. oryzae* are expected to give negative results by PCR. To ensure that the isolated genomic DNAs yield products by PCR, RAPD-PCR was applied using short random primers. These 9 base long primers (Appendix C) were efficiently used in previous studies, to amplify fungal DNA fragments (Ören and Ögel, 1997).

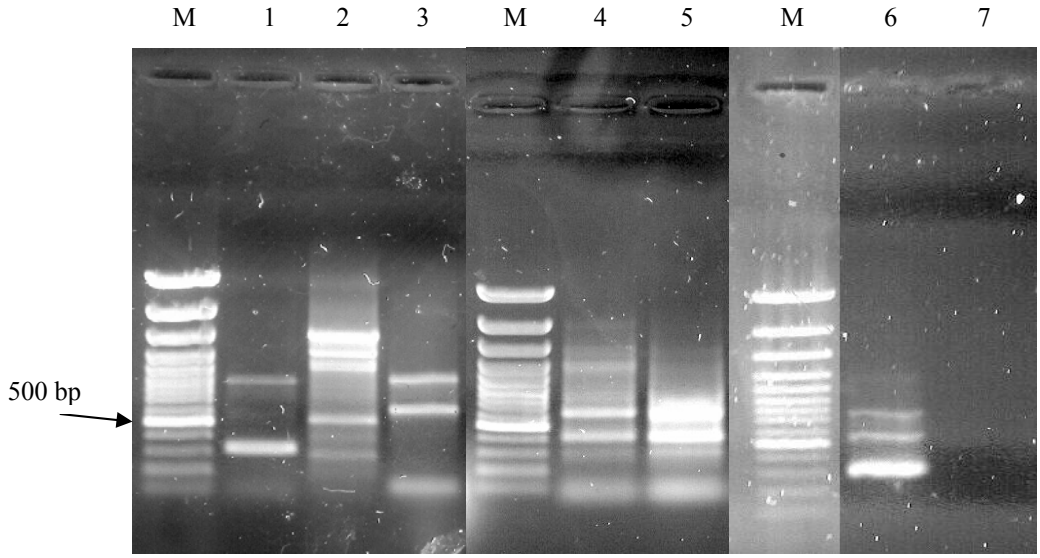


Figure 3.33 Amplification of closely related *Aspergillus* species' genomic DNAs with RAPD primer. **M**, GeneRuler™ 100 bp DNA Ladder Plus; **1**, *A. nidulans*; **2**, *A. flavus*; **3**, *A. niger*; **4**, *A. parasiticus*; **5**, *A. oryzae*; **6**, positive control; **7**, negative control

As shown in Figure 3.33, all the genomic DNAs yielded products with the RAPD primer indicating that all the genomic DNAs are pure enough for being used against the detection primers.

3.12 PCR with Closely Related *Aspergillus* Species and Detection Primers

3.12.1 PCR with Closely Related *Aspergillus* Species and Afmp1-2 Primers

A. fumigatus, A. nidulans, A. flavus, A. niger, A. parasiticus and *A. oryzae* genomic DNAs were amplified with Afmp1-2 primers using PCR conditions outlined in Section 2.2.3 with an annealing temperature of 60°C (Figure 3.34).

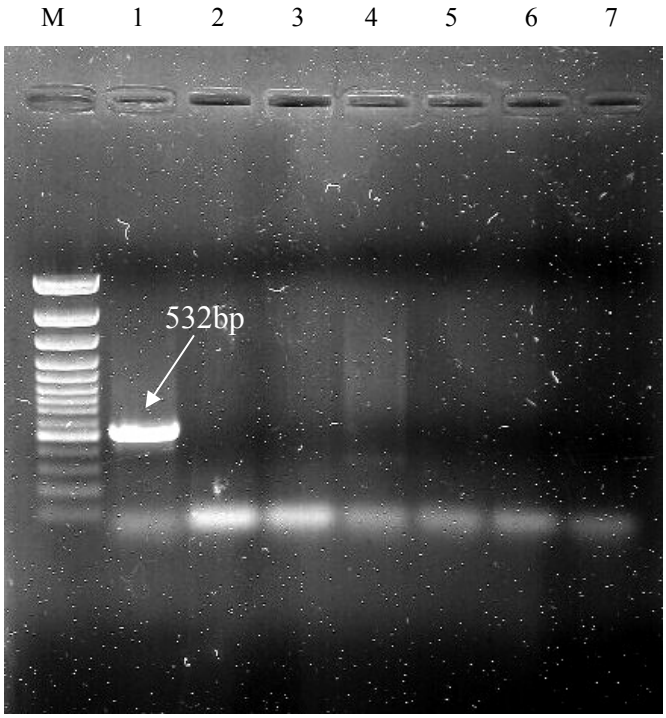


Figure 3.34 Amplification of closely related *Aspergillus* species with primers Afmp1-Afmp2. **M**, GeneRuler™ 100 bp DNA Ladder Plus; **1**, *A. fumigatus*; **2**, *A. nidulans*; **3**, *A. flavus*; **4**, *A. niger*; **5**, *A. parasiticus*; **6**, *A. oryzae*; **7**, negative control

Amplifications showed that designed primers are unique for *A. fumigatus* genomic DNA. *A. niger* gave a 300 bp band with primers Afmp1-2 but it was not bright enough and its size was less than the expected size. So, this band is likely to be unspecific and may be eliminated by methods like "hotstart" PCR.

3.12.2 PCR with Closely Related *Aspergillus* Species and Prms1-2 Primers

The same conditions described in section 3.12.1 were applied for Prms1-2 primers. Amplifications showed that the designed primers yield a specific and unique product only from *A. fumigatus* genomic DNA (Figure 3.35). *A. niger* gave a 700 bp band with primers Prms1-2 but it was not bright enough and its size was more than

the expected size. So, this band is likely to be unspecific and may be eliminated by methods like “hotstart” PCR.

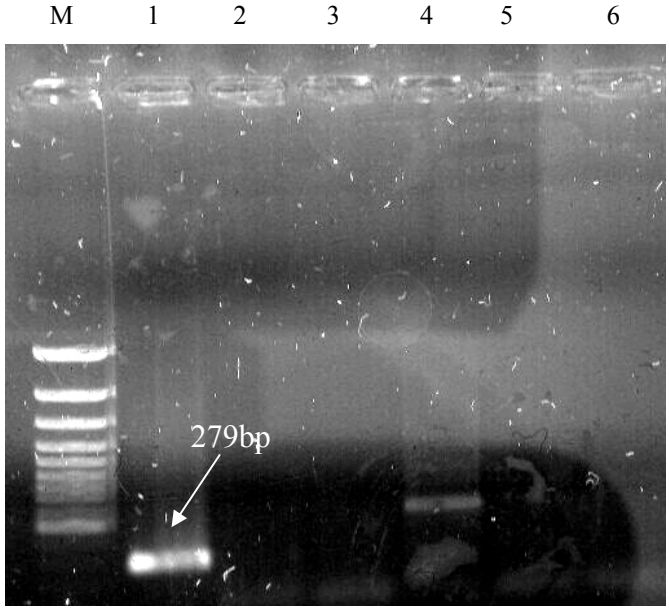


Figure 3.35 Amplification of closely related *Aspergillus* species with primers Prms1-Prms2. **M**, GeneRuler™ 100 bp DNA Ladder Plus; **1**, *A. fumigatus*; **2**, *A. nidulans*; **3**, *A. flavus*; **4**, *A. niger*; **5**, *A. parasiticus*; **6**, *A. oryzae*

3.12.3 PCR with Closely Related *Aspergillus* Species and Ribopro 1-2 Primers

The same conditions described in section 3.12.1 were applied for Ribopro 1-2 primers. Amplifications showed a specific product from *A. fumigatus,* of expected size, however, this fragment was very small and was indistinguishable from primer dimers (Figure 3.36). If these primers will be used for detection, it is essential that the primer dimers are eliminated by hotstart PCR.

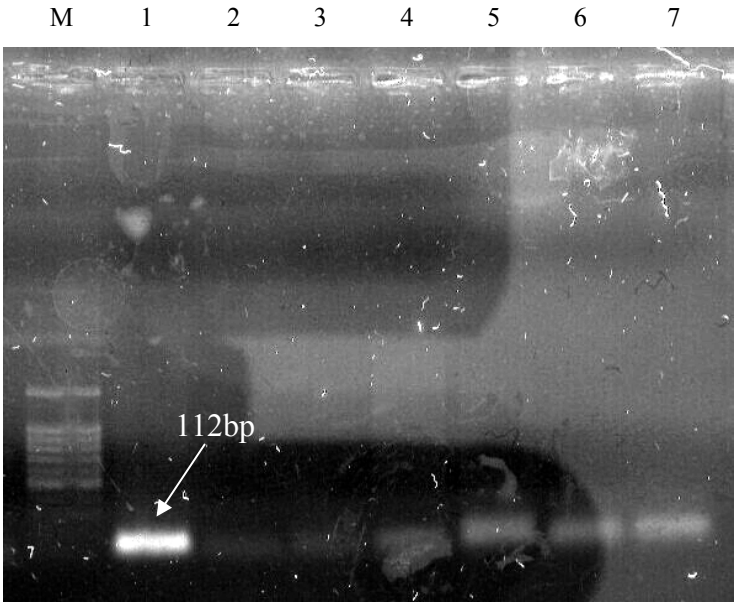


Figure 3.36 Amplification of closely related *Aspergillus* species with primers Ribopro1-Ribopro2. **M**, GeneRuler™ 100 bp DNA Ladder Plus; **1**, *A. fumigatus*; **2**, *A. nidulans*; **3**, *A. flavus*; **4**, *A. niger*; **5**, *A. parasiticus*; **6**, *A. oryzae*; **7**, negative control

3.13 Design of Nonconserved Sequence Region Finding Program

During the design of primers, there were a number of difficulties. ClustalW software can be used for finding homologous sequences by multiple sequence alignment. However, for designing species-specific primers for detection purposes, nonconserved sequences were needed and these had to be determined manually.

For the purpose of finding nonconserved sequences by computer, a C++ program was written in this study. The program used ClustalW output file which was saved a as text file (.txt) and the program converted it into an output text file. Output text file contains multiple sequence alignment with stars under the non-conserved sequences. The program written is shown in Figure 3.37.

```
#define MAX_LENGTH 1000
#include <stdio.h>
main()
{
FILE *input,*output;
char s1[MAX_LENGTH];
char s2[MAX_LENGTH];
int j,i,l1=0,l2=0,numberReadFlag=0;
char name1[20];
char name2[20];
char temp[200];
char temp1[100];
char temp2[100];
/*ignore first three lines*/
input=fopen("alper.txt","r");
output=fopen("out.txt","w");
fgets(temp,99,input);
fgets(temp,99,input);
fgets(temp,99,input);
while(1)
      {
      if (!fscanf(input,"%s",name1))
            break;
      fscanf(input,"%s",temp1);
      i=0;
      numberReadFlag=0;
      while((temp1[i])  )
            {
            if (temp1[i]!='-')
                  numberReadFlag=1;
            s1[l1++]=temp1[i++];
            }
      if (numberReadFlag)
            fscanf(input,"%d",temp);


      fscanf(input,"%s",name2);
      fscanf(input,"%s",temp2);
      i=0;
      numberReadFlag=0;
      while(temp2[i]   )
            {
            if (temp2[i]!='-')
                  numberReadFlag=1;
            s2[l2++]=temp2[i++];
            }
      if (numberReadFlag)
            fscanf(input,"%d",temp);
      if (i!=60)
            break;
      fgets(temp,199,input);
      fgets(temp,199,input);
      }
i=0;
while(i<l1)
      {
      fprintf(output,"%15s     ",name1);
      for(j=i;j<((i+60>l1)?l1:i+60);j++)
            fprintf(output,"%c",s1[j]);
```

```
      fprintf(output,"\n");
      fprintf(output,"%15s     ",name2);
      for(j=i;j<((i+60>l1)?l1:i+60);j++)
            fprintf(output,"%c",s2[j]);
      fprintf(output,"\n");
      for(j=0;j<2;j++)
            fprintf(output,"          ");
      for(j=i;j<((i+60>l1)?l1:i+60);j++)
            if  ((s1[j]!=s2[j])&& (s1[j]!='-') &&(s2[j]!='-') )
                  fprintf(output,"*");
            else
                  fprintf(output," ");
      fprintf(output,"\n\n\n");
      i+=60;
      }
}
```

Figure 3.37 Nonconserved sequence finding program

The output text file can be seen as shown in Figure 3.38.

```
A.fumigatus  ------------------------------------------------------------
Neurospora   ATGTCCAAGATTTCCGTCGCCGCTGTGCGTCAGCAGTTACTGACCTTCTTGAGTACTCTA


A.fumigatus  ------------------------------------------------------------
Neurospora   ACGAGACCAAGAAGCGCAACTTCCTCGAGACCGTCGAGCTCCAGATCGGCCTCAAGAACT


A.fumigatus  ------------------------------------------CCACCGTTCCCCGCC
Neurospora   ATGACCCCCAGCGTGACAAGCGTTTCTCTGGCACCATTCGCCTGCCCAGCATTCCCCGCC
                                                        * *

A.fumigatus  CCAACATGACCATCTGTGTTCTTGGTGACCAGCACGATCTCGACCGTGCCAAGCACCACG
Neurospora   CCAACATGAGCATCTGCATTCTCGGTGACCAGCACGATATCGACCGTGCCAAGCACGGCG
                   *      **    *                *                   **

A.fumigatus  GCATCGATGCCATGTCCGCTGATGACCTGAAGAAGCTCAACAAGAACAAGAAGCTCATCA
Neurospora   GTGTTGACGCCATGTCCGTCGACGATCTCAAGAAGCTCAACAAGAACAAGAAGCTCATCA
              ** *  *          **  *  *  *

A.fumigatus  AGAAGCTGGCTCGCAAGTACGATGCTTTCCTTGCTTCCGACACCCTCATCAAGCAGATTC
Neurospora   AGAAGCTTGCTCGCAAGTACGATGCCTTCGTCGCCTCCGAGGCCCTTATCAAGCAGATCC
                    *                *   * *  *     **    *           *

A.fumigatus  CTCGTCTCCTGGGTCCCGGTCTGTCCAAGGCTGGTAAATTCCCTACCCCCGTCTCTCACA
Neurospora   CCCGTCTGCTCGGTCCCGGTCTTTCCAAGGCTGGCAAGTTCCCCACCCCCGTCTCCCACT
              *     *  *           *           *  *     *           *   *

A.fumigatus  GCGAGGACATGGCCAACAAGGTCACCGAGATCAAGTCTACCATCAAGTTCCAGCTCAAGA
Neurospora   CCGACGACCTTACCGGCAAGCTCAACGAGGTCAAGTCTACCATCAAGTTCCAGCTCAAGA
             *   *   * **  **    *   *    *
```

```
A.fumigatus  AGGTTCTCTGCCTCGGTGTTGCCGTTGGCAACGTCGGCATGACCAAGGAAGAGCTCGTCG
Neurospora   AGGTTCTCTGCATGGGTGTCGCCGTCGGCAACGTTGGCATGACCCAGGAGCAGCTTGTTG
                       * *     *     *        *          *    **    *  *


A.fumigatus  CCAACGTCATGCTGGCCATCAACTACCTCGTCTCCCTGCTGAAGAAGGGTTGGCAGAACG
Neurospora   GTAACATCATGTTGGCCATCAACTACCTCGTCTCCCTCCTCAAGAAGGGCTGGCAGAACG
             **   *     *                              *  *        *

A.fumigatus  TTGGCAGCCTTGTCCTCAAGGCTACCATGTCTCCCCCCAAGCGTCTCTACTAGATTCTCT
Neurospora   TTGGTAGCCTTACCATCAAGGCTACCATGTCTCCCCCCAAGCGCCTCTACTAA-------
                  *      ** *                                 *        *

A.fumigatus  GCGCAATTAGCTTTGTGACAGGGGTTTTTAGGCGCTCAGATGTGGGATTGTATATGCCAT
Neurospora   ------------------------------------------------------------


A.fumigatus  AGATAGGAAATGTGATGTCCACGACTGGTACCTAAA
Neurospora   ---------------------------------
```

Figure 3.38 Output file of the program. (*) indicates the nonconserved regions.

3.14 Detection of Overlapping 60S Ribosomal Protein L1-b

As shown in blast results in Appendix H, there are two 60S ribosomal proteins of *Caenorhabditis elegans* that have 155 amino acid of their sequence in common. The larger protein is 217 amino acid long. The same situation is also observed in *Drosophila melanogaster*. Clone 15 of *A. fumigatus* corresponded to the 155 amino acid long small protein.

In this study, it was shown that the genes of the two ribosomal proteins are overlapping in *A. fumigatus*, as well as in *C. elegans* and *D. melanogaster*. This finding is new to the literature and suggests that some ribosomal protein genes may indeed be expressed in an overlapping manner, creating multiple proteins from the same DNA region, and in fact, the same strand of the DNA.

ACCTAGGCCTCAATCAGGCTAGTGCCATCCACACCCACAAGCGGAGAACGGGATTTT
CTCAAGGCGGATAGCGAGCCGCAGGCTTTTTCTCTCGTCCTTGCACCGATATTCAGC
GCAGAGCCACACCATAAGAGTTATTCTTTTGGAAGACTGTCAGCAAG**ATGTCTAAGA**
TCACAGTCGGTATGCATCAGAATTTAACTGGCTTCTTTTGGGAAGCTGCAACTAATA
TGTCTCCTGGTTACAGCCGGAGTGCGCGAGAATGTCGAGCAGCTGCTCAACTACTCT

CAGAATGAGAAGAAGAGAAACTTCCTCGAGACCGTCGAGCTTCAGATCGGTCTGAAG
AACTACGACCCCCAGCGTGACAAGCGTTTCTCTGGCACCATCAAGCTGCCCACCGTT
CCCCGCCCCAACATGACCATCTGGTACGTCAACTTTGCCGACCGACCGATCCTTCGG
TGGAATGTCTTGATGCGCATCATCGGGAGAAGGATGAGGTAACGCACAAGTTATGGG
ACTAAGGGTTGCTGACGGTGTTGTCTGTAGTGTTCTTGGTGACCAGCACGATCTCGA
CCGTGCCAAGCACCACGGCATCGATGCCATGTCCGCTGATGACCTGAAGAAGCTCAA
CAAGAACAAGAAGCTCATCAAGAAGCTGGCTCGCAAGTACGATGCTTTCCTTGCTTC
CGACACCCTCATCAAGCAGATTCCTCGTCTCCTGGGTCCCGGTCTGTCCAAGGGTAC
GTATCTTCGGTTCCCGCAGAACGCCCAGCGCGCGGCATAGCCTCTAGCAAATGCTAA
CTCGGCGCAGCTGGTAAATTCCCTACCCCCGTCTCTCACAGCGAGGACATGGCCAAC
AAGGTCACCGAGATCAAGTCTACCATCAAGTTCCAGCTCAAGAAGGTTCTCTGCCTC
GGTGTTGCCGTTGGCAACGTCGGCATGACCAAGGAAGAGCTCGTCGCCAACGTCATG
CTGGCCATCAACTACCTCGTCTCCCTGCTGAAGAAGGGTTGGCAGAACGTTGGCAGC
CTTGTCCTCAAGGCTACCATGTCTCCCCCCAAGCGTCTCTACTAGATTCTCTGCGCA
ATTAGCTTTGTGACAGGGGTTTTTAGGCGCTCAGATGTGGGATTGTATATGCCATAG
ATAGGAAATGTGATGTCCACGACTGGTACCTAAATCTTTTTTTCGGGAATTCCGTAG
TGTGTGCTGGCATACTGCACATGTTCCAATCTTCATCTATTCTAGGATATACGTCCG
CTGCATAGAGAACATAAACAGTACCGTCCGTAAAGATAAATATACAAGTGGGATAAA
GTAGGGTAAAAAGTTACTGCATTCCGGGCATAAGGTCCG

Figure 3.39 Two overlapping ribosomal protein L1-b genes. Intron and exon sequences of *A. fumigatus* genome for clone 15; yellow highlighted red sequences denote exon sequences and blue highlighted black sequences denote intron sequences. Bold-red sequences denote exon sequences and blod-blue sequences denote intron sequences.

Figure 3.39 shows the overlapping genes. Highlighted sequences are previously found sequences from cDNA. 217 amino acid long 60S ribosomal protein L1-b gene starts from the first underlined ATG codon and 155 amino acid long 60S ribosomal protein L1-b gene starts from the second underlined ATG codon. Both 60S ribosomal protein L1-b genes are terminated by the underlined TAG codon.

3.15 Northern Blot Analysis of 60S Ribosomal Protein L1-b

In order to find support for the overlapping genes theory, total RNA isolation and northern blot analysis were performed using the 112 bp PCR fragment amplified by Ribopro1-2, as a probe.

In a previous study by Banu Yalçındağ, *A. fumigatus* was grown in medium containing locust bean gum for six days. The mycelia from each day were filtered and part of them was used in RNA isolation and the rest was stored at -80 ˚C.

For the preparation of fresh mycelium, *A. fumigatus* was cultivated as on glucose explained in section 2.2.1. From the above mentioned six samples and one new sample from glucose, total RNA was isolated according to the procedure given in section 2.2.9. RNA was visualized on agarose gel (Figure 3.40).

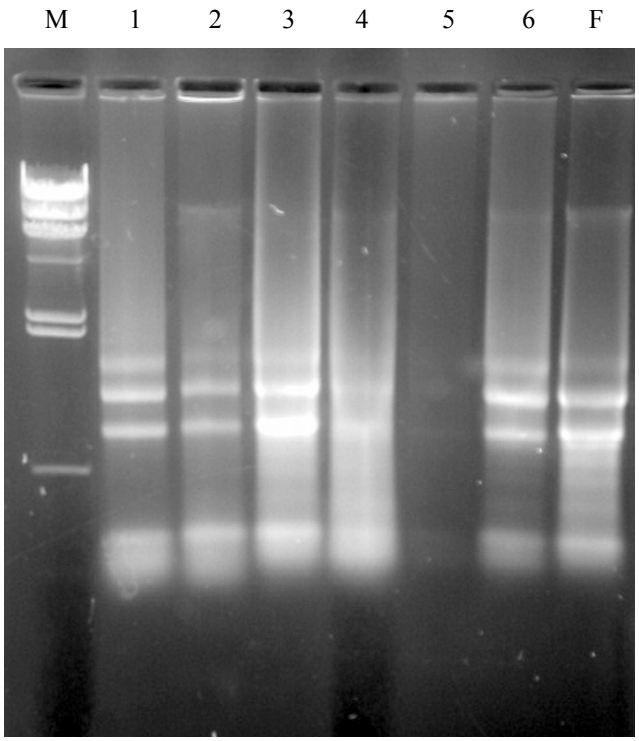


Figure 3.40 M, λ DNA/*Hin*dIII Marker; **F,** Fresh mycelium; The numbers denote for the cultivation days on which RNA isolation was performed.

Then, RNAs were blotted for northern transfer as explained in section 2.2.10, hybridized and detected by the DIG-chemilumiscence method (Figure 3.41).

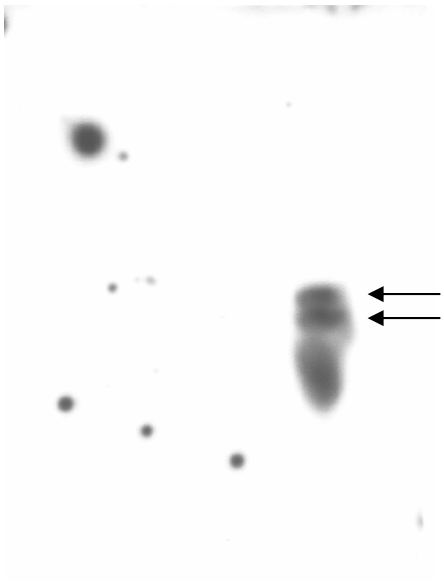


Figure 3.41 Northern blot analysis of RNAs

In the northern analysis, two bands were observed on the RNA obtained from the glucose-grown mycelium. This suggests that 217 and 155 amino acid 60S ribosomal proteins are indeed overlapping genes and expressed at the same time. However, this suggestion requires further experimental proof. In the other RNAs interestingly, no signals were observed.

CHAPTER 4

CONCLUSIONS

Aspergillus fumigatus is a foodborne and airborne human pathogen. In addition, it produces mycotoxins such as gliotoxin, helvolic acid, fumigallin, and fumigaclavin. The most common disease caused by *A. fumigatus* is aspergillosis, which is often fatal, especially among AIDS, cancer, and organ-transplant patients. In this study, random cDNA cloning was performed from a cDNA library of *A. fumigatus*. Some of these clones were selected according to their insert sizes and were further subjected to sequence analysis. The sequences were then analyzed by gene bank and relative software to determine the possible function of these genes. Two of the clones were identified as primase and 60S ribosomal protein L1-b genes. These genes and a third gene corresponding to the antigenic cell wall galactomannoprotein gene of *A. fumigatus* were used in the design of 3 primer pairs. For primer design a software was written to differentiate the nonconserved regions by multiple sequence alignment. Designed primers were experimented by PCR on different *Aspergillus* species and a unique band was obtained only against *A. fumigatus* DNA with these primers. It is planned that this PCR based method developed, will be used in the rapid detection of *A. fumigatus* from food samples, human blood or tissue, and spores from air.

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APPENDIX A

CHEMICALS, ENZYMES AND THEIR SUPPLIERS

Chemical or Enzyme	Supplier
λ.DNA/*Eco*RI+*Hin*dIII	MBI Fermentas
λ.DNA/*Hin*dIII	MBI Fermentas
β-mercaptoethanol	Merck
Agar	Oxoid
Agarose	Sigma
Anti-digoxygenin-AP	Roche
Blocking Reagent	Roche
Calf Intestine Alkaline Phosphatase	MBI Fermentas
Calcium Chloride	Merck
Casein Hydrolysate	Sigma
Chloroform	Merck
CSPD	Roche
DEPC	Sigma
Developer and Replenisher	Kodak
DIG Easy Hyb	Roche
dNTP mix	MBI Fermentas
*Eco*RI	MBI Fermentas
EDTA	Merck

Ethanol	Gurup Deltalar
Ethidium Bromide	Sigma
Fixer and Replenisher	Kodak
Formaldehyde	Merck
Formamide	Merck
Gene Ruler™ 100bp DNA Ladder Plus	MBI Fermentas
Glacial Acetic Acid	Merck
Glycerol	Merck
Glucose	Merck
Hexanucleotide Mixture	Roche
Hydrochloric Acid	Merck
Isopropanol	Merck
Kanamycin Monosulfate	Sigma
KCl	Merck
K_2HPO_4	Merck
Klenow Enzyme	Boehringer Mannheim
Maleic Acid	AppliChem
Maltose	Difco
Mineral Oil	Sigma
$MgSO_4.7H_2O$	Merck
MOPS	Merck
NaCl	Merck
NaOH	Merck
Phenol:Chloroform:Isoamylalcohol	AppliChem
RNase A	Roche
SDS	Merck
Sodium Acetate	Merck
Sodium Citrate	Merck
Soluble Starch	Sigma
Spermidine	Sigma

Sucrose	Merck
Taq DNA Polymerase	MBI Fermentas
Tetracycline	Mustafa Nevzat İlaç San.
Tris	Merck
Triton X-100	Sigma
Tryptone	Difco
Tween 20	Sigma
*Xho*I	MBI Fermentas
Yeast Extract	Merck

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1. Agarose (0.8 % w/v, 100 ml)

0.8 g agarose is dissolved in 100 ml TAE buffer by heating and stirring.

2. Antibody Solution

Anti-digoxygenin-AP is centrifuged for 1 minute at 10 000 rpm prior to each use and the necessary amount is carefully pipetted from the surface. Antibody solution is freshly prepared by diluting anti-digoxygenin-AP 1:10000 (75 mU/ml) in blocking solution.

3. Blocking Solution

10X blocking solution is diluted 1:10 with maleic acid buffer.

4. Buffer P1 (Resuspension Buffer)

50 mM Tris-Cl, pH = 8.0
10 mM EDTA
100 µg/ml RNase A

5. **Buffer P2 (Lysis Buffer)**

200 mM NAOH
1 % SDS

6. **Buffer P3 (Neutralization Buffer)**

3.0 M potassium acetate, pH = 5.5

7. **Buffer QBT (Equilibration Buffer)**

750 mM NaCl
50 mM MOPS, pH = 7.0
15 % isopropanol
0.15 % Triton X-100

8. **Buffer QC (Wash Buffer)**

1.0 M NaCl
50 mM MOPS, pH = 7.0
15 % isopropanol

9. **Buffer QF (Elution Buffer)**

1.25 M NaCl
50 mM Tris·Cl, pH = 8.5;
15 % isopropanol

10. **$CaCl_2$ (1 M, 50 ml)**

5.55 g of $CaCl_2$ is dissolved in 40 ml H_2O. The volume is adjusted to 50 ml and sterilized by filtration.

11. Detection Buffer

0.1 M Tris-HCl
0.1 M NaCl, pH = 9.5

12. DNase free RNase

RNase A is dissolved in 0.01 M Sodium acetate (pH 5.2) to give a final concentration of 10 mg/ml. The solution is heated to 100ºC for 15 min in a boiling water bath for the inactivation of DNase. It is cooled slowly to room temperature. 0.1 volume of 1 M Tris-HCl (pH 7.4) is added until the pH of the solution is 7.0. The solution is dispensed into aliquots and stored at -20ºC.

13. EDTA (0.5 M, pH 8.0, per Liter)

186.1 g of disodiumethylenediaminetetraacetate is added to 800 ml of distilled water. It is stirred vigorously on a magnetic stirrer while the pH is adjusted to 8.0 with NaOH pellets. The volume of the solution is then adjusted to 1 liter with distilled water, dispensed into aliquots and sterilized by autoclaving.

14. Ethanol (70%, 100 ml)

70 ml ethanol is mixed with 30 ml sterile distilled water.

15. Extraction Wash Buffer (12 ml)

300 µl Concentrated Wash Buffer (DNA Extraction Kit - Fermentas)
5.7 ml distilled water
6 ml 95 % ethanol

16. Formaldehyde Gel-Loading Buffer (10X)

50% (v/v) glycerol
10 mM EDTA (pH 8.0)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF

17. Glucose (25% w/v, 10 ml)

2.5 g of glucose is dissolved in 8 ml H_2O. The volume is adjusted to 10 ml and sterilized by filtration.

18. High Stringency Wash Buffer

0.5X SSC
0.1 % SDS

19. Kanamycin Solution (0.1 g/ml, 10 ml)

1 g of kanamycin monosulfate is dissolved in 8 ml H_2O. The volume is adjusted to 10 ml and sterilized by filtration.

20. LB Kanamycin Agar (per Liter)

10 g NaCl
10 g tryptone
5 g yeast extract
20 g agar

Final volume is adjusted to 1 liter with water. After adjusting pH to 7.0 with NaOH, the medium is autoclaved. 500 µl of 0.1 g/ml filter-sterilized

kanamycin is added when it cools to 55ºC, and poured to petri dishes (~25 ml/100 mm plate). The plates are covered with foil and stored at 4ºC.

21. LB Kanamycin Broth (per Liter)

10 g NaCl
10 g tryptone
5 g yeast extract

Final volume is adjusted to 1 liter with water. After adjusting pH to 7.0 with NaOH, the medium is autoclaved. 500 µl of 0.1 g/ml filter-sterilized kanamycin is added when it cools to 55ºC.

22. LB Medium Supplemented with 0.2% (w/v) Maltose and 10mM $MgSO_4$ (per Liter)

10 g NaCl
10 g tryptone
5 g yeast extract
2.46 g $MgSO_4.7H_2O$

Final volume is adjusted to 1 liter with water. pH is adjusted to 7.0 with NaOH. After autoclaving, 500 µl 20% filter-sterilized maltose solution is added.

23. LB Tetracycline Agar (per Liter)

10 g NaCl
10 g tryptone
5 g yeast extract
20 g agar

Final volume is adjusted to 1 liter with water. After adjusting pH to 7.0 with NaOH, the medium is autoclaved. 1.5 ml of 10 mg/ml tetracycline is added when it cools to 55ºC, and poured to petri dishes (~25 ml/100 mm plate). The plates are covered with foil and stored at 4ºC.

24. Low Stringency Wash Buffer

2X SSC
0.1 % SDS

25. Lysis Buffer

40 mM Tris-HCl (pH = 8.0)
10 mM EDTA (pH = 8.0)
0.2 M NaCl
1.5 % (w/v) SDS

26. Maleic Acid Buffer

0.1 M maleic acid
0.15 M NaCl

The pH is adjusted to 7.5 with NaOH pellets and sterilized by autoclaving.

27. Maltose (20% w/v, 10 ml)

2.0 g of maltose is dissolved in 8 ml H_2O. The volume is adjusted to 10 ml and sterilized by filtration.

28. $MgSO_4$ (10 mM, per Liter)

2.46 g $MgSO_4.7H_2O$ is dissolved in 800 ml H_2O. The volume is adjusted to 1 liter and sterilized by autoclaving.

29. MOPS electrophoresis buffer (10X, per Liter)

41.8 g of MOPS is dissolved in 700 ml of sterile DEPC treated H_2O. The pH is adjusted to 7.0 with 2 N NaOH. Then, 20 ml of DEPC treated 1 M NaAC and 20 ml of DEPC treated 0.5 M EDTA (pH 8.0) are added. The solution is sterilized by passing through a 0.45 μm milipore filter, and stored at room temperature protected from light.

30. NaAC (3 M, per Liter)

408.3 g of $NaAC.3H_2O$ is dissolved in 800 ml of H_2O. The pH is adjusted to the desired value with glacial acetic acid. The volume of the solution is then adjusted to 1 liter with distilled water, dispensed into aliquots and sterilized by autoclaving.

31. NaCl (5 M, per Liter)

292.2 g NaCl is dissolved in 800 ml distilled water. The volume is adjusted to 1 liter and sterilized by autoclaving.

32. NaOH (10 N, 100 ml)

40g of NaOH pellets is added slowly to 80 ml of H_2O. When the pellets have dissolved completely, the volume is adjusted to 1 liter with H_2O. The solution is stored at room temperature. Sterilization is not necessary.

33. NE Buffer

0.3 M NaAC (pH 7.0)
1 mM EDTA

34. NZY Agar (per Liter)

5 g NaCl
2 g $MgSO_4.7H_2O$
5 g yeast extract
10 g casein hydrolysate
15 g agar

The final volume is adjusted to 1 liter with water. After adjusting pH to 7.5 with NaOH, autoclaved and poured into petri dishes (~80 ml/150 mm plate).

35. NZY Broth (per Liter)

5 g NaCl
2 g $MgSO_4.7H_2O$
5 g yeast extract
10 g casein hydrolysate

The final volume is adjusted to 1 liter with water. After adjusting pH to 7.5 with NaOH, autoclaved.

36. NZY Top Agar (per Liter)

5 g NaCl
2 g $MgSO_4.7H_2O$
5 g yeast extract

10 g casein hydrolysate
7 g agarose

The final volume is adjusted to 1 liter with water. After adjusting pH to 7.5 with NaOH, autoclaved.

37. SDS (10% w/v, per Liter)

100 g of SDS is dissolved in 900 ml of H_2O. The solution is heated to 68°C and stirred with a magnetic stirrer to assist dissolution. If necessary, pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume of the solution is adjusted to 1 liter with H_2O. It is stored at room temperature. Sterilization is not necessary. Do not autoclave.

38. SM Buffer (per Liter)

5.8 g NaCl
2.0 g $MgSO_4.7H_2O$
50 ml 1 M Tris-HCl (pH 7.5)
5 ml 2% (w/v) gelatin

39. Solution A

50 mM $CaCl_2$
10 mM Tris-HCl (pH 8.0)

40. Solution 1

50 mM Glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA

41. Solution 2

0.2 N NaOH
1% SDS

42. Solution 3

3 M NaAC (pH 4.8)

43. SSC (20X, per Liter)

175.3 g of NaCl and 88.2 g of sodium citrate are dissolved in 800 ml DEPC treated distilled water. The pH is adjusted to 7.0 with NaOH. The volume is adjusted to 1 liter and sterilized by autoclaving.

44. TAE Buffer (50X, per Liter)

242 g of Tris base is dissolved in 600 ml of distilled water. The pH is adjusted to 8.0 with approximately 57 ml glacial acid. Then 100 ml of 0.5 M EDTA (pH 8.0) is added and the volume is adjusted to 1 liter.

45. TE buffer (pH 8.0)

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

46. TTE Buffer

0.5 M sucrose
10 mM EDTA (pH = 8.0)
10 mM Tris-HCl (pH = 8.5)
4 mM spermidine

36 mM KCl

0.25 % Triton X-100

10 mM β-mercaptoethanol

47. Tris-HCl (10 mM, pH 8.0, per Liter)

121.1 g Tris base is dissolved in 800 ml of distilled water. The pH is adjusted to the desired value with concentrated hydrochloric acid. The solution is cooled to room temperature before making final adjustment to pH. The volume of the solution is then adjusted to 1 liter with distilled water, dispensed into aliquots and sterilized by autoclaving.

48. Washing Buffer

0.1 M maleic acid

0.15 M NaCl

pH is adjusted to 7.5 with solid NaOH, the solution is sterilized by autoclaving. After autoclaving, 0.3 % (v/v) Tween 20 is added.

49. YpSs Agar (per Liter)

4 g yeast extract

1 g K_2HPO_4

0.5 g $MgSO_4.7H_2O$

15 g soluble starch

20 g agar

The final volume is adjusted to 1 liter with water, and autoclaved.

50. YpSs Broth (per liter)

4 g yeast extract
1 g K_2HPO_4
0.5 g $MgSO_4.7H_2O$
10 g glucose

The final volume is adjusted to 1 liter with water, and autoclaved.

APPENDIX C

SEQUENCES OF THE PRIMERS

Name of the primer	Sequence of the primer (5’ to 3’)
315	AGG GTT TTC CCA GTC ACG AC
316	GAT AAC AAT TTC ACA CAG G
RAPD	CGT GCA CGC

APPENDIX D

pBK-CMV PHAGEMID VECTOR

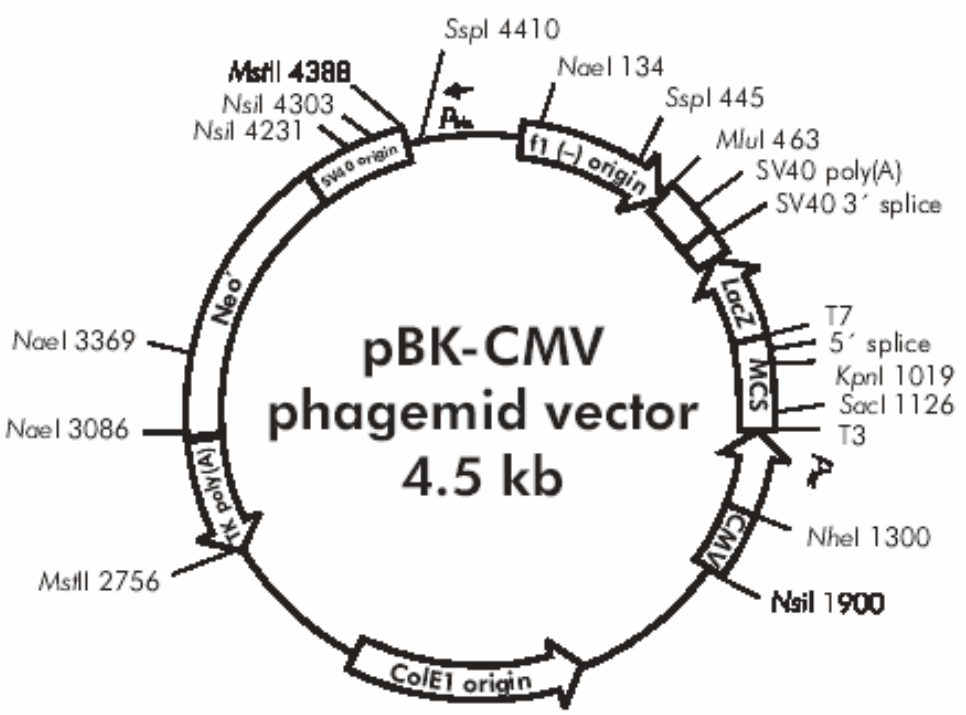

Figure D.1 pBK-CMV phagemid vector restriction map

APPENDIX E

DNA SIZE MARKERS

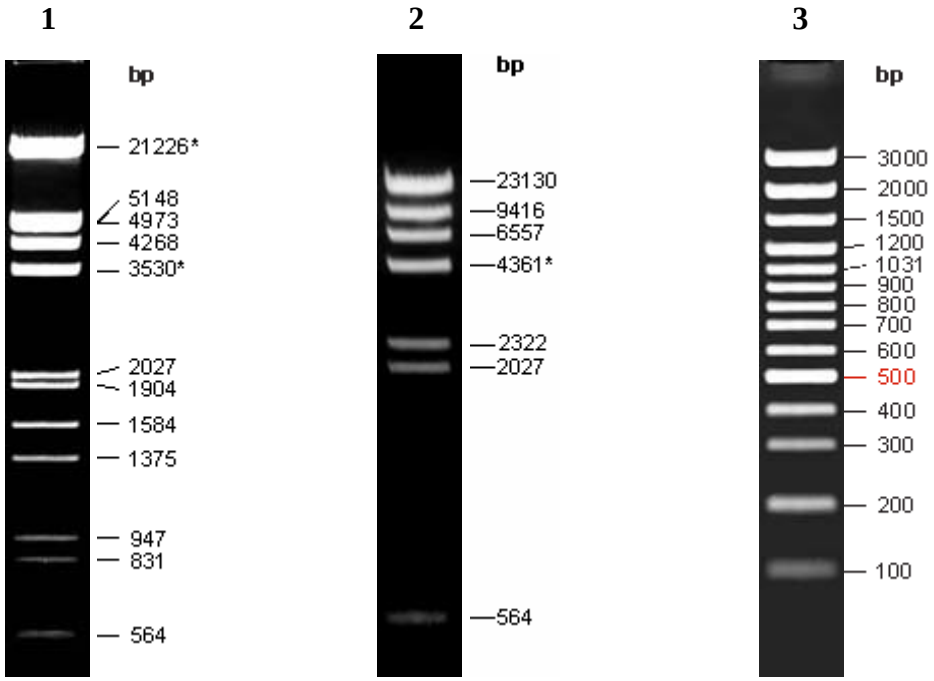

Figure E.1 DNA Size Markers. **1**, Lambda DNA/*Eco*RI+*Hin*dIII Marker; **2**, Lambda DNA/*Hin*dIII Marker; **3**, GeneRuler 100bp DNA Ladder Plus

APPENDIX F

SEQUENCES OF PLASMIDS

CCCTTTGACG ACNGCCNTTG AATTGTAATA CGACTCACTA TAGGGCGAAT TGGGTACACT
TACCTGGATA CCCCACCCGG GTGGAAAATC ATGGGCCCGC GGCCGCTCTA GAAGTACTCT
CNAGCTTTTT TTTTTNTTTT TTTTTTCTGC TCATCAAAAT CATGATGTCT ATTTCTACTG
ACTTGCAAAT CGGATCGATG AACTAGATGC ATACTTTTGA TAGTTGGAAT CACAGTGCGC
GCCTTGAACG CCTCCCAGGA ATATAGACTG CTGAACTAAA AGATAATACC AACGTGAATG
CGGACTTCTG GAACGGAACT GGTATCAAGG TGCGGAGGGC CGCTTAAGGA CAAGATTAGA
CCAGGAGGAC TGCATTGACC GTGCCATCGT TCTCAGGAGT ATTGGTGATC TGCGCAGATT
TACCCCATAC GACCTTTCCG CTTGTCGTGA CGAGACCAAT TCGGAGACGT TGACTTCGAT
GATGGTTCCC TTGGTCAGGA NCCCAGTTGG GTGTAAGAGG GTCTGNGGGT CTCTTGACCC
CAGGATGGGC AGTTGCACGG TGACCNAGCC CGGTGCGTGA CATTGGCTTC TGTAACGANA
CCATCGGCGN AGAAAGCCGT ACTGACGGTN CNCGGTGAAN

Figure F.1 5'—> 3' sequence of clone 9. Underlined sequences include oligo dT and vector sequences.

CCCTTTAACG ACGGCCNNTT GAATTGTAAT ACGACTCACT ATAGGGCGAA TTGGGTACAC
TTACCTGGTT ACCCCACCCG GGTTGGAAAA CGATGGGCCC GCGGCCGCTC TAGAAGTACT
CTCGAGTTTT TTTTTTTTTT TTTTTTTTAG GTACCAGTCG TGGACATCAC ATTTCCTATC
TATGGCATAT ACAATCCCAC ATCTGAGCGC CTAAAAACCC CTGTCACAAA GCTAATTGCG
CAGAGAATCT AGTAGAGACG CTTGGGGGGA GACATGGTAG CCTTGAGGAC AAGGCTGCCA
ACGTTCTGCC AACCCTTCTT CAGCAGGGAG ACGAGGTAGT TGATGGCCAG CATGACGTTG
GCGACGAGCT CTTCCTTGGT CATGCCGACG TTGCCAACGG CAACACCGAG GCAGAGAACC
TTCTTGAGCT GGAACTGATG GTAGACTTGA TCTCGGTGAC CTTGTTGGCA TGTCCTCCTG
TGANAACGGG GGTAGGAAAT TTACCANCTT GGACAGACCG GGACCAGGAG ACGAGGAATC

TGCTGATAGG GTGTCGAAGC AGAAGCTCGT CTTGCGAGCA GCTNTTGAGA CTTTTGTCTG
GTTGAGCTCT CAGGTANAGG GAATGATCAG CTGTGT

Figure F.2 5'—> 3' sequence of clone 15. Underlined sequences include oligo dT and vector sequences.

CCNTTAAACG ACNGCCAGNT GAATTGTAAT ACGACTCACT ATAGGGCGAA TTGGGTACAC
TTACCTGGTT ACCCCACCCG GGTTGGAAAA CGATGGGCCC GCGGCCGCTC TAGAAGTACT
CTCGAGTTTT TTTTTTNTTT TTTTTTTTTT TTTTTTTTTT GATAACGGAT GAACATGTTA
TAAGCATGTA TATGCAATAC AGTACATTGA TCGCTCATTC TTACACCGAT NTGCCAAACC
TCATATGCAA CAATTGCTGT TTTGANACTT CATATCGAAG GAGGAGAAGC ACCAAGAACT
CATAAAACGC GACCGAGGAA CGAACAAAAC ATNTAGAAAA AGTAGAAATC GAAATACGGA
GAACAAAGAG TCCGTCAAAA ACACGCCTCT CAGGACCTTC TCCATCTCCT CCTCCGTCAG
NGTCTCATAC TCGATCAGTG CCTTGGTCAG AAGCTCAGTT CGTGTCTCCG TTCAGTCANA
ATCTTGGTGG CACGCATCCG GCTTCTCACA GTCGTCGACT CGGATTCATT CTGGTTGGTT
CAATGATTAA TACATATTGG CNANAGGTCA CTTACAGTTC TNGGGTTCCN ATCTGGGTGA
CANGTAACCG TTCAGGGCTT GTGATTCCCC A

Figure F.3 5'—> 3' sequence of clone 17. Underlined sequences include oligo dT and vector sequences.

GGCCTTTGAC ACNGCCTTTG AATTGTAATA CGACTCACTA TAGGGCGAAT TGGGTACACT
TACCTGGTTA CCCCACCCGG GTTGGAAAAT GATGGGCCCG CGGCCGCTCT AGAAGTACTC
TCGAGTTTTT TTTTTTTTTT TTTTGGTATA TTGGTATGAA CTGCGGTGAG CAGTGCAAAC
TGGTATTAAC TCGCTATTTT CGGTTGCCGC AGCTGGTTGC CACTCTAAAA CAAAATCAAA
CGCCATCCCA ATGTCTATCA TGATTTGTTG AATCGGATCA TGCTGCTCAT GCCACCGTAA
ATGCCTCAAG GCAAGTGTTG AAAAGGGTCA AAGTAGAACT GAATTAGAAG AATGTTGAAA
CATGGGTATC ATTTAATCCG TCTCCTCCAC GGGGCCACAA GTGCAGAACA GGTTCTGATC
ACCGTATGCA TCATCTACTC TGGTCACCGA AGGCCAGAAC TTCTTCTCCA CCAAATATGG
AAGGGGATAC GCCGCAGCCT CCCGCGAGTA GGGACGGTTC CACTCACTGG ACAGCAGATC
GCNCTGCGTG TGAGGGCGTC TTGAAACTTA CCTCCGAGCT GTCGCGNTCT CACAGCGGCA
TCTCTGCAAT GGAATGACCT CAAAATCGGT CACTCGCTGT C

Figure F.4 5'—> 3' sequence of clone 20. Underlined sequences include oligo dT and vector sequences.

CCTTTAACGA CGGCCAGCGA ATTGTAATAC GACTCACTAT AGGGCGAATT GGGTACACTT
ACCTGGTACC CCACCCGGGT GGAAAATCGA GGGCCCGCGG CCGCTCTAGA AGTACTCTCG
AGTTTTTTTT NNTNTTNNNT TTTCCCTTGA AAANGNANCC CTANTTGNAG GGNAANNAAA
NTGGNTNAGG TNNNGNNTTT CNTAANCCCA TNNATTGGGG NNNNNAAANG NAAACNAAGN
GGGCCNAGCG GGTCCGNTGG TNACCCAANC CANTNCCTTG NGGNCCNAAC CNGTTNCTGG
AAACCCAAAG TTAAGAACTC NCNGTTTNAA AGTNTAAATT CTCTNNAAAC TTTNCGAGAN
TATTTAACCC GANANTTTNG GAAANGGGNN TTGTNNNCCN NNANCTCGGG GAAATTTTNN
GGGNTAATAA AGGGGTTNTG AANTTGAAAN CCNNNTGGNG TGGCCCANTA CCCCCTCNTT
GGNGNTAGCT NNAAAGNNNG NNTAAAGCTG GGNNTATTCC NCGGNANACN GGTNCACCNN
TNTTCTACTA NGTNCCNNAN AATTTTCTTT ANNATAAACT CTTNTTCANA AAACANNTTN
CCCGGTNTTT NNCCCCNN

Figure F.5 5'—> 3' sequence of clone 21. Underlined sequences include oligo dT and vector sequences.

CCTTTAACAC GGCCAGNGAA TTGTAATACG ACTCACTATA GGGCGAATTG GGTACACTTA
CCTGGTACCC CACCCGGGTG GAAAATCGAT GGCCCGCGGC CGCTCTAGAA GTACTCTCGA
GTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTN CGTCTTGAAA AGTCAGTCAA
TATGCATATA TAATATATAT CTCTATCGNG GGACACAACC ATCAAGGTCA TCCGCCTGAT
ACACCTTCTC TTCCATCAGG ATATTATGCT CCGTANATCA ATCCCGATCC CAGTCCAGTC
CAGNGCAATT CCAAGTCCAT ATCAATATAT AAATCACAGN TTCGCAGCCT CTTTCACCTC
CCCAATCTTC AACTNGGGCG AATACTTCTT CAACACGCCC TCATNGNGGT ACTACAACCA
TCAACATCAG NTCAGCCCTC TGTNCATATG CNCGTTGGTA AGGTANAACG CACCTTCAAA
ACTGCTTCGA GGCATCCTNC CCGCCAACGC TNANAATCTT GGCGCCGCCG GGATGTCGTC
GATAACTGGG CAGTCGTAAC CTGTTGTCGA TATATGTAAG GCCTGGTCGG AGTTTGTGGG
TGGACTCGCA NGGGTAAGAT GGGCATCGTC NC

Figure F.6 5'—> 3' sequence of clone 22. Underlined sequences include oligo dT and vector sequences.

CCTTTAACGA CGGCCNGCGA ATTGTAATAC GACTCACTAT AGGGCGAATT GGGTACACTT
ACCTGGTACC CCACCCGGGT TGGAAAATCG TGGGCCCGCG GCCGCTCTAG AAGTACTCTC
GAGTTTTTTT TTTTTTTTTT TTACGAATTT NGGCATCGGG TCGTTGATTC AATTGCGTGG
CTTATATACA GCCTAACCAA CACCTATAAA CGCCTTTACT TCGCCAGCAT AAACCAATAC
TTCCGCTAAG AATAGAAACC TGCAAATCCC AATATCAAGA ACCCAGCCAT ATATCGGAAC

GAATGCAAAC AGTGAATGCC CTTAAAATTC CATGCTTTCC GATTTTACCT CATCCGGATC
TTCATTTCCT CGTTTGCCGA CGCGTTCCTC TTTGTTTAGA GAAGCGATGA ACGAACGGAA
GTAGTCAATA TATGGCTTAA GGCTGGTCTT CTCGTAATCT TGCAATTTGC GACTTCCCTG
GGAGTCGCTA GGTGTGTGGC TTCATCTTGA CCAGCTTCGG CGCCTGCATT ACGCTAAGTG
CCAGCATCCA GGCATCATCT CCGCAGTAAT GAGTGATGTT GGGACGGAAA GAGATCAATC
GCCGTTTTTG GGTCATGGGA CAAAAAGCCA GTCGGGN

Figure F.7 5'—> 3' sequence of clone 23. Underlined sequences include oligo dT and vector sequences.

GNNCNNAGGC CTTTGACGAC GGCCTTTGAA TTGTAATACG ACTCACTATA GGGCGAATTG
GGTACACTTA CCTGGNTACC CCACCCGGGT GGAAAATCGA TGGGCCCGCG GCCGCTCTAG
AAGTACTCTC GAGTTTTTTT TTTTTTTTTT TTACGAATTT NGGCATCGGG TCGTTGATTC
AATTGCGTGG CTTATATACA GCCTAACCAA CACCTATAAA CGCCTTTACT TCGCCAGCAT
AAACCAATAC TTCCGCTAAG AATAGAAACC TGCAAATCCC AATATCAAGA ACCCAGCCAT
ATATCGGAAC GAATGCAAAC AGTGAATGCC CTTAAAATTC CATGCTTTCC GATTTTACCT
CATCCGGATC TTCATTTCCT CGTTTGCCGA CGCGTTCCTC TTTGTTTAGA GAAGCGATGA
ACGAACGGAA GTAGTCAATA TATGGCTTAA GGCTGGTCTT CTCGTAATCT TGCAATTTGC
GACTTCCCTG GGAGTCGCTA GGTGTGTGGC TTCATCTTGA CCAGCTTCGG CGCCTGCATT
ACGCTAAGTG CCAGCATCCA GGCATCATCT CCGCAGTAAT GAGTGATGTT GGGACGGAAA
GAGATCAATC GCCGTTTTTG GGTCATGGGA CAAAAAGCCA GTCGGGN

Figure F.8 5'—> 3' sequence of clone 24. Underlined sequences include oligo dT and vector sequences.

APPENDIX G

THE GENETIC CODE AND SINGLE-LETTER AMINO ACID DESIGNATIONS

5'-OH terminal base	Middle base				3'-OH terminal base
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val*	Ala	Glu	Gly	G

*Codes for Met if in the initiator position

Figure G.1 The genetic code

Alanine	A		Leucine	L
Arginine	R		Lysine	K
Asparagine	N		Methionine	M
Aspartic acid	D		Phenylalanine	F
Cysteine	C		Proline	P
Glycine	G		Serine	S
Glutamic acid	E		Threonine	T
Glutamine	Q		Tryptophan	W
Histidine	H		Tyrosine	Y
Isoleucine	I		Valine	V

Figure G.2 Single-letter amino acid designations

APPENDIX H

PROTEIN DATABASE SEARCH RESULTS

Search results for clone 9:

- hypothetical protein AN2926.2 [Aspergillus nidulans FGSC A4]
  Accession number : EAA63497
  Length = 282
  Identities = 66/76 (86%)

- hypothetical protein [Neurospora crassa]
  Accession number : XP_325161
  Length = 261
  Identities = 62/75 (82%)

- hypothetical protein FG01135.1 [Gibberella zeae PH-1]
  Accession number : XP_381311
  Length = 260
  Identities = 63/75 (84%)

- conserved hypothetical protein [Schizosaccharomyces pombe]
  Accession number : NP_588561
  Length = 260
  Identities = 62/73 (84%)

- hypothetical protein CaO19.7398 [Candida albicans SC5314]
  Accession number : EAK97644
  Length = 129
  Identities = 66/75 (88%)

- similar to TGF beta-inducible nuclear protein 1 (L-name related LNR42) [Mus musculus]
  Accession number : XP_289688
  Length = 260
  Identities = 59/75 (78%)

- TGF beta-inducible nuclear protein 1; hairy cell leukemia protein 1 [Homo sapiens]
  Accession number : NP_055701
  Length = 260
  Identities = 59/75 (78%)

- hypothetical protein MG10835.4 [Magnaporthe grisea 70-15]
  Accession number : EAA47024
  Length = 260
  Identities = 59/75 (78%)

- protein YR-29 (29.7 kD) (1O220) [Caenorhabditis elegans]
  Accession number : NP_493387
  Length = 259
  Identities = 54/75 (72%)

- ribosomal protein S8e family protein [Arabidopsis thaliana]
  Accession number : NP_196254
  Length = 260
  Identities = 56/75 (74%)

- similar to TGF beta-inducible nuclear protein 1; hairy cell leukemia protein 1 [Rattus norvegicus]
  Accession number : XP_223612
  Length = 224
  Identities = 54/75 (72%)

- putative TGF(transfoming growth factor) beta inducible nuclear protein TINP1 [Oryza sativa (japonica cultivar-group)]
  Accession number : BAC79847
  Length = 259
  Identities = 55/75 (73%)

- conserved protein, COG SSU ribosomal protein S8E [Cryptosporidium parvum]
  Accession number : EAK88959
  Length = 260
  Identities = 55/75 (73%)

- constituent of 66S pre-ribosomal particles, involved in 60S ribosomal subunit biogenesis; Nsa2p [Saccharomyces cerevisiae]
  Accession number: NP_011052
  Length = 261
  Identities = 66/87 (75%)

Search results for clone 15:

- 60S ribosomal protein l1-b, putative [Aspergillus fumigatus]
  Accession number: CAE47895
  Length = 217
  Identities = 147/161 (91%)

- hypothetical protein [Neurospora crassa]
  Accesion number: XP_322380
  Length = 217
  Identities = 122/161 (75%)

- 60s ribosomal protein l10a. [Schizosaccharomyces pombe]
  Accession number: NP_587891
  Length = 216
  Identities = 113/160 (70%)

- L10A ribosomal protein [Candida albicans]
  Accession number: CAB56219
  Length = 217
  Identities = 102/160 (63%)

- ribosomal Protein, Large subunit (24.1 kD) (rpl-1) [Caenorhabditis elegans]
  Accession number: NP_491061
  Length = 216
  Identities = 95/160 (59%)

- ribosomal Protein, Large subunit (17.1 kD) (rpl-1) [Caenorhabditis elegans]
  Accession number: NP_491062
  Length = 155
  Identities = 91/155 (58%)

- ribosomal protein L10a [Homo sapiens]
  Accession number: BAC16802
  Length = 217
  Identities = 92/161 (57%)

- 60S ribosomal protein L10A (RPL10aA) [Arabidopsis thaliana]
  Accession number: NP_563813
  Length = 216
  Identities = 86/160 (53%)

- 60S RIBOSOMAL PROTEIN L10A (L1 in yeast) [Encephalitozoon cuniculi]
  Accession number: NP_597402
  Length = 219
  Identities = 26/89 (29%)

- Homology to rat L10a, eubacterial L1, and archaebacterial L1; identical to S. cerevisiae L1A (Ssm1p); Rpl1bp [Saccharomyces cerevisiae]
  Accession number: NP_011380
  Length = 217
  Identities = 97/159 (61%)

Search results for clone 17:

- hypothetical protein AN5588.2 [Aspergillus nidulans FGSC A4]
  Accession number: EAA62231
  Length = 802
  Identities = 38/54 (70%)

- hypothetical protein ((AF323913) intermembrane space AAA protease IAP-1 [Neurospora crassa])
  Accession number: XP_322617
  Length = 738
  Identities = 24/56 (42%)

- metalloprotease [Agrobacterium tumefaciens str. C58]
  Accession number: NP_534204
  Length = 648
  Identities = 23/53 (43%)

- metalloprotease [Bradyrhizobium japonicum USDA 110]
  Accession number: NP_773786
  Length = 640
  Identities = 22/53 (41%)

- COG0465: ATP-dependent Zn proteases [Rhodospirillum rubrum]
  Accession number: ZP_00014331
  Length = 643
  Identities = 21/51 (41%)

- ATP-dependent Zn proteases [Thermoanaerobacter tengcongensis]
  Accession number: NP_623928
  Length = 611
  Identities = 22/52 (42%)
- hypothetical protein CaO19.8836 [Candida albicans SC5314]
  Accession number: EAK97509
  Length = 687
  Identities = 20/50 (40%)

- cell division protein FtsH [Caulobacter crescentus CB15]
  Accession number: NP_422020
  Length = 626
  Identities = 21/52 (40%)

Search results for clone 20:

- putative glycine dehydrogenase (decarboxylating) [Schizosaccharomyces pombe]
  Accession number: NP_592832
  Length = 1017
  Identities = 38/56 (67%)

- probable glycine decarboxylase P subunit [Neurospora crassa]
  Accession number: CAE76410
  Length = 1100
  Identities = 41/65 (63%)

- hypothetical protein AN7136.2 [Aspergillus nidulans FGSC A4]
  Accession number: EAA61388
  Length = 1625
  Identities = 35/42 (83%)

- glycine dehydrogenase (decarboxylating; glycine decarboxylase, glycine cleavage system protein P) [Homo sapiens]
  Accession number: NP_000161
  Length = 1020
  Identities = 34/58 (58%)

- similar to Glycine decarboxylase [Rattus norvegicus]
  Accession number: XP_219785
  Length = 1007
  Identities = 34/58 (58%)

Search results for clone 22:

- hypothetical protein AN5828.2 [Aspergillus nidulans FGSC A4]
  Accession number: EAA58337
  Length = 84
  Identities = 41/46 (89%)

- predicted protein [Neurospora crassa]
  Accession number: XP_322905
  Length = 83
  Identities = 32/43 (74%)

- hypothetical protein FG10193.1 [Gibberella zeae PH-1]
  Accession number: XP_390369
  Length = 84
  Identities = 29/41 (70%)

- cytochrome b5 [Helicoverpa armigera]
  Accession number: AAC33731
  Length = 127
  Identities = 27/45 (60%)

- cytochrome b5 [Sorghum bicolor]
  Accession number: AAO17707
  Length = 133
  Identities = 21/44 (47%)

- cytochrome b5 (15.6 kD) (1H317) [Caenorhabditis elegans]
  Accession number: NP_491931
  Length = 141
  Identities = 22/44 (50%)

- cytochrome b5 [Arabidopsis thaliana]
  Accession number: NP_199692
  Length = 140
  Identities = 21/40 (52%)

- probable cytochrome b5 [Schizosaccharomyces pombe]
  Accession number: NP_587997
  Length = 129
  Identities = 23/44 (52%)

Search results for clone 23:

- hypothetical protein AN3033.2 [Aspergillus nidulans FGSC A4]
  Accession number: EAA63604
  Length = 521
  Identities = 136/182 (74%)

- hypothetical protein FG06339.1 [Gibberella zeae PH-1]
  Accession number: XP_386515
  Length = 498
  Identities = 99/179 (55%)

- hypothetical protein [Neurospora crassa]
  Accession number: XP_331008
  Length = 576
  Identities = 99/183 (54%)

- probable DNA primase small subunit [Schizosaccharomyces pombe]
  Accession number: NP_593765
  Length = 454
  Identities = 75/165 (45%)

- DNA primase catalytic subunit [Coprinopsis cinerea]
  Accession number: BAC76768
  Length = 416
  Identities = 69/169 (40%)

- DNA primase small subunit, 49kDa; primase polypeptide 1; primase p49 subunit; DNA primase, subunit 48; DNA primase 1 [Homo sapiens]
  Accession number: NP_000937
  Length = 420
  Identities = 54/154 (35%)
- DNA polymerase alpha subunit IV (primase) [Rattus norvegicus]
  Accession number: CAA09723
  Length = 415
  Identities = 57/154 (37%)

- p48 polypeptide of DNA primase; Pri1p [Saccharomyces cerevisiae]
  Accession number: NP_012273
  Length = 409
  Identities = 65/176 (36%)

- DNA primase small subunit, 49kDa [Mus musculus]
  Accession number: NP_032947
  Length = 417
  Identities = 51/154 (33%)

- DNA primase (EC 2.7.7.-) 46K chain – mouse
  Accession number: A33269
  Length = 417
  Identities = 51/154 (33%)

- DNA primase small subunit family [Arabidopsis thaliana]
  Accession number: NP_199003
  Length = 407
  Identities = 46/87 (52%)

- DNA polymerase subunit A [Drosophila melanogaster]
  Accession number: CAA56196
  Length = 438
  Identities = 57/158 (36%)

- DNA PRImase homolog (48.1 kD) (pri-1) [Caenorhabditis elegans]
  Accession number: NP_499126
  Length = 410
  Identities = 42/115 (36%)