MOLECULAR CHARACTERIZATIONS OF NITRATE ASSIMILATION IN *ASPERGILLUS FUMIGATUS*

by

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M.Sc. Simon Fraser University, 1992

B.Sc. University of Victoria, 1989

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in the Department

of

Biological Sciences

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SIMON FRASER UNIVERSITY

June 1997

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Date Approved: June 23 1997

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0-612-24287-0

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Abstract

The molecular characterization of the nitrite reductase gene cluster, from the opportunistic fungal pathogen (of humans and animals), *Aspergillus fumigatus* Fresenius and the creation of a *niiA*⁻ mutant (suitable for transformation studies) were achieved. Specifically, a genomic fragment containing the entire structural gene encoding nitrite reductase (*niiA*), plus segments of the nitrate reductase gene (*niaD*) and the nitrate transporter gene (*crnA*), was isolated. The complete genomic sequence of the *A. fumigatus niiA* gene was 5293 bp. The *niiA* gene sequence encodes a protein sequence of 1110 amino acids. The *niiA* open reading frame is interrupted by 8 small introns. The *niiA* putative protein sequence was found to contain regions corresponding to the FAD, NADPH, FeS and siroheme binding sites. The *niaD-niiA* intergenic regulatory region was found to contain promoter consensus sequences very similar to those identified in the *A. nidulans niaD-niiA* intergenic regulatory region. These consensus sequences include TATA and CAAT as well as for the *areA* gene product (general transcription factor) binding sites GATA, and the nirA gene (nitrate assimilation pathway specific transcription factor) product binding site (TCCGCGGA). Partial sequences corresponding to the *A. fumigatus niaD* and crnA genes were also obtained from the same genomic fragment and these showed extensive sequence homology (*niaD* = 60% identity; *crnA* = 60%) to their homologs from *Aspergillus nidulans*. The three genes for nitrate assimilation in *A. fumigatus* were determined to be physically linked and transcribed in the same direction as those found in *A. nidulans*. The nitrate assimilation gene cluster was found to reside on the largest chromosome band (5.3 Mb). Northern analysis indicated

that the expression of the nitrate assimilation gene cluster in *A. fumigatus* was induced by nitrate and repressed by ammonium at the transcriptional level.

To obtain strains mutant for nitrite reductase, gene disruptions of the *niiA* gene were created by using a transformation plasmid (pYA10). This Bluescript® derivative was contained a 1.9 kb internal fragment of the *niiA* gene. As well as the bacterial hygromycin B resistance gene (*hph*) as a selectable marker for transformation into *A. fumigatus* . Two *niiA* mutants were obtained (transformants:T3 and T6) which grew poorly on minimal media containing nitrate as the sole nitrogen source, but had normal growth on minimal medium containing ammonium and showed resistance to the antibiotic hygromycin B. Moreover, the two mutants were found to be mitotically stable. Southern analysis of DNA from T6, using three probes namely, the same *niiA* internal fragment, the plasmid Bluescript and the *hph* gene, confirmed that the *niiA* gene was disrupted. Southern analysis also revealed that the plasmid was inserted at the *niiA* gene locus on the chromosome. The mutant of the T6 strain was rescued by transformation with plasmids containing the entire sequence of the *niiA* gene of *A. fumigatus* (pYA2-3) and *A. nidulans* (p*niiA*), conclusively identifying it as *niiA*$^-$.

Contributions to our knowledge of the molecular characterization of *A. fumigatus* in general, and to the nitrate assimilation gene cluster in particular, have been achieved in this study. In addition, the *niiA* mutant strain could be extremely useful in future studies of *A. fumigatus* which to date has seen little genetic characterization.

Dedication

I dedicate this thesis to my wife Salma.

Acknowledgments

I would like to thank my senior supervisor Dr. Margo Moore for her support throughout the course of this thesis. I am very grateful to Dr. Etta Kafer for her guidance, advice especially with the transformation studies, providing me with references, useful editorial comments and suggestions, and enlightening discussions. I am also very grateful to Linda Pinto for her tremendous support and help with the preparations of this thesis. I would like to thank Charlene Mayes for her very useful editorial comments and suggestions. I would like to thank Loren Launen for her support and help with the preparations of this thesis. I would like to thank Dr. Radka Melanova for her support and warm friendship. Many thanks to Jason Grant for his help with the drawing of some of the figures of this thesis, and Rupinder Bagri for some of the typing of this thesis. I would like to thank the other people who helped me during my writing of this thesis.

I would like thank Dr. M. Monod (Laboratoire de Mycologie, Center Hospitalier University Vaudois, Lausanne, Switzerland) for kindly providing me with the genomic and the cDNA libraries. I would like to thank Dr. D. W. Holden (Dept. of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, London, UK) for kindly providing me plasmid PID21. I would like to thank Dr. R. van Gorcom for being external examiner (Dept. of Molecular Genetics and Gene Technology, TNO Nutrition and Food Research Institute, Rijswijk, The Netherlands). I would like to thank Dr. B. Brandhorst and Dr. E. Kafer for being members of my supervisory committee. I would like to thank Dr. D. Baillie for being the internal examiner. Many thanks to Dr. C. kennedy for being the chair of my defense.

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INTRODUCTION

Aspergillus fumigatus Fresenius is an opportunistic fungal pathogen that poses serious medical problems as this fungus is the most common cause of invasive and non-invasive pulmonary aspergillosis in immunocompromised patients (Holden *et al.*, 1994). Aggressive cytotoxic chemotherapy, organ transplants, and AIDS all predispose patients to fungal infections (Holden *et al.*, 1994). Little is known regarding the pathogenesis of *A. fumigatus*. To date, the virulence factors important in the initiation and spread of aspergillosis have not been identified (Holden *et al.*, 1994). However, in parallel with bacterial virulence, researchers postulate that virulence of *A. fumigatus* may be related to the production of extracellular proteases. A serine alkaline protease (ALP) and a metalloprotease (MEP) have been cloned and investigated as potential virulence factors (Monod *et al.*, 1993, Ramesh *et al.*, 1995). However, disruption of the ALP or MEP genes did not decrease the virulence of the *A. fumigatus* in murine models of aspergillosis when compared to wild-type strains (Monod *et al.*, 1993 and Remesh *et al.*, 1995). In fact, the only mutant of *A. fumigatus* that has failed to cause systemic infection when injected into the bloodstream of steroid-treated mice was a paba (*p*-aminobenzoic acid) requiring mutant (Sandhu *et al.*, 1979, Holden *et al.*, 1994).

Although several genes encoding potential epitopes responsible for the allergenicity of *A. fumigatus* have been cloned (Crameri *et al.*, 1996, Moser *et al.*,1992), few housekeeping genes have been cloned to date (Mellado *et al.*, 1995). Therefore, molecular/genetic characterization of housekeeping genes provides a good starting point for increasing our understanding of *A. fumigatus*. The cloning of gene clusters opens the doorway to facilitate future studies of a diverse nature in *A. fumigatus* including those involving pathogenicity. The most suitable gene cluster that can be cloned in *A.*

fumigatus and disrupted without affecting the viability of the organism, is the dispensable nitrate assimilation gene cluster (since other nitrogen sources such as ammonium, glutamine, and glutamate can be used; Marzluf, 1993). Therefore, the goal of this research was to clone and characterize the nitrate assimilation gene cluster from *A. fumigatus*. Information regarding its nitrate assimilation gene cluster would be useful in contributing to the general molecular characterization of this organism and in providing insights into the overall structural organization of this gene cluster with respect to its corresponding genes investigated in other species of filamentous fungi. Ultimately, development of a homologous transformation system for this medically important fungal pathogen can be achieved. Such a transformation system has been proven to be very practical in other *Aspergillus* species of agricultural and industrial importance (Daboussi *et al.*, 1989). Therefore, an effective homologous transformation system will facilitate the study of other *A. fumigatus* genes of interest, for example, potential virulence factors.

The specific objectives of this work are as follows:

1) to clone and characterize the nitrate assimilation gene cluster from *A. fumigatus* which consists of three genes: nitrate reductase (*niaD*), nitrite reductase (*niiA*), and the nitrate transporter (*crnA*)
2) to carry out complete molecular characterization of the nitrite reductase gene (*niiA*)
3) to create a *niiA*$^-$ strain of *A. fumigatus*, which can rescued by complementation with cloned *niiA*$^+$ genes of *A. fumigatus* and *A. nidulans*.

Biology of Fungi

Apergillus fumigatus (Fresenius) is a dikaryomycotan fungus of the subdivision Ascomycotina (Kendrick, 1992). The subdivision comprises two diverse classes (Ascomycetes and Saccharomycetes), among which are beneficial decomposers or saprobes as well as parasites and detrimental, opportunistic pathogens (Kendrick, 1992). Ascomycetous fungi derive their name from sac-shaped meiosporangia (asci) which form 8 endogenous ascospores. Although ascomycetes may reproduce sexually, the more frequent means of fungal propagation involves the asexual production of conidia (non-motile, dust-like mitospores) borne on stalked conidiophores (Raper, 1965). These dust-like spores are easily transported on air currents and in fact, the air we breath is estimated to contain several million fungal spores per cubic meter (Kendrick, 1992), making exposure to fungal pathogenic spores for immunosuppressed patients, inevitable.

The vegetative structure of a fungus (the thallus) consists of either unicells (in fungi expressing a 'yeast-like' morphology) or of long, tubular, threadlike filaments of cells called hyphae. Fungi which produce hyphae are generally referred to as filamentous fungi (Bos, 1996). Only the asexual or anamorphic phase of *A. fumigatus* can infect humans while its benign teleomorph or sexual phase, classified as *Sartorya fumigata* Vuillemin (Kendrick, 1992) is found associated with tree roots. Humans infected with *Aspergillus fumigatus* have inhaled conidia, which germinate initially in the lung tissue and develop invasive hyphae (which if left unchecked can spread other parts of the body; Kendrick, 1992).

Filamentous Fungi and Nitrogen Metabolism:

Filamentous fungi (ff) possess complex nitrogen catabolic pathways which function to ensure a constant nitrogen supply for growth (Marzluf, 1993). Specific primary nitrogen sources preferred by ff include ammonia, glutamate or glutamine. However, several secondary nitrogen sources such as nitrate, purines, amino acids and amides can be utilized once primary sources are depleted (Marzluf, 1993). The utilization of such secondary nitrogenous compounds requires *de novo* synthesis of premeases and catabolic enzymes, which are controlled at the transcriptional level by a nitrogen regulatory circuit (Cove, 1966 and 1979, Hawker *et al.*, 1992).

Nitrogen Cycle:

The nitrogen cycle includes processes such as nitrogen assimilation, nitrogen fixation, nitrification, and denitrification. Nitrogen assimilation is the process where by inorganic nitrogen is utilized as a nutrient to synthesize important nitrogen containing compounds. Nitrogen assimilation requires energy expenditure depending on the redox potential of the inorganic source used. For example, ammonium is a low cost source of nitrogen. In contrast, nitrate assimilation requires much greater energy expenditure. Energy is required to reduce nitrate to nitrite by the enzyme nitrate reductase, and for the further reduction of nitrite to ammonium by the nitrite reductase. The process of nitrogen fixation (fixing atmospheric nitrogen) is carried out by both aerobic and anaerobic bacteria, and is catalyzed by the enzyme nitrogenase and it requires great energy expenditures. Nitrification is an aerobic process that involves the oxidation of

ammonium to nitrite and the subsequent oxidation of the nitrite to nitrate. Denitrification is a process in which nitrate is used as an oxidant in anaerobic respiration leading to the release of nitrogen gas and nitrous oxide (Prescott *et al.*, 1993).

Genome Structure in Filamentous Fungi:

Gene organization in filamentous fungi (ff) include gene clusters, nonclustered genes, or cluster genes. To date an increasing number of gene clusters have been discovered including the *A. nidulans prn* gene cluster, four genes involved proline catabolism (Arst and MacDonald, 1975), the *N. crassa qa* gene cluster, seven genes for quinic acid break down (Giles *et al.*, 1986a); and the corresponding *A. nidulans qut* gene cluster, seven genes for quinic acid utilization (DaSilva *et al.*, 1986), the *A. nidulans alcA-alcR* alcohol cluster for ethanol utilization (Pateman *et al.*, 1985, Gwynne *et al.*,1987) and the nitrate assimilation gene cluster, three genes for nitrate utilization from *A. nidulans* and *A. parasiticus* (Cove, 1963, Chang *et al.*, 1996). Characterization of these gene clusters has revealed that individual transcripts are synthesized (as in higher eukaryotes) rather than the multi-cistronic mRNA species, which occur in prokaryotes. More recently, twenty–five co-regulated transcripts have been found to define a sterigmatocystin (a toxic secondary metabolite) gene cluster in *A. nidulans*. The transcripts of the sterigmatocystin (ST) gene cluster range in size from 0.6 to 7.2 kb and they are coordinately induced under ST-producing conditions (Brown *et al.*, 1996).

A number of fungal "cluster" genes have also been discovered which specify a single mRNAs from which multifunctional polypeptides are translated; these include the Neurospora *his-3* and the *A. nidulans aromA* and (Legerton and Yanofsky, 1985,

Neurospora *his-3* and the *A. nidulans aromA* and (Legerton and Yanofsky, 1985, Hawkins, 1987). In *A. nidulans*, the *aroA* cluster gene is transcribed as a single mRNA species encoding the pentafunctional AROM protein (Charles *et al.*, 1985, Hawkins, 1987). Comparison of the *aromA* cluster genes product to its homologue from bacteria suggested that the *aromA* cluster genes may have arisen as a result of a fusion of five individual prokaryotic units (Hawkins *et al.*, 1993, Charles *et al.*, 1985, 1986, Hawkins, 1987).

Gene Structure and Regulation:

As found in all eukaryotes, fungal genes have promoter sequence regions to which transcription factors bind and initiate transcription (Gurr *et al.*, 1987). Although most genes of ff have only one transcription initiation site, it is not unusual for some genes to contain multiple transcription initiation sites (both major and minor sites) which may significantly influence the size of the mRNA. For example, two transcripts 4.1 and 3.4 kb, of the *A. nidulans* homologue of *qa-3* gene are made due to the presence of two initiation sites 700 bp apart (Gurr *et al.*, 1987).

The TATAAA sequence is a signature promoter sequence of most higher eukaryotic and prokaryotic genes and is usually located about 30 bp (eukaryotic) upstream of the major transcriptional initiation site (Ballance, 1986). However, this TATAAA consensus sequence is not present in all ff promotors. When present it is located 30-60 bp upstream of the major initiation site in ff promotors, and it is present in a single copy as in the yeast *Schizosaccharomyces pombe* and in higher eukaryotes. In *Saccharomyces*

1986). TATAA sequence serves as a binding site for transcription factors.

Removal of the AT-rich motif from the *A. nidulans trpC* gene did not affect expression which suggests that little functional significance exists for the AT-rich motif for this particular gene (Gurr *et al.*, 1987). However, the TATAAA sequence in other ff genes is thought to be important in binding transcription factors (Bruchez *et al.*, 1993b). Another promoter consensus sequence is the CAAT sequence, often located between 60 bp and 120 bp upstream from the cap site in the promoter region. The CCAAT box is also an important promoter sequence. For example, deletion of the CCAAT sequence reduces the level of *A. nidulans amds* gene expression under either carbon or nitrogen limiting conditions. CCAAT box containing-oligonucleotide was able to restore expression of the *amds* gene (Littlejohn and Hynes, 1992). The CCAAT box sequence of the *A. nidulans* Taka-amylaseA (TAA) gene was found to confer starch inducibility on the gene (Nagata *et al.*, 1993). The CCAAT sequence was found to bind two proteins designated AnCP1 and AnCP2 in nuclear extracts from starch-grown and glucose-grown cells, respectively. A third protein designated AnNP1 was also found to bind to a 25 bp region upstream of AnCP2 in nuclear extracts from cells grown under repressive conditions. The AnNP1 protein is proposed to function as a repressor and hence impede the binding of AnCP2 to the CCAAT box (Nagata *et al.*, 1993).

Eukaryotic gene transcription involves the production of a primary RNA transcript which is subsequently processed and is followed by the addition of a cap at the 5' end and a poly (A) tail at the 3' end. The 5' end is capped by the addition of a 7-methyl-Guanidine to one of the very first nucleotides of the mRNA. The cap is thought to stabilize the mRNA and to guide the mRNA to ribosomes. The 3' end of mRNA is modified

poly (A) tail at the 3' end. The 5' end is capped by the addition of a 7-methyl-Guanidine to one of the very first nucleotides of the mRNA. The cap is thought to stabilize the mRNA and to guide the mRNA to ribosomes. The 3' end of mRNA is modified by the addition of poly (A) tail downstream of the consensus sequence AAUAAA which is found in most of the eukaryotic mRNAs and is thought to play a role in polyadenylation (Lewin, 1995). Some ff genes contain a similar or abbreviated form (AUAA). Other genes lack the AAUAAA consensus (Gurr *et al.*, 1987). Another consensus sequence, CCTGTTCC which may function as a termination signal, is found downstream from the poly(A) site of mammalian mRNA (McClauchlan *et al.*, 1985). Some ff genes contain a similar block (CATGGTTCT) downstream of the AAUAAA consensus sequence, but whether or not the CATGGTTCT sequence plays a role in transcription termination remains to be determined (Montague, 1987).

Translation of most ff genes begins at the first ATG, and the sequence around the ATG is crucial for guiding the ribosomes to the correct translation start site (Kozak, 1981). Higher eukaryotic mRNAs contain the consensus sequence CCACC**ATG**GC in which the -2 position is usually a purine and often is adenine. Similarly, in ff mRNAs, 80% of the sequence around the ATG contain adenine at -3 position (Kozak, 1981, Bruchez *et al.*, 1993a).

Introns:

Introns are non-coding sequences found interrupting the coding sequence in most eukaryotic genes (Watson *et al.*, 1987). Introns are removed from the primary RNA transcript in a process known as RNA splicing to produce mRNA. Introns are often

identified by utilizing S1 mapping, electron heteroduplex analysis or by comparison of genomic to cDNA sequences (Watson *et al*, 1987). In ff, most genes cloned and sequenced have been found to contain introns. In contrast, very few yeast genes have introns. Yeast genes carry only a single intron with the only exception being the MATa 1 gene with two introns (Miller, 1984). Intron sizes can vary from 50 bp to 10 kb depending on the organism (Lewin, 1995).

Ff introns are much shorter than those found in mammalian and yeast genes with an average intron size of approximately 100 bp, although some introns may be as large as 300 bp. The number of introns interrupting the non-coding sequence varies from one in *N. crassa H3* gene (Woudt *et al.*, 1983), to two in *N. crassa am* gene (Kinnaird and Fincham, 1983), to as many as seven in the *A. nidulans niiA* (Johnstone *et al.*, 1990) gene or eight in the *A. nidulans ben-A* gene (May *et al.*, 1985). Examples of ff genes which do not have introns, include genes of the *N. crassa qa* cluster (Giles *et al.*, 1985) and *pyr-4* (Glazebrook *et al.*, 1987). Furthermore the yeast introns are often positioned toward the 5' end of the nuclear protein encoding genes whereas ff introns are positioned toward the 5' end, 3' end or randomly distributed throughout the gene (Gurr *et al.*, 1987).

Ff introns display the same intron boundaries found in vertebrates, plants, and yeast. They start with GT at 5' end and terminate with AG at the 3' end. It should be noted that an exception to this has been observed in intron 2 of the *N. crassa fes-1* where the 5' boundary is CT not GT. In addition, the 5' boundaries of intron 1 of the genes *qa-1S* and *des-1* are GC instead of GT (Bruchez *et al.*, 1993b, Gurr *et al.*, 1987). Analyses of ff introns have shown there is a strong preference for the bases ANGT following GT but there is no preference for particular nucleotides preceding the GT splice site (Gurr *et al.*, 1987). In

addition, ff introns lack pyrimidine-rich sequences preceding the AG splice site, and the 3'-end splice sites terminate with PYAG in most introns. Few introns terminate with AAG (Bruchez *et al.*, 1993b).

The presence of introns in the middle of coding sequence raises the question of their physiological significance. They may be required so that a gene can be differentially spliced to give rise to two or more forms of the gene product (Krainer and Maniatis, 1988). For example, the *A. nidulans amdS* gene is alternatively spliced, two *amdS* transcripts are made: a short one which results from splicing all of three introns and a long transcript which retains intron 3 as a part of the coding sequence (Corrick *et al.*, 1987). The *A. nidulans niiA* gene may also be alternatively spliced. PCR analysis has shown that two mRNA species are made and that the shorter transcript retains the last intron, which contains a stop codon (intron 7) as part of its coding sequence (Johnstone *et al.*, 1990). Alternative splicing is wide spread in higher eukaryotes (Maniatis, 1991).

Introns divide coding sequences into segments known as exons which vary in size from 3 to 850 nucleotides long in *N. crassa* and *A. nidulans*. For example, the *N. crassa tub*-2 gene coding sequence is made up of seven exons ranging in size from 4 to 264 nucleotides long (Orbach *et al.*, 1986), and the *A. nidulans* actin gene coding sequence is made up of six exons ranging in size from 3 to 257 nucleotides in length (Kinghorn, 1987). Compared to higher eukaryotic exon sizes, which can be classified in groups that are 50, 140, 200, 300, and >300 bp long, exon sizes in ff are found to exhibit random size variations (Montague, 1987).

Genetic and Molecular studies on Nitrate Assimilation Genes in Filamentous Fungi:

Disruption of the structural genes encoding the nitrate or nitrite reductases renders the cell unable to use nitrate as a nitrogen source. Mutants defective in nitrate assimilation are easily identified and assigned to either gene by growth on defined media (Cove, 1979). Mutants grow poorly on nitrate as the sole nitrogen source. In addition mutants with a disrupted nitrate reductase (*niaD*) gene are resistant to chlorate (Cove, 1979). Phenotypic selection of mutants is very useful since it allows identification of mutants through a double selection process. However, it should be noted that mutants which grow poorly or can not grow on either nitrate or nitrite as a nitrogen source do not arise solely from mutations disrupting the *niaD* and *niiA* genes, but they may also result from mutations in a number of other genes involved in the regulation of the nitrate assimilation pathway in *Aspergillus* (Cove, 1966, 1967). That is, chlorate resistant mutations are obtained also in *cnx* genes which encode products involved in the biosynthesis of the molybdenum cofactor and lead to an inability of the fungus to utilize nitrate as a nitrogen source (Cove, 1979).

The expression of the nitrate assimilation gene cluster is regulated in several ways. It is induced by nitrate and repressed by ammonium. Suppression of the expression of nitrate assimilation genes by ammonium is known as nitrogen metabolite repression. The induction by nitrate and repression by ammonium of the nitrate assimilation gene cluster, are two independent events and their respective effects are mediated by products of different regulatory genes (Cove, 1967, Hurlburt and Garret, 1988). Induction of the nitrate reductase and nitrite reductase by nitrate in wild-type cells has been investigated at the translation

level by comparing enzyme levels from induced and uninduced cells (Crawford and Arst, 1993). Induction of these enzymes has also been investigated at the transcriptional level by Northern analysis of total RNA from induced and uninduced cells (Hawker *et al.*, 1992).

Genetic studies using *A. nidulans* and *N. crassa* have shown that the induction of nitrate assimilation genes is mediated by the positively-acting gene products of the *nirA* or *nit-4* (a pathway specific transcription factor) and *areA* or *nit-2* (a general transcription factor; Burger *et al.*, 1991, Crawford and Arst, 1993, Fu and Marzluf, 1987b, Fu *et al.*, 1989). In *A. nidulans*, loss-of-function *nirA*⁻ mutations affect inducibility and lead to the inability of cells to utilize nitrate or nitrite as nitrogen sources. In contrast, gain-of- function mutations $nirA^c$ result in constitutive expression of the nitrate reductase gene while the $nirA^d$ mutations relieve nitrogen metabolite repression (Cove, 1967, Cove, 1979, Rand and Arst, 1978, Tollervey and Arst, 1981, Cove, 1993). In addition, the double mutant alleles $nirA^{c/d}$ have been found to suppress the loss-of-function mutation in the regulatory gene *areA* which mediates nitrogen metabolite repression in *A. nidulans* (Tollervey and Arst, 1982). Mutants of $nirA^c$ and $nirA^d$ have been identified either by staining colonies for nitrate reductase activity or monitoring hypersensitivity to chlorate toxicity under appropriate conditions (Cove, 1979 and 1993, Pateman and Cove, 1967, Rand and Arst, 1978, Tollervey and Arst, 1982).

The *areA* gene from *A. nidulans* has been extensively studied and several *areA* mutant phenotypes have identified (Arst and Cove, 1973, Arst and Scazzocchio, 1985, Arst *et al.*, 1989, Caddick and Arst, 1990, Hynes, 1975, Kudla *et al.*, 1990, Stankovich *et al.*, 1993). Some of the *areA* mutants studied exhibit loss-of-function mutations while other mutants possess altered expression of other genes that are regulated by the *areA* gene (Arst,

1982, Arst and Cove, 1973, Caddick and Arst, 1990, Crawford and Arst, 1993). Loss-of-function mutations of the *areA* (*areA*^{r}) gene render the cell unable to utilize nitrogen sources other than ammonium or glutamine. The *areA*^{r} mutation results in a pleiotropic effect by reducing the expression of a number of genes under the control of the *areA* gene, which implies the *areA* gene has a role in gene regulation (Arst and Bailey, 1977, Arst and Cove, 1973, Arst and Scazzocchio, 1985). It has been reported that the *N. crassa nit-2* gene can rescue the *areA*^{r} mutant which suggests that both genes share similar functional domains (Davis and Hynes, 1987). The *nit-2* gene mutations described in the literature are all loss-of-function mutations (Fu and Marzluf, 1990, Marzluf, 1981, Perkins *et al.*, 1982).

Some mutants which carry point mutations, duplications, or deletions within the putative DNA binding domain of the *areA* gene have also been described. One mutation in the *areA* gene is *areA*^{102} which carries a leucine to valine change in the zinc-finger loop (Kudla *et. al.*, 1990). This mutation was found to elevate the expression of a number of genes including acetamidase, citrulline permease, and histidase, and to reduce the expression of other genes such as the nitrate reductase, nitrite reductase, and formamidase. The *areA*^{102} mutation also abolishes two purine permeases (Arst, 1977, Arst and Cove, 1973, Arst and Scazzocchio, 1985, Polkinghorne and Hynes, 1982, Stankovich *et al.*, 1993, Crawford and Arst, 1993).

The regulatory genes *nirA* from *A. nidulans* and *nit4* from *N. crassa* have been cloned and characterized (Burger and Scazzocchio, 1991, Fu and Marzluf, 1989), and the regions of the NIRA and NIT4 proteins which bind DNA have been found to reside within the N-termini and to share 90% identity at the amino acid level (Burger and Scazzocchio, 1991, Yuan *et al.*, 1991). Both proteins contain seven conserved cysteine residues in their DNA binding

domains which are thought to participate in Zn^{+2} chelation to form a zinc finger (Campbell and Kinghorn, 1990). They have also been found to contain a very acidic region that might be involved in gene activation. In addition, the NIRA protein sequence contains two proline-rich regions while the NIT4 contains glutamine-rich and polyglutamine-rich regions. Proline-rich, glutamine rich, and polyglutamine-rich regions may also be candidates for activation domains (Marzluf, 1993, Crawford and Arst, 1993). Studies which involved deletion of the glutamine-rich region of the NIT4 sequence have shown that this region is essential for NIT4 function (Yuan *et al.*, 1991). In contrast, deletion of the polyglutamine-rich region of the NIT4 sequence resulted in a reduction of but not in a loss of function of this protein (Yuan *et al.*, 1991, Yuan and Marzluf, 1992). The ability of *nirA* and *nit-4* genes to complement mutants of each other has been explored: it was found that although the *nirA* gene did not complement a mutant of the *nit-4* gene, the *nit-4* gene was able to complement a mutant of *nirA* gene (Yuan *et al.*, 1991, Hawker *et al.*, 1991). The inability of the *A. nidulans nirA* gene to complement its *N. crassa* homolog could be attributed to a lack of expression of the *nirA* in *N. crassa* (Yuan *et al.*, 1991). However, a hybrid gene in which the C-terminus (573 amino acids) of the NIT4 protein was replaced with the C-terminal (397 amino acids) of the NIRA protein was partially functional in *N. crassa* (Hawker *et al.*, 1991), suggesting that at least the glutamine- and polyglutamine-rich regions of the NIT4 protein can be replaced by the proline-rich regions of the NIRA protein. The partial functionality of the hybrid protein indicates that there must be similar motifs contained within the C-terminal region of the *A. nidulans* NIRA protein which can bind to similar sequences of *N. crassa* DNA and induce some activity (Hawker *et al.*, 1991). Both NIT4 and NIRA proteins contain the motif S/TPXX, a common motif found in DNA-binding

proteins (Campbell and Kinghorn, 1990). The C-terminus of the NIRA protein contains seven SPXX and two TPXX motifs whereas the C-terminus of the NIT4 protein contains six SPXX and one TPXX. It has been speculated that phosphorylation of the S/TPXX motif might modulate activity of the protein by fitting into a DNA groove (Suzuki, 1989, Suzuki and Yagi, 1991 Crawford and Arst, 1993, Marzluf, 1993).

The *areA* and *nit-2* are two global positive-acting regulatory proteins which activate the expression of a number of other unlinked structural genes in the nitrogen regulatory circuit in *A. nidulans,* and *N. crassa*, respectively. The *areA* and *nit-2* regulatory genes have been cloned and characterized and they encode protein products of 876 (*areA*) and 1036 (*nit-2*) amino acids (Kudla *et al.*, 1990, Fu and Marzluf, 1990). The AREA and NIT2 protein sequences were found to share significant homology only in some regions. One of these is a domain of 52 amino acids: a putative DNA binding region which contains a zinc finger (Cys-X2-Cys-X-Cys-X2-Cys). The Cys-X2-Cys-X17-Cys-X2-Cys region shares very significant sequence similarity to the DNA-binding domain of the GATA family. Evidence has been obtained which shows that sequence changes in the putative DNA binding domain decrease or abolish DNA binding (Kudla *et al.*, 1990, Lee *et al.*, 1990, Wootton *et al.*, 1991, Stankovich *et al.*, 1993). In addition to a zinc finger domain, the AREA and NIT2 proteins are also rich in the S/TPXX motif and in acidic regions which may be involved in gene activation (Fu and Marzluf, 1990, Kudla *et al.*, 1990).

A more interesting mutation of the *areA* gene is $areA^{300}$ which possesses an in-frame tandem duplication of 417 bp, which results in an AREA protein with two zinc fingers. The mutation not only leads to nitrogen metabolite de-repression of nitrate reductase and nitrite reductase, it also relieves the repression of thymine-7-hydroxylase synthetase and

asparaginase by atmospheric oxygen (Caddick and Arst, 1990, Shaffer and Arst, 1984, Shaffer *et. al.*, 1988). It has been postulated that the two zinc fingers contained in the $AREA^{300}$ protein function independently and alternatively, and that the two zinc fingers may allow a trans-acting protein to diversify and establish control over a new set of genes (Crawford, 1993).

Studies which involved deletions of 30 to 140 residues from the *areA* C-terminus resulted in nitrogen metabolite de-repression (Crawford and Arst, 1993). Therefore, the C-terminal region of the *areA* gene may play a crucial role in nitrogen metabolite repression (Kudla *et al.*, 1990, Stankovich *et al.*, 1993). In contrast, studies which involved transformation with deletion clones have shown that the 214 C-terminal residues of NIT2 and about 499 N-terminal residues of *AREA* are not essential (Fu and Marzluf, 1990, Arst *et al.*, 1989, Kudla *et al.*, 1990). However, it should be noted that the transformation with deletion clones performed in *N. crassa* were determined using only nitrate as the sole nitrogen source. The work has been criticized because the authors did not use a range of nitrogen sources in their work, so that the results were less informative than was the case in the *AREA* studies in *A. nidulans*. It has been argued that residues unnecessary for expression of one structural gene may be essential for the expression of other gene(s), and therefore using a single assay of functionality can not be generalized (Stankovich *et al.*, 1993).

Structure of Nitrate Assimilation Genes in Filamentous Fungi:

The nitrate assimilation pathway has been extensively studied in *Aspergillus nidulans* and *N. crassa* by means of genetic and physiological analyses (Cove, 1979, Cove and Pateman, 1963, Kinghorn, 1989 Marzluf, 1993, Crawford, 1993). Moreover, the structural genes encoding nitrate (*niaD*) and nitrite (*niiA*) reductase, as well as the gene encoding a nitrate transporter (*crnA*) have been cloned from *A. nidulans*. The genes were found to be physically linked on chromosome VIII and that the nitrate and nitrite reductase genes are divergently transcribed from an intergenic promoter region of 1200 bp (Cove, 1979, Johnstone *et al.*, 1990). The nitrate transporter (*crnA*) gene is transcribed in the same direction as the *niiA*. In addition, the *niaD* gene has been cloned from other *Aspergillus* species including *A. niger* (Unkles *et al.*, 1992), *A. parasiticus* (Chang *et al.*, 1996), and *A. oryzae* (Kitamoto *et al.*, 1995). The *niaD* and *niiA* genes in the ascomycete *Leptosphaeria maculans* were found to be transcribed in the same direction (Williams *et al.*, 1994). In contrast, the nitrate reductase (*nit-3*) and nitrite reductase (*nit-6*) gene have also been cloned from *N. crassa*; and these were found to reside on separate chromosomes but were regulated in a parallel fashion (Exley *et al.*, 1993).

The organization of nitrate assimilation genes in *A. nidulans* is conserved in all *Aspergillus* species investigated so far. The gene organization suggests that the nitrate and nitrite reductases may be co-regulated in all of the examined species. Studies have shown that the nitrate reductase gene regulates its own expression and it may also regulate the expression of the nitrite reductase (*niiA*) and the nitrate transporter (*crnA*) genes (Cove, 1969, Hawker *et al.*, 1991, Maloy, 1993).

heme-siroheme domains.

The nitrate reductase enzyme transfers two electrons donated by two NADPH molecules, and to FAD, to heme/Fe^{2+} cofactor, to molybdopetrin, and finally to nitrate thereby reducing nitrate to nitrite. The nitrite reductase then transfers six electrons from six NADPH molecules in sequence from FAD, to heme-siroheme cofactors, and finally to nitrite reducing it to ammonium ions (Campbell and Kinghorn, 1990).

a) Nitrate reductase:

The structural gene for nitrate reductase has been cloned and characterized from a number of organisms representing prokaryotes, eukaryotes, plants, and algae (Kinghorn and Campbell, 1989). These clones have allowed the use of the predicted putative amino acid sequences (from different organisms) to be compared so that sequence similarities can be inferred. Studies which involved alignment of nitrate reductase protein sequence from *Arabidopsis thaliana* (Crawford *et al.*, 1988), *Nicotiana tabacum* (Calza *et al.*, 1987), *A. nidulans* (Johnstone *et al.*, 1990), *Neurospora crassa* (Exley *et al.*, 1993), *A. niger* (Unkles *et al.*, 1991) and *A. parasiticus* (Chang *et al.*, 1996) show that there is extensive sequence homology between fungal and plant proteins. The sequence homology among plant and fungal nitrate reductase gene sequences suggests that these genes originated from a common ancestral gene (Kinghorn and Campbell, 1989).

Biochemical evidence shows that the nitrate reductase enzyme possessed three domains; FAD (flavoreductase), heme (cytochrome b577) and Molybdenum cofactor (Molybdo-reductase) with a SH group functioning as the primary electron acceptor from NADH/NADPH. Comparisons of the *A. nidulans* nitrate reductase (*niaD*) protein sequence

Biochemical evidence shows that the nitrate reductase enzyme possessed three domains; FAD (flavoreductase), heme (cytochrome b577) and Molybdenum cofactor (Molybdo-reductase) with a SH group functioning as the primary electron acceptor from NADH/NADPH. Comparisons of the *A. nidulans* nitrate reductase (*niaD*) protein sequence to yeast flavocytochrome b_2 (heme binding protein) and bovine and human NAD cytochrome b_2 (known to contain FAD cofactor domain) showed regions of similarity around the mid region and at the C-terminus, respectively (Ozls *et al.*, 1985, Kinghorn and Campbell, 1989). The *A. nidulans* nitrate reductase (NIAD) protein also shares sequence similarities with other enzymes containing molybdenum cofactor, heme, or FAD domains. Based on protein sequence alignments, the Molybdenum-cofactor, heme, and FAD domains of fungal nitrate reductases are positioned from N-terminus to C-terminus, respectively (Campbell and Kinghorn, 1990).

b) Nitrite reductase:

The structural gene for the nitrite reductase (*niiA*) has also been cloned from filamentous fungi, plants, bacteria, and algae (Campbell and Kinghorn, 1990). The nitrite reductase (NIIA) catalyzes a six step electron reduction of nitrite to ammonium. The NIIA protein accepts six electrons from three equivalents of NADPH and transfers them via bound FAD, iron-sulfur center, and siroheme to nitrite. While plant assimilatory nitrite reductase utilizes ferredoxin as the electron donor, fungal NIIA and *Escherichia coli* respiratory NIR utilize NADH/NADPH as electron donors. Plant, fungal, and *E. coli* nitrite reductase enzymes contain both an iron-sulfur center and the prosthetic group siroheme. The Fe_4S_4 domain represents tetranuclear iron-sulfur centers and the siroheme denotes a

tetrahydroporphyrin. Four cysteine residues found conserved in the nitrite reductase enzymes are thought to bind the tetranuclear iron cluster. The siroheme is also suggested to bind to one of these well conserved four cysteine residues (Back *et al.*, 1988).

c) Nitrate transporter:

The structural gene for a fungal nitrate transporter (*crnA*) gene has been cloned and characterized from *A. nidulans*. The gene encodes a 1.8 kb mRNA transcript which translates to a membrane protein of 507 residues with 12 putative transmembrane helices (Unkles *et al.*, 1991 and 1995). Mutants of the *A. nidulans crnA* gene grew on nitrate as the only nitrogen source implying that an alternative nitrate transport system exists in *A. nidulans* (Crawford and Arst, 1993). In fact, a second nitrate transporter gene (*crnB*) has been identified in *A. niger* (Debets *et al.*, 1990b) and has recently been cloned from *A. nidulans* (A. Glass, pers. comm.). The *A. nidulans crnA* gene shares significant homology with two genes encoding high-affinity NO_3^- transporter: *nar-3* and *nar-4* isolated from the alga, *Chlamydomonas reinhardtii* (Quesada *et al.*, 1994).

MATERIALS AND METHODS

Bacterial strains and growth conditions used

The *E. coli* strain KW251 (Promega) was used to propagate the λEMBL3 genomic library. The strain DH5α was used to propagate the plasmid Bluescript® and subclones in this vector. KW251 and DH5α (BRL) were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). The plasmid PJD21 containing the bacterial hygromycin B phosphotransferase (*hph*) gene was kindly provided by Dr. Holden (Dept. of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, London, UK). The plasmid p*niiA* containing the *A. nidulans* nitrite reductase gene (*niiA*) was obtained from the Fungal Genetics Stock Center (FGSC).

Fungal strains and growth media and conditions

Wild-type *Aspergillus fumigatus* isolates ATCC 13073 and 42202 were used in this study. Stock cultures were grown on MYPD medium slants and plates (0.3% Malt extract, 0.3% Yeast extract, 0.5% Bacto Peptone, 1.0% Dextrose, with 2% agar) as recommended by ATCC. The slants were stored at 4° C under sterile mineral oil. The *A. fumigatus* ATCC 13073 strain was used to prepare genomic DNA and total RNA, and to prepare protoplasts for pulsed field gel electrophoresis and transformations. *A. fumigatus* ATCC 42202 was also used to prepare genomic DNA while the genomic library DNA was from *A. fumigatus* strain CHUV 192-88 of Jaton-Ogay *et al.* (1992). The *A. fumigatus* isolates were grown in MYPD medium. Cells in liquid cultures were grown for 2 to 3 days at 28° C with shaking at 240 rpm, and cells on plates were grown for a week at 28° C. Minimal growth media containing various N-sources were prepared as described by Cove (1966).

Genomic Library Screen

An *Aspergillus fumigatus* (CHUV 192-88) λEMBL3 genomic library was provided by Dr. M. Monod [Laboratoire de Mycologie (Service de Dermatologie), Center Hospitalier Universitaire Vaudois, Lausanne, Switzerland]. A titre of about 2 x 10^9 pfu/ml was used. Approximately twenty thousand phage plaques were screened with a 0.8 kb EcoRI/EcoRV cDNA fragment corresponding to the nitrite reductase gene (*niiA*) of *A. fumigatus*. Three rounds of screening were carried out according to standard procedures as outlined in Sambrook *et al.*, (1989).

Phage DNA extraction

Phage DNA was extracted using the Qiagen phage prep kit (Qiagen).

Plasmid DNA isolation

Plasmid DNA was isolated either using the alkaline plasmid prep method (Sambrook *et al.*, 1989) or using a plasmid prep kit (Qiagen). The DNA extracted with either of these method was used for both restriction digests and sequencing reactions.

Restriction enzymes

All restriction enzymes were purchased from either from Bethesda Research Laboratories (BRL) or from Pharmacia. The amount of restriction enzyme used per reaction was generally about 2 units of enzyme per 1 µg of DNA. The digestion reactions were carried out at 37° C for at least one hour, using the reaction buffers

provided by the supplier.

Agarose gel electrophoresis

Genomic and cloned DNA digested to completion was loaded on 0.7% agarose gel and run in 0.5X TBE buffer as described in Sambrook *et al.* (1989). DNA was visualized under ultraviolet light (UV) by addition of ethidium bromide, (1 µl of a 10_{mg}/ml stock solution per 100 ml of agarose), before pouring the gel. Gels were photographed using a UVP gel documentation system.

Southern blots

Gels containing DNA to be transferred were soaked in four volumes of 0.25 M HCl for 15 minutes, then washed with distilled water, and soaked in four volumes of 0.5 NaOH with 1.5 M NaCl for 30 minutes. The gels were subsequently soaked in four volumes of 1.5 M NaCl and 0.5 M Tris-HCl for 45 minutes. The transfer of DNA to a nylon membrane (Amersham) was carried out using standard procedures as described by Sambrook *et al.*(1989). When the transfer was complete, the blots were soaked in 2X SSC and the DNA was cross-linked to the nylon membrane by UV irradiation using a Strata Linker (Stratagene).

Labeling of DNA probes

All of the probes used in this study were labeled with ^{32}P-dATP or dCTP using the oligolabeling technique of Feinberg and Voglestein (1983). Random hexamer primers were obtained from Pharmacia. The labeling reaction was carried out overnight

at room temperature. Then the probe was put through a G-25 Sephadex packed in a 1 ml syringe. The specific activity of the probe was measured using a liquid scintillation counter (Beckman) and the required amount of probe was used (1 $x10^6$ counts per ml of hybridization buffer).

Hybridization of probes to DNA blots

DNA blots were first prehybridized overnight in the hybridization buffer (5XSSC, 0.5% SDS), which contained 2.5% Denhardt's solution (50X stock solution: 1X Denhardt's is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone) at 68°C. Then freshly filtered hybridization buffer with the probe was added and hybridization was carried out at 68°C overnight (sometimes the hybridization was carried out in a hybridization buffer containing 50% formamide). Next day the blots were washed in 1X SSC and 0.1% SDS buffer at 68°C four periods of 15 minutes. Kodak X-OMAT or Dupont films were placed on the blot with DuPont lighting-plus-intensifying screen and kept at –70° C or room temperature overnight. Generally autoradiographs were developed the next day.

Subcloning

Genomic DNA fragments to be subcloned were purified from agarose gel using a Sephaglas® BandPrep Kit (Pharmacia). DNA fragments were ligated to the plasmid Bluescript® using the enzyme T4 ligase, as recommended by the supplier (BRL). The *E. coli* transformation procedure was carried out as described in the BRL transformation protocol. Transformed cells containing plasmids with inserts were detected as white

colonies on agar plates containing ampicillin, X-gal (5-bromo-4 chloroindoyl-Beta-D-galatoside) and IPTG (isopropyl thiogalactoside); Mini-preps of DNA from a sample of white colonies was digested with diagnostic restriction enzymes and suitable subclones were identified in agarose gels.

Preparation of a nested set of deletions

Some of the *niiA* gene sequencing made use of nested deletions. The deletions were created using the Erase-a-Base System (Promega) from a 2.8 kb EcoRI/PstI genomic subclone containing a portion of the *A. fumigatus niiA* gene cloned in pYA22/3, Fig. 3). The resulting fragments were sequenced using the reverse primer of the vector plasmid (Bluescript®).

Oligonucleotides

Commercially available forward and reverse primers were used to sequence the ends of the inserts from the various subclones generated in this study. Oligonucleotide primers (18 nucleotides in length) were designed from genomic sequence for either PCR or for subsequent sequencing as it became available, using the program OLIGO (Rychlik and Rhoads, 1989). All oligonucleotides were obtained from the GIBCO BRL Custom Primers service (GIBCO). Such primers were used to obtain the *niiA* gene sequence contained in pYA2-3 and pYA61.

Sequencing methodology and sequence analysis

DNA sequencing was performed using the dideoxy termination method of Sanger *et al.* (1975). All DNA fragments to be sequenced were submitted as DNA, or as "ready reactions", and were sent to the automated sequencing services at either the Biotechnology Laboratory, IMBB, Simon Fraser University, Burnaby, BC., or the University Core Services, The University of Calgary, Calgary, AB. Both strands of the *niiA* gene were sequenced.

DNA sequences determined were used to search the data banks using THE BLAST SERVICE NET WORK, NCBI. Subsequent DNA and protein sequence analyses were conducted using the following computer programs: (I) FASTA (Pearson and Lipman, 1988); (II) ESEE (The Eyeball Sequence Editor) (Cabot and Beckenbach, 1989); and (III) Clustal W (Higgins and Sharp, 1988).

Polymerase chain reaction (PCR)

PCR reactions were carried out in 25 µl reaction volumes with 25-50 ng of DNA as template, 25 pmol of both primer sets, 1.25 mM dNTPs, and 0.5 to 1.0 units of TAQ polymerase (Promega). The conditions used were as follows: 35 cycles of 30 seconds at 96° C, 60 seconds at 55° C, 60 seconds at 72° C. PCR products were separated on a 1% agarose gel.

Preparation of total RNA and Northern blotting

Mycelia were collected by filtration under sterile conditions. 100 mg (from 100 ml culture) of wet mycelia were frozen in liquid nitrogen and ground to powder in a pre-cooled mortar and pestle. Total RNA was isolated from the cell powder using the Trizol reagent (BRL) as described by the manufacturer. Approximately 20 µg samples were Electrophoresed on a 1.2% formaldehyde denaturing agarose gel. The RNA was transferred to Hybond^{+} nylon membrane (Amersham International) by Northern blotting as described in Sambrook *et al.* (1989) and RNA was cross-linked to the nylon membrane by UV irradiation using Strata Linker (Stratagene).

Hybridization to ^{32}P-labelled probes was carried out at 42° C in 50% formamide. A 0.8 kb cDNA fragment was used as a probe for nitrite reductase (*niiA*) gene, a 1.6 kb EcoRI genomic fragment for nitrate reductase (*niaD*) gene, and a 0.6 kb EcoRI genomic fragment for the nitrate transporter (*crnA*) gene. Filters were washed at 55° C in 0.1 x SSC, 0.1% SDS. Autoradiography was carried out overnight at room temperature.

Protoplast preparations for pulsed field gel analysis and transformation

For *Aspergillus fumigatus* the sorbitol method for preparations of mycelial protoplasts was used, exactly following the protocol devised by Debets and Bos (1986) for *Aspergillus niger* (for details, see Appendix section II).

Pulsed field electrophoresis

Fungal protoplasts and protoplast-agarose plugs for pulsed field gel electrophoresis were prepared as described by Ehninger *et al.* (1990). Pulsed field electrophoresis was performed using the Gene Navigator™ System (Pharmacia). A 15 x 15 cm 0.8 % gel was prepared as described in the user's manual (Pharmacia). DNA-agarose plugs were loaded on the gel following the direction described in the user's manual. The gel was run at 50 V with a pulse duration of 72 h, 48 h, 24 h, 24 hr and with a pulse switch of 55, 47, 40, and 37 min respectively, in 0.5 x TBE at 4°C. Two consecutive runs of the program were required to obtain a good separation of the chromosomes. The gel was stained in 5µg /ml ethidium bromide for 1 h, de-stained for 1 h and photographed under UV illumination. For transfer of DNA, the gel was treated twice with 0.25 M HCl for 15 min and then was denatured in a solution containing 0.5 M NaOH and 1.5 M NaCl for 1 h. It was subsequently neutralized in a solution containing 1.5 M NaCl, 0.5 M Tris-HCl for 1 h. The DNA was transferred to Hybond™ N^+ nylon membrane (Amersham) by capillary action as described in Sambrook *et al.* (1989). The DNA was cross-linked to the nylon membrane by UV irradiation using the Strata Linker apparatus (Stratagene). The *Schizosaccharomyces pombe* chromosomal DNA was used as a size marker (BRL). To identify the DNA molecule carrying the nitrate assimilation gene cluster, a 2.8 kb EcoRI/PstI *niiA* genomic fragment was used as a hybridization probe.

Construction of pYA10

Plasmid pYA22-3 contains a 2.8 kb EcoRI/PstI genomic fragment corresponding to part of the *A. fumigatus* intergenic *niiA-niaD* region plus the 5' half of the *niiA* gene. This plasmid was double digested with HindIII and SalI, and a 0.9 kb HindII/SalI fragment (corresponding to the 5' end of the *niiA* locus) and 4.9 kb fragment were released. The 4.9 kb HindIII/SalI fragment therefore corresponds to the Bluescript® vector with the rest of the genomic *niiA* DNA (i.e. a 5' internal fragment) was purified. A 2.4 kb HindIII/SalI fragment containing the bacterial hygromycin gene was obtained by digesting the plasmid pID21 of Tang *et al.* (1992) with HindIII/ SalI. The purified 2.4 kb HindIII/SalI containing the hygromycin gene was ligated to the 4.9 kb HindIII/SalI to produce pYA10. The plasmid pYA10, therefore, contains a 1.9 kb HindIII/PstI fragment corresponding to some of the 5' coding sequence of the *A. fumigatus niiA* gene and the bacterial hygromycin gene which flanked by 5' transcription initiation sequences and 3' termination sequences of the *A. nidulans trpC* gene for expression in *Aspergillus* (for an overview see Fig. 22, section III).

Protoplasting and Transformation procedures for gene disruption

Mycelial protoplasts of *A. fumigatus* wild type were obtained using the protocol of Debets and Bos (1986; see Appendix II). For genetic transformation the method of Osmani *et al.* (1989) was used (see Appendix II).

Analysis of putative *niiA*⁻ mutants

Transformants, resulting from disruption of the *niiA* gene, expected to be defective in nitrate assimilation and resistant to the antibiotic hygromycin B such types were identified by their poor growth on test media, i.e. minimal media = MM containing 10 mM sodium nitrate as the sole nitrogen source compared to MM with ammonium tartrate (10 mM) and by resistance to the antibiotic hygromycin B (250 μg/ml). Potential transformants were tested for normal growth in minimal media containing ammonium. Plates were incubated at 28° C for 3 to 5 days, and corresponding tests using liquid cultures were incubated at the same temperature with shaking at 200 rpm for 1-3 days.

Test for *niiA* mutant phenotypes and mitotic stability of disruption transformants

To check the stability of transformants, protoplasts from one nitrate-defective and hygromycin B resistant transformant (T6) were prepared and were plated to obtain single colonies deriving from single protoplast on minimal media containing ammonium tartrate without hygromycin B. Conidia from such colonies were inoculated into the same non-selective medium and incubated for 3 to 5 days. After vegetative growth, conidia were then transferred onto test minimal media containing hygromycin B (250 μg /ml) and either ammonium or nitrate as the sole nitrogen source. Such tests were scored for growth and conidiation after 3 to 5 days.

Fungal DNA extraction

DNA from wildtype and mutant cells was isolated using the phenol chloroform method as described by May *et al.* (1985, updated in 1991, see Appendix section II).

Southern analysis of transformants; probes used

To confirm the gene disruption postulated in transformant T6, based on its phenotype, the genotype was determined by Southern analysis. A 1.9 kb HindIII/ PstI genomic fragment corresponding to part of the 5' coding sequence of the *A. fumigatus niiA* gene was used as a probe to compare DNA from both wild-type and transformant T6 cells. In addition, the plasmid Bluescript® and the hygromycin B phosphotransferase (*hph*) gene were also used as probes to characterize the DNA insert into the *niiA* locus of the *niiA*⁻ transformant (T6).

Chlorate toxicity assay

To obtain a measure of chlorate sensitivity, conidia of wild-type *A. fumigatus* (ATCC 13073) were inoculated into minimal medium containing ammonium tartrate, urea, or L-arginine as the sole nitrogen source, with and without potassium chlorate, at concentrations of 100 mM, 200 mM, and 400 mM. After incubating the plates for 5 days at 28° C, the radial diameters of colonies were recorded.

Transformation of the *A. fumigatus niiA*-defective Hyg^r transformant T6 with cloned *niiA* DNA from *A. fumigatus* (pYA2-3) and *A. nidulans* (p*niiA*)

Protoplasts from *A. fumigatus* nitrite reductase-defective Hyg^r transformant (T6) were transformed with the plasmids pYA2-3 and p*niiA* (obtained from the Fungal Genetics Stock Center). The pYA2-3 contains the entire structural gene of the *A. fumigatus niiA* gene plus a part of the *niiA-niaD* intergenic region. The p*niiA* contains an *A. nidulans* genomic fragment corresponding to the entire *niiA* gene structure including the *niiA-niaD* intergenic region and the *niaD* gene. Transformed protoplasts were plated on sucrose minimal medium containing nitrate (5 mM) as the sole nitrogen source. The plates were incubated at 30° C for 7 days. Colonies appearing on the selection plates were tested for their ability to grow on nitrate as the sole nitrogen source using minimal medium. In addition, untransformed protoplasts from the *niiA*$^-$ mutant were plated on sucrose minimal medium containing either ammonium or nitrate as the sole nitrogen source.

RESULTS

Section I

Cloning and characterization of the nitrate assimilation gene cluster (*crnA-niiA-niaD*) of *A. fumigatus*

Originally 1.5 kb EcoRI cDNA clone (pYA06) corresponding to the *A. fumigatus* nitrite reductase (*niiA*) gene was isolated (for details see Appendix p. 152).

a) Screening of an *A. fumigatus* a genomic library with a 0.8 kb *niiA* cDNA

A 0.8 kb EcoRI/EcoRV cDNA fragment (derived from pYA06) of the *A. fumigatus niiA* gene [see Appendix, Fig. 33] was used as a probe to screen an *A. fumigatus* (ACHUV 192-88) λEMBL3 genomic library, and one genomic clone λYA4-3 was isolated. When λYA4-3 was completely digested with EcoRI, fragments of 6-7 kb, 1.6, and 0.7 kb were observed (Figure 1a). Southern blot analysis using the 0.8kb EcoRI/EcoRV cDNA as a probe resulted in hybridization with a genomic 6-7 kb fragment (Figure 1b). Subcloning of the 6-7 kb (double) band into the pBluescript® vector resulted in two clones (pYA1-2 and pYA2-3) with 6.5 kb and 7 kb EcoRI inserts, respectively. Similarly, the two smaller fragments were subcloned (clones pYA60 and pYA61). Only one of these subclones, pYA2-3 with the 6.5 insert, was subsequently found by Southern dot blot analysis to hybridize to the cDNA probe (Figure 2), while the other large fragment apparently is located elsewhere in the genome (see Discussion I)

b) Identification of the entire nitrite reductase gene (*niiA*)

Based on sequencing data and homology to *A. nidulans*, the orientation of clone pYA2-3 with respect to the flanking small EcoRI fragments (pYA60 with the 0.7 kb insert

on the left and pYA61 with the 1.6 kb insert on the right) as well as the location of a PstI site in the middle of pYA2-3 were identified (Figure 3). Sequences of region 3 had no similarity to known data bank protein sequences.

The presence of the PstI site in the middle of the 6.5 kb EcoRI insert of pYA2-3 allowed the subcloning of the two PstI/EcoRI fragments with 3.7 kb and 2.8 kb insert in clones pYA21-3

(= left half) and pYA22-3 (= right half), respectively. The sequence obtained from regions 4 and 5 (see Figure 3) showed extensive homology with the nitrite reductase gene from *A. nidulans* (Johnston *et al.*, 1990) making it possible to conclude that whole gene was present. Complete characterization of the *A. fumigatus* nitrite reductase gene is presented below (Section II of Results).

c) Identification of partial *crnA* (nitrate transporter) gene coding sequence

The sequence obtained from region 6 showed extensive homology (60% identity over 85 amino acids) to the 5' end of the *A. nidulans* nitrate transporter gene (*crnA*) (Unkles *et al.*, 1991, 1995). Regions 7 and 8 of pYA60 were sequenced using the reverse and forward primers, respectively, and the genomic sequences obtained were used to search the protein database. The results showed extensive homology with a nitrate transporter gene (*crnA*) from *A. nidulans* (Unkles *et al.*, 1991). The adjacent sequences obtained from region 7 of pYA60 and region 6 of pYA2-3 were combined (Figure 3) to give a total of 657 bp (Figure 4a). The 657 bp sequence corresponds to a part of the N-terminus region of the *A. fumigatus* *crnA* and translated (148 putative amino acids) it aligned to the corresponding part of *A. nidulans crnA*. The alignment of this polypeptide from *A. fumigatus* showed extensive

amino acid homology (80% identity, Figure 4b) (Unkles *et al.*, 1991, 1995). The genomic sequence was also found to contain two small putative introns, 50 and 69 bp in length (Figure 4a). In addition, the genomic sequence of 351 bp obtained from region 8 of pYA60 (Figures 3 and 5a) was translated (116 putative amino acids) and was aligned with the *A. nidulans CRNA* protein sequence from amino acid 221 to 340 (Figure 5b). Figure 6 shows the position of the two parts translated from the sequencing of regions 6-7 and 8, (Figure 3) obtained from the *A. fumigatus crnA* gene with respect to the *A. nidulans* CRNA amino acid sequence.

d) Identification of the *niiA-niaD* intergenic region and partial *niaD* (nitrate reductase) gene sequence.

Initial sequence data obtained from region 2 of pYA61 (1.6 kb in Figure 3) did not show any amino acid homology to known protein sequences in the databanks. More nucleotide sequence was subsequently obtained using a primer designed from the sequenced part of region 2, resulting in a total of 767 bp (Figure 7a) identified from left end of pYA61. Results identified part of the N-terminal coding sequence of the *A. fumigatus* nitrate reductase gene (*niaD*) plus part of the *niaD-niiA* intergenic region (promoter and regulatory region). The N-terminal coding sequence was found to contain three putative introns, 38, 53, and 75 bp in length (Figure 7a). Figure 7b shows the alignment of the 91 N-terminal deduced amino acids from *A. fumigatus* with that of *A. niger* (Unkles *et al.*, 1992), *A. parasiticus* (Chang *et al.*, 1996), and *A. nidulans* (Johnstone *et al.*, 1990).

The genomic sequence obtained from region 1 of pYA61 using the reverse primer (Figure 3) showed extensive homology to (AA 129-310) of the nitrate reductase (NIAD) protein

sequence from *Aspergillus niger* (Unkles *et al.*, 1992). More sequence was identified from this end of pYA61 using a primer designed from the region 1 sequence; resulting in a total of 598 bp coding sequence which was found to contain two small introns, 57 and 39 bp in length (Figure 8a). The sequence encodes a 179 putative amino acids which shares 79.9 % identity and 89.9% similarity with the corresponding protein sequence from NIAD proteins from *A. parasiticus* and *A. niger* (Figure 8b). Figure 8c shows a multiple alignment of the 197 deduced amino acids from *A. fumigatus* nitrate reductase with protein sequences from *A. niger*, *Ustilago maydis* (Banks *et al.*, 1993), *N. crassa* (Okamoto *et al.*, 1991), *Hordeum vulgare* (Schnorr *et al.*, 1991), and *Arabidopsis thaliana* (Crawford *et al.*, 1988).

e) Sequence analysis of the of the remaining 7 kb EcoRI fragment in pYA1-2

The ends of the 7 kb EcoRI genomic insert of pYA1-2 (Figure 10) were sequenced. These (R and F) DNA sequences did not show any significant homologies with known gene sequences in the data banks (Figure 11a and b). To further investigate this 7 kb EcoRI genomic fragment, two adjacent fragments were subcloned; a 2.2 EcoRI/EcoRV and a 2.8 kb ClaI/EcoRV fragments into the plasmid vector pBluescript® producing plasmids pYA211-2 and pYA211-3 (Figure 10). The DNA sequence of the ends of these subclones were determined and used to search the data banks using Blastx. The genomic sequences derived from the ends of pYA211-3 did not show any significant homology with known protein sequences in the databanks (Figure 11c and d)

Figure 1. Southern analysis of the *A. fumigatus* λYA4-3 genomic clone with 0.8 kb EcoRI/EcoRV *niiA* cDNA:

(a) Ethidium bromide stained gel showing an EcoRI digest of λYA4-3. (b) Southern analysis of the EcoRI digest of λYA4-3 probed with the 0.8 kb EcoRI/EcoRV *niiA* cDNA. The probe detected a genomic fragment between 6 and 7 kb in size. M = 1 kb marker.

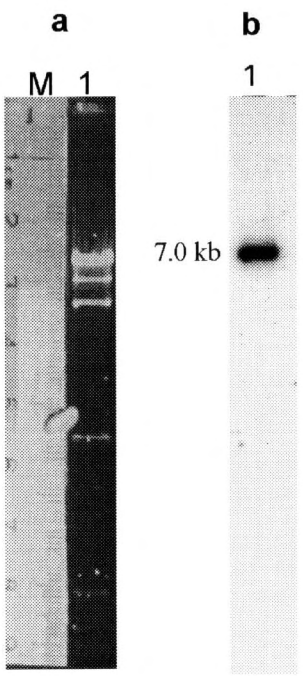
a
b
M
1
1
7.0 kb

Figure 2. Dot blot analysis of pYA1-2 (7 kb insert) and pYA2-3 (6.5 kb) using the same cDNA probe as in Fig. 1. The *niiA* cDNA probe hybridized to subclone pYA2-3.

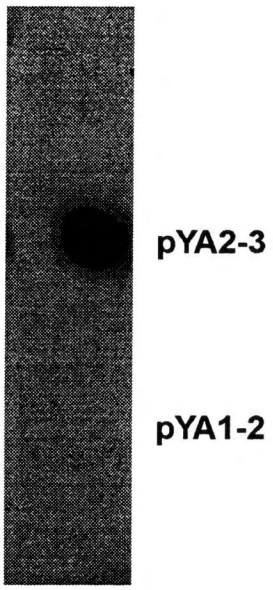
pYA2-3
pYA1-2

Figure 3. Sequencing of the ends of the 6.5 kb EcoRI genomic insert in pYA2-3 from *A. fumigatus* and the two ends adjacent EcoRI fragments reveals that this insert contains the entire gene sequence for the nitrite reductase gene and partial sequences of a nitrate transporter, while the nitrate reductase gene is located in the adjacent EcoRI fragment (on the right) and the nitrate transporter genes.

The following homologies to *A. nidulans* sequence were identified: regions 1 and 2 of pYA61 on the right correspond to the *niaD* (nitrate reductase) gene coding region and part of the *niaD-niiA* intergenic sequences, respectively. Region 3 (of pYA2-3) also corresponds to the *niiA-niaD* intergenic region. The middle regions 4 (of pYA22-3) and 5 (of pYA21-3) correspond to the *niiA* (nitrite reductase) gene coding sequence. The three Regions 6 (of pYA21-3), 7 and 8 (of pYA60) were found to correspond to the *crnA* (nitrate transporter) gene coding sequence. Bold arrows indicate the deduced direction of transcription of *niaD*, *niiA*, and *crnA* of *A. fumigatus* below. F = Forward primer, R = Reverse primer, E = EcoRI, and P = PstI.

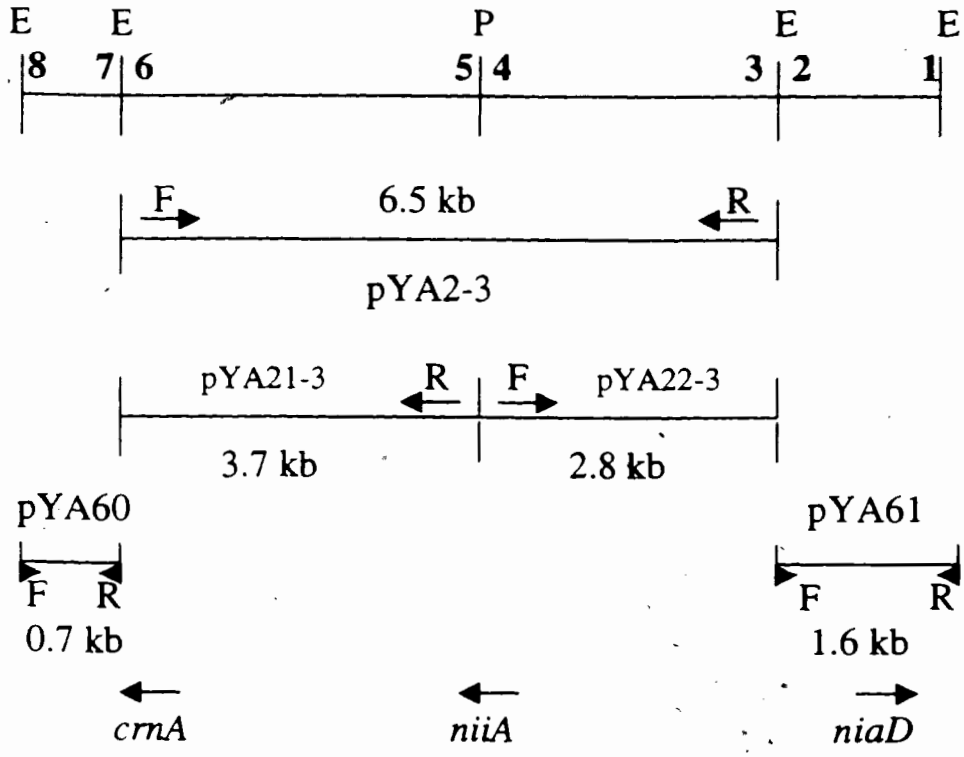
E E P E E
8 7 6 5 4 3 2 1
F
6.5 kb
R
pYA2-3
pYA21-3
R
F
pYA22-3
3.7 kb
2.8 kb
pYA60
pYA61
F R
F R
0.7 kb
1.6 kb
crnA
niiA
niaD

Figure 4. Analysis of DNA sequence obtianed from regions 6 and 7 of figure 3.

(a) Nucleotide sequence and deduced amino acid sequence from regions 6 and 7 (see Figure 3).

(b) Alignment of the *A. fumigatus* (*Af*) amino acid sequence with the corresponding region of the *A. nidulans* (*An*) nitrate transporter (CRNA) protein sequence indicating extensive sequence homology (52% identity). The (*) denotes an identical amino acid, and the (·) denotes a conserved amino acid. The gaps were inserted to maximize the sequence alignments, using the program "Clustal W".

a

```
GGTCAATACCAGTCCTCAATCCCCTCGACCGATATGTCCGGGTATTCTTCTTCCTCGTGG
 G  Q  Y  Q  S  S  I  P  S  T  D  M  S  G  Y  S  S  S  S  W   20
CTTGGCTTCATGGTTGCATACCTGTCATGGTATGCGTTTCCACCTCTGgtaaggcaatgg
 L  G  F  M  V  A  Y  L  S  W  Y  A  F  P  P  L               36
tctcctatacggcaagcacttggaaacgtggcacaattgactgagaatggctgtctagTT
                                                             L  37
AACGGTGACCATTCGCAAAGATCTTCATATGACACAGCAACAAGTCGCAAAATCGAACAT
  T  V  T  I  R  K  D  L  H  M  T  Q  Q  Q  V  A  K  S  N  I  57
TGTTGCTCTTCTGGCTACgtaagtattgaacggaaagttctttcggaaaaccatgctgac
  V  A  L  L  A  T                                           63
gagcccagATTCCTTGTTCGATTCATCGCTGGGCCACTATGTGACCGCTTCGGACCTCGA
         F  L  V  R  F  I  A  G  P  L  C  D  R  F  G  P  R   80
CTTGTCTTTATCGGCCTCTTGCTTTGTGGCTCTATTCCTACAGCTATGGCTGGTCTAGTG
L  V  F  I  G  L  L  L  C  G  S  I  P  T  A  M  A  G  L  V   100
ACCACCCCTAATGGTTTAATCGCTTTGCGTTTCTTCGTTGGCATCCTTGGTGGCACATTT
 T  T  P  N  G  L  I  A  L  R  F  F  V  G  I  L  G  G  T  F  120
GTCCCTTGCCAAGTATGGTGCACCGGTTTCTTCGATAAGAACATTGTGGGAACCGCCAAC
 V  P  C  Q  V  W  C  T  G  F  F  D  K  N  I  V  G  T  A  N  140
TCTCTGGCCGCAGGCTGGGGTAATGCGGGAGGTGGG
 S  L  A  A  G  W  G  N  A  G  G  G                         148
```

b

```
An    1   MDFAKLLVASPEVNPNNRKALTIPVLNPFNTYGRVFFFSWFGFMLAFLSWYAFPPLLTVT
Af        ----------------GQYQSSIPSTDMSGYSS----SSWLGFMVAYLSWYAFPPLLVT
                          ..   .**     .          ** *** * ************

An        IRDDLDMSQTQIANSNIIALLATLLVRLICGPLCDRFGPRLVFIGLLLVGSIPTAMAGLV
Af        IRKDLHMTQQQVAKSNIVALLATFLVRFIAGPLCDRFGPRLVFIGLLLCGSIPTAMAGLV
          ** ** * * * * ********* ***** ****************** ***********

An        TSPQGLIALRFFIGILGGTFVPCQVWCTGFFDKSIVGTANSLAAGLGNAGGG 161
Af        TTPNGLIALRFFVGILGGTFVPCQVWCTGFFDKNIVGTANSLAAGWGNAGGG
          * * ******** ********************* *********** ******
```

Figure 5. Analysis of DNA sequence obtained from region 8 of figure 3:

(a) Nucleotide sequence and deduced amino acid sequence obtained from region 8. (see Fig. 3).

(b) The *A. fumigatus* sequence showed extensive sequence homology (60 % identity) with the 3' half of the *A. nidulans* nitrate transporter (CRNA) protein sequence. The (*) denote an identical amino acids, and the (·) denotes a conserved amino acid. The gaps were inserted to maximize the sequence alignments using the program Clustal W.

a

```
GAATACCCCTACTGCCAAATGGTGGGAGCGCCACATCTGGATGAAGGAGAATAATGAATC 60
  N  T  P  T  A  K  W  W  E  R  H  I  W  M  K  E  N  N  N  E 20
CCAAGCCAATATCGTCAACCTCATGTCAGGCAGCCCCTCTACCCGCCCATCTGGTCCTCC 120
  Q  A  N  I  V  N  L  M  S  G  S  P  S  T  R  P  S  G  P  P 40
ATCTATCAATATATCCACTCCGCAATCTGAGAAGAAGGGTGCCCAGACACCTCAGATGGT 180
  S  I  N  I  S  T  P  Q  S  E  K  K  G  A  Q  T  P  Q  M  V 60
CGATCCTGAAGCTCAAGCTGTTGGACAGGTTGACATCCTCAGAGCAGACACCGTGGTGGC 240
  D  P  E  A  Q  A  V  G  Q  V  D  I  L  R  A  D  T  V  V  A 80
CCCCAGCCGCAAGGAGGTCATGAACGTTCTTCTCAGTCTCTCCACGGCCGCTGTCGCCAT 300
  P  S  R  K  E  V  M  N  V  L  L  S  L  S  T  A  A  V  A  I 100
CCCATACGCCTGTTCCTTTGGATCCGAGCTGGCTATCAACTCCATTCTGGG          351
  P  Y  A  C  S  F  G  S  E  L  A  I  N  S  I  L             116
```

b

```
An 181 IFDSLIRDQGLPAHKAWRVAYIVPFILIVAAALGMLFTCDDTPTGKWSERHIWMKEDTQT
Af     ----------------------------------------NTPTAKWWERHIWMKENNES
                                                *** ** ******** ...

An     ASKGNIVDLSSGAQSSRPSGPPSIIAYAIPDVEKKGTETP--LEPQSQAIGQFDAFRANA
AF     Q--ANIVNLMSGSPSTRPSGPP-SINISTPQSEKKGAQTPQMVDPEAQAVGQVDILRADT
          *** * **. *.****** *  . *  ****..**  ..*..**.** *  ** .

An   VASPSRKEAFNVIFSLATMAVAVPYACSFGSELAINSIL 380
Af   VVAPSRKEVMNVLLSLSTAAVAIPYACSFGSELAINSIL
     *. ***** **. **.* ***.****************
```

Figure 6. Position of the putative amino acid sequences (indicated by bold bars) obtained from the *A. fumigatus* nitrate transporter gene (*crnA*) with respect to that of *A. nidulans*. Numbers 1-507 correspond to the amino acid sequence of the *A. nidulans* CRNA.

A. nidulans CRNA sequence

Figure 7. Analysis of the *A. fumigatus niaD* geneomic sequence obtained from region 1 of the plasmid pYA61:

(a) Nucleotide sequence and its corresponding putative amino acid sequence obtained from pYA61. The partial sequence of the *niaD-niiA* intergenic region and the nontranslated 5' end of the transcript [-270 to –1] are shown, and the three introns in the *niaD* gene are indicated by lowercase letters in bold.

(b) Multiple alignment of the partial N-terminal putative amino acid sequence of *A. fumigatus* (*Af*) *niaD* with that of *A. niger (Anig)* (Unkles *et al.*, 1992), *A. parasiticus* (*Ap*) (Chang *et al.*, 1996 and *A. nidulans* (*An*) (Johnstone *et al.*, 1990. The (*) and (·) denote identical and similar amino acids, respectively. The gaps were inserted to maximize the sequence alignment using the program "Clustal W".

a

```
TACTAGCTGCTTTTCAACACAATACTACCTTGAGATCCCCGCGGGAGATCGTCATCATGA -270
AGATATCCCTGGCCTGCTTCTAGCCTTTCTCCGAGGCCATTTGTTTATACAGACAAATAT -210
GGCAATATCTGAGTCAAGACAATGTGTCTGACTACTGGCTTCCTCACTTGTAAACAGAAT -150
GCGTCATTTTCCTGATTCATTCCATCCTGACTATGGTTGCCTTCTTCTGTCTGGCCTATC  -90
CTTGAGGGATTTCTAGTCTAGAAAAGCTGGCTCGCTTCTCCGTGTTTCCTTCATTCTATA  -30
CCGACATTATCAGCTGTAGTACTCTGAGCAATGGCCACCATAACCCAAGTTCAGACACAG   30
                                M  A  T  I  T  Q  V  Q  T  Q    10
GGAGACACACACGGTCTTGCCGAAGAGCCTTACGgtagtgccaggccagatcaccgtcaa   90
 G  D  T  H  G  L  A  E  E  P  Y                               21
gggagatctcgaatctcgacctgccagATATCCCCCTTCCACCACCATCCACGAACCCAT  150
                          D  I  P  L  P  P  P  S  T  N  P      32
CTGAGATCCTCGCTCAAGACAAGGGCACACCAGACAATCATgttcctccgagaccctcgg  210
S  E  I  L  A  Q  D  K  G  T  P  D  N  H                      47
ctcatccagattaacgggcgtccacccattcaatgtcgaacctccactggacagCTTTGT  270
                                                   L  C       49
TCAATGAAAGTATGCACTGCCTCCTTGAAGCTCACTTTGTTGCAATTGGCACGGCGCTCG  330
 S  M  K  V  C  T  A  S  L  K  L  T  L  L  Q  L  A  R  R  S   69
GCTCACAACCCCACgttaaggtttccttgaaatcaccggaactcttctacagGAACCATG  390
 A  H  N  P  T                                     N  H       76
GGCCTGTCCCTCTGGTCCGCGAAGAAGAATATTCCCAACTGGGAAAT               437
G  P  V  P  L  V  R  E  E  E  Y  S  Q  L  G  N                91
```

b

```
Af    1 -MATITQVQTQGDTHG---LA-------EEPY------DIPLPPPSTNPSEILAQDKGTP
Anig  1 -MATVTEVLTEPFTAQGVTLKSGPIKVHQEELPAVELSDIPLPPPSKEPTEVLSIDKPTP
Ap    1 -MATITEVRTDALVPTDLVLKTGQIKIQSEEISTKDLSDIPLPPPSKRPTEVLSVDKGTP
An    1 MSTTVTQVRTGSIPKT---LKTSQIRVEEQEITELDTADIPLPPPSKEPTEVLSLDKTTP
           .*.*.* *          *                  ********  *.*.*. ** **

Af      DNHLCS-------MKVCTASLKLTLLQLARRSAHNP------TNHGPVPLVREEEYSQLG 90
Anig    DYHVPRDPRLIRLTGVHPFNVEPPLTALYDEGFLTSPELFYVRNHGPVPLVKDEDIPNWE 123
Ap      DSHVPRDPRLIRLTGVHPFNVEPPLTDLYKEGFLTSPELFYVRNHGPHVKD--EDIPHWE 121
An      DSHVPRDPRLIRLTGVHPFNVEPPLTALFQQGFLTPPELFYVRNHGPVPHVRDEDIPNWE 181
         * *.          *     .  *  *       .                      *
```

Figure 8. Analysis of genomic sequence obtained from region 1 of pYA61:

(a) Nucleotide sequence and its corresponding amino acid sequence obtained from pYA61 using both the reverse primer and one specific designed primer. The sequence obtained contains two small introns (shown by lowercase letters in bold).

(b) Multiple alignment of the *A. fumigatus* (*Af*) translated amino acid sequence with the nitrate reductase (NIAD) protein sequence from *A. parasiticus* (*Ap*), and *A. niger* (*Anig*). The cysteine residue identified as essential in binding the molybdenum atom in *A. nidulans* NIAD is underlined.

(c) Shows multiple alignment of the *A. fumigatus* amino acid sequence obtained from region 1 of pYA61 with the nitrate reductase protein sequences from *Ap* (Chang *et al.*, 1996), *Anig* (Unkles *et al.*, 1992), *Ustilago maydis* (*Um;* Banks *et al.*, 1993), *N. crassa* (*Nc;* Okamoto *et al.*, 1991), *Hordeum vulgare* (*Hv;* Schnorr *et al.*, 1991), and *Arabidopsis thaliana* (*At*; Crawford *et al.*, 1988). The numbers correspond to the positions of the *Anig*, *Um*, *Nc*, *Hv*, and *At* nitrate reductase protein sequences. The (*) denotes identical amino acids and the (·) denotes conserved amino acids. Dashes were inserted to maximize the homology using the program "Clustal W".

a

```
CCCGTCGTCTTGAACTTTCGTCAGATTTTGCAGAAATTTAATCAGATCACAGCCCCCATT 60
 P  V  V  L  N  F  R  Q  I  L  Q  K  F  N  Q  I  T  A  P  I     20
ACACTCGTATGCGCCGGAAACAGACGTAAGGAGCAAAACACAGTGCGGAAATCAAAAGGT 120
 T  L  V  C  A  G  N  R  R  K  E  Q  N  T  V  R  K  S  K  G     40
TTTTCTTGGGGACCAGCTGGTCTTTCAACTGCCCTTTTTACCGGGCCCTTGATGGCGGAT 180
 F  S  W  G  P  A  G  L  S  T  A  L  F  T  G  P  L  M  A  D     60
GTTCTGCGGTATGCGAAGCCACTGCGTCAAGCCAAGTACGTCTGCATGGAGGGAGCAGAT 240
 V  L  R  Y  A  K  P  L  R  Q  A  K  Y  V  C  M  E  G  A  D     80
AAGCTGgtcagtattcctcctggaattgcccatgccattctctggtccactgacttagct 300
 K  L                                                           82
atagCCTAATGGCTACTATGGCACATCAGTCAAGCTCAATTGGGCCATGGATCCAAACAA 360
       P  N  G  Y  Y  G  T  S  V  K  L  N  W  A  M  D  P  N  K   101
ATTGATCATGCTTGCGCATAAGATGAATGGCGAGCCTCTTCGTCCGGATCACGTCGCCCC 420
  L  I  M  L  A  H  K  M  N  G  E  P  L  R  P  D  H  V  A  P   131
TAGAGCCGTTGTACCTGCCCAAATCGGCGGCCGAAGCGTGAAGTGGATCAAGAAACTGAT 480
  R  A  V  V  P  A  Q  I  G  G  R  S  V  K  W  I  K  K  L  I   151
CTTGACTGATGCACCCAGTGACAACTGGTACCACATCTATGACAACCGAGTATTACCgta 540
  L  T  D  A  P  S  D  N  W  Y  H  I  Y  D  N  R  V  L  P     170
agtgaaccggggtacgtcagataaccacttgctgagCCGTTGCACCAGAACGACCGTCTC 600
                                     R  C  T  R  T  T  V  S   178
TCCCGAAATGGCAGCTTCAGATCCGATGTGGTGGCGTGACGAGCGCTATGCAATTTAT   658
  P  E  M  A  A  S  D  P  M  W  W  R  D  E  R  Y  A  I  Y     197
```

b

```
Af        1  --------PVVLNFRQILQKFNQITAPITLVCAGNRRKEQNTVRKSKGFSWGPAGLSTAL
Ap      121  TIEGLVEKPLVLNFRQVLQQYDQITAPITLVCAGNRRKEQNIVRKTKGFSWGSAGLSTAL
Anig    120  SIEGLVEKPLVLNFRDILQQYDQITAPITLVCAGNRRKEQNVVRKTKGFSWGSAGLSTAL
                    *.*****. .**.. ********************* ***.****** *******

Af              FTGPLMADVLRYAKPLRQAKYVCMEGADKLPNGYYGTSVKLNWAMDPNKLIMLAHKMNGE
Ap              FTGPLLADILRSGKPLRQAKYVCMEGADKLPNGHYGTLIKLNWALDPNRGIMLAHKMNGE
Anig            WTGPMMADILRSAKPLRKAKYVCMEGADKLPNGYYGTSIKLNWAMDPNRGIMLAHKMNGE
                 ***..**.**  ****.*************** *** .*****.***. **********

Af              PLRPDHVAP-RAVVPAQIGGRSVKWIKKLILTDAPSDNWYHIYDNRVLPRCTRTTVSPEM
Ap              SLRPDHGRPLRAVVPGQIGGRSVKWLKRLILTDAPSDNWYHINDNRVLP----TMVSPDM
Anig            DLRPDHGRPLRAVVPGQIGGRSVKWLKKLILTDAPSDNWYHIYDNRVLP----TMVSPEM
                 *****  * ***** *********.*.************** ******    • ***.*

Af              AASDPMWWRDERYAIY-197
Ap              ASNNRNWWHDERDAIY 311
Anig            SSSDPNWWRDDRYAIY 310
                ..    **.*.• ***
```

c

```
Af          TLVCAGNRRKEQNTVRKSKGFSWGPAGLSTALFTGPLMADVLRYAKPLRQ------
Ap     149  TLVCAGNRRKEQNIVRKTKGFSWGSAGLSTALFTGPLLADILRSGKPLRQ------
Anig   148  TLVCAGNRRKEQNVVRKTKGFSWGSAGLSTALWTGPMMADILRSAKPLRK------
Um     115  TLVCAGNRRKEQNMVAKGLGFNWGAAGVSTGLFTGVYLADILDYCKPKNPLLSSFP
Nc     238  TLVCAGNRRKEQNVLRKSKGFSWGAGGLSTALWTGVGLSEILARAKPLTKKGG---
At     182  TLVCAGNRRKEQNMVKKSKGFNWGSAGVSTSVWRGVPLCDVLRRCGIFSRKGG---
Hv     188  TLVCAGNRRKEQNMVQQTVGFNWGAAGVSTSVWRGARLRDVLLRCGVMSKKGQ---
            ************  .  .  ** **  *.**  .  *  . ..*

Af          ----------AKYVCMEGA-D
Ap          ----------AKYVCMEGA-D
Anig        ----------AKYVCMEGA-D
Um          SYDVAVLDRARHVVFEGA-D
Nc          ---------GARYVCFEGA-D
At          ----------ALNVCFEGSED
Hv          ----------ALNVCFEGAED
                     *  *  ** . *
```

Figure 9. The position of the *A. fumigatus* putative amino acids sequence from the ends of the subcloned EcoRI fragment (on the right = pYA61) with respect to the *A. niger* nitrate reductase (NIAD) protein sequence of 883 amino acids.

A. niger NIADsequence

Figure 10. A restriction map of the 7 kb EcoRI genomic insert of pYA1-2 derived from the original *A. fumigatus* genomic clone, λYA4-3. Two subclones with inserts of 2.8 kb (ClaI/EcoRV) and the 2.2 kb (EcoRV/EcoRI) in plasmids pYA211-3 and pYA211-2, respectively, were partly sequenced (as indicated by arrows)

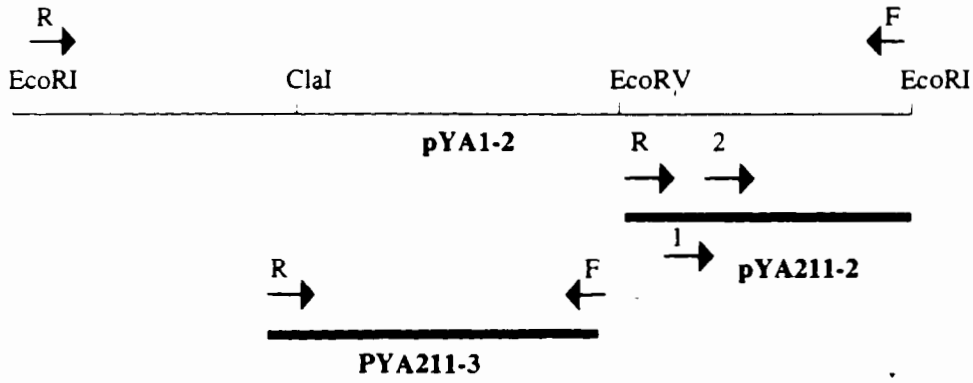
R
F
EcoRI
ClaI
EcoRV
EcoRI
pYA1-2
R
2
1
pYA211-2
R
F
PYA211-3

Figure 11. Nucleotide sequences obtained from two genomic insert (see Fig. 10) using the forward primers (a and c) or reverse primers (b and d). (a) 300 bp and (b) 312 bp obtained from pYA1-2. (c) 300 bp and (d) 317 bp obtained from pYA211-3. These sequences were used to search the protein data banks but no significant matches were found.

a

```
AATTCGCAGCGTTCAAGAAGGCCAAGGTCGATACCCACCAAGTTACCGGCGCAGACCTGT  60
ACGGCAGCCGCGACCACCTGCAAAACAACTACCTGTACCGCTACGCCGGTGCCGAGCTGG 120
GAATGTTCGGCAACTCCAGTGATGAAGCTGCGCACTTGAGCTACGTCATCGACAGCAAGG 180
GCAAGCCGGCCAATGGTGCGCGCCACAGCTACACCGTGCATTTTGCCAAGGACCAGTTAC 240
CGTCGGCCGATGCGGTCTGGTCGGTTGACGGTTGTACGTCGGTCAAGGATTAAAATCGGG 300
```

b

```
AATTCGAAGCCGACTTGGGTATAGCGCTTGTGGGTGTCGTCGAGGCTGCGGTTGTTGAGG  60
CGTAGCGCGCCACCGTCGGCCTGGATCAGGCGCAATGCGCCGTAAGTGGCGGCGCTGAAG 120
TCATACGAGGCGAAGGCCTGTAAGCGATACAGCGGCGCTTGCTTGAGTTCGCTGCCGAAG 180
TAATCGTCATTGCGGCCATAGAGCTGGGCTTCCAGGTTGGTTTCCAGTACCCATTTGTCG 240
CCGATGCCGTGGGTGTAGTTGTAGACAAAGGTGGCGCCCCAGCGATTGGCACCGGGGGAC 300
ACGTCCGGCTTTTGGCTGTGGTACTCACCCACCGGCACGGTGATCAGGTTCAACAGGCCG 360
CTGTAGGTACGC                                                  312
```

c

```
ACTGCCTCGCCGGGCGAGGCATGCACCCGTGCGACGGTCACGTCCAGATGGCGGATCGAC  60
TGCCAGTCCGCCAGATAGTCGGGGGGCAACTGGTAGAGGAAGCTGTTGTGCTCGTGCGGC 120
ACCCCGTTGATGACCGCCGAGCGCCCCCACCAGGCGCTGTCGAAGGCCAGCAATTGGTTG 180
AGGCGCGCCAGGGCATGGTGTTGAAAGTGTTCGATGTCCTGGTGTTGGGTCAGGCGTTGC 240
AGGTCCAGGGTCAGGGTGCTGAAAGCCTGTAACAGGCTCGCGGGATCGAGACGGTTCATC 300
```

d

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CCATCATCGGTGTACTTGAATATTGATTGTCTTGGCCATGGGTTTTATTAAAGCGATGGT  60
CTGAGCGGGTTTCCAGAAAAGGACTCCTTACCGTGTAAGCCAGCGTATTTTCCGCATACT 120
TATTTTCCGGTGTTGCGGACGTCGGGTCAGGAAGCCCCAATTTAAAGGCCGCAGCTTTTG 180
CAATGACGTGACGGCCAAAATTATTGGGTTTTTCCAGCCCATCGGCATGGCTCTGGGTCA 240
GCGTTACCTGCATCCTGCCGGGCTCGTCATCGGACACCGGTGCATACCAAGGGGCTTGGC 300
ACTTAAAGCTGCTATCA                                              317
```

A total of 993 bp were obtained from pYA211-2 as shown in figure 10. Approximately, 625 bp of this genomic sequence was found to contain an open reading frame of 218 amino acids (starting with an ATG codon) (Figure 12a). The genomic sequence was used to search the protein data banks using Blastx and the results showed 64% identities and 76% similarity, and 61% identities and 72% similarity over the first 120 putative amino acids with protein sequences of secreted proteinase b, c, and a from *Erwinia chrysanthemi* (amino acids 256-376) and alkaline protease from *Pseudomonas aeruginosa* (amino acids 250-370), respectively (Delepelaire and Wandersman, 1989, Dahler *et al.*, 1990, Kawasaki *et al.*, 1990). Figure 12b show multiple alignment of the *A. fumigatus* putative amino acid sequence with protease b from *E. chrysanthemi* and alkaline protease from *P. aeruginosa*. More genomic sequence (317 bp) up stream of the EcoRV fragment was obtained from pYA211-3 using the forward primer (Figure 11d). The complement of this sequence was found to contain potential promoter consensus sequences such as a TAATA box located at -62 to –58 and two CAAT boxes located at positions -219 to -216 and -43 to -35 (Figure 12a).

f) Southern analysis of genomic DNA from two *A. fumigatus* isolates and two *E. coli* strains using probes derived from pYA1-2

To determine the source of the 7 kb EcoRI genomic fragment in pYA1-2, genomic DNA from *A. fumigatus* strains (ATTCC13073 and 42202) and two *E. coli* strains (DH5λ and KW251) were digested EcoRI for Southern analysis (Figure 13a). The 2.2 kb EcoRI/EcoRV and 4.8 EcoRI/EcoRV genomic fragments derived from pYA1-2 were as probes. No signals were detected by either probe from the genomes tested (Figure 13b). In

order to determine if a specific band can be amplified from the genomes being tested, PCR analysis was carried out using primers, 500 bp a part , designed from the 993 bp sequence (derived from pYA211-2) with genomic DNA from *A. fumigatus* (ATCC 13073 and 42202), DH5α, and KW251. The PCR primers amplified multiple products from all the templates used including a band (500 bp) of the expected size (Figure 14).

This results section dealt with a brief general characterization of the *A. fumigatus* 15.8 kb EcoRI genomic insert in λYA4-3. An 8.8 kb EcoRI genomic fragment was found to contained most of the nitrate assimilation gene cluster, while the rest of the 15.8 kb EcoRI genomic insert (7 kb EcoRI fragment in pYA1-2) contained unrelated sequences to the nitrate assimilation gene cluster. This 7 kb EcoRI fragment in pYA1-2 may contain a novel fungal protease gene.

Figure 12. (a) Nucleotide sequence obtained from pYA211-2 and pYA211-3 as outlined in figure 10. The initiation codon ATG marks the start of the open reading frame identified in the sequence obtained from pYA211-2. The longest open reading frame is shown below the nucleotide sequence. The sequence upstream of the ATG codon (obtained from pYA211-3) contains a potential TAATA box at position -62 to -58, two potential CAAT boxes at positions -219 to -216 and -43 to –35.

(b) Multiple alignment of the *A. fumigatus* putative amino acid sequence with that of secreted proteinase b precursor and alkaline metalloproteinase from *E. chrysanthemi* (*Ec*) *P. aeruginosa* (*Pa*), respectively. The *A. fumigatus* amino acid sequences shares 64% identities and 76% similarity, and 61% identities and 72% similarities over the first 120 putative amino acids sequence. Three motifs corresponding to the calcium binding domain GGXGDXUX (X = any amino acid, U = bulky hydrophobic amino acid) are boxed.

a

```
TATTCGGGGAGACCTGAATAATTTCAGGGGATGCTGCAAATATCGGGTTCAAAGAGTTGC -363
CAATGCACAATCCCATCATATGCAGTTTAAGGTTGATAGCAGCTTTAAGTGGCAAGCCCC -303
TTGGTATGCACCGGTGTCCGATGACGAGCCCGGCAGGGTGCAGGTAACGCTGACCCAGAG -243
CCATGCCGATGGGCTGGAAAAACCCAATAATTTTGGCCGTCACGTCATTGCAAAAGCTGC -183
GGCCTTTAAATTGGGGCTTCCTGACCCGACGTCCGCAACACCGGAAAATAAGTATGCGGA -123
AAATACGCTGGCTTACACGGTAAGGAGTCCTTTTCTGGAAACCCGCTCAGACCATCGCTT  -63
TAATAAAACCCATGGCCAAGACAATCAATATTCAAGTACACCGATGATGGATGATATCGC   -4
CATCATGCAAGATAAATTCGGGGCGAACCTCCAAACGCGTACGGGAGATACGACGTATGG  56
     M  Q  D  K  F  G  A  N  L  Q  T  R  T  G  D  T  T  Y  G  20
ATTCAACTCAAATGCCGACCGTGAAGTATTCAAATTGGAATCAGTCGATGATTTACCGGT 116
  F  N  S  N  A  D  R  E  V  F  K  L  E  S  V  D  D  L  P  V  40
ATTTTGCGTGTGGGACGCCGGTGGTAGAGACACATTCGATTTCTCCGCATTCAAGCAAGA 176
  F  C  V  W  D  A  G  G  R  D  T  F  D  F  S  A  F  K  Q  D  60
TCAGGTTATCAATTTGAGATCGGGCAGCTTCTCTAATGTAGGGGGAGGCATAGGCAACGT 236
  Q  V  I  N  L  R  S  G  S  F  S  N  V  G  G  G  I  G  N  V  80
CTCGATCGCCAACGGCGTCACCATCGAACGGGCTATTGGCGGTGCTGGCAACGACATTCT 296
  S  I  A  N  G  V  T  I  E  R  A  I  G  G  A  G  N  D  I  L 100
TATTGGGAATGACTCGGATAACGAACTGGAGGGCGGCGAGGGCGATGACATTCTCTATGT 356
  I  G  N  D  S  D  N  E  L  E  G  G  E  G  D  D  I  L  Y  V 120
CAAGGCGAACTCTGGCTTTAACCGGCTGTCAGGCGGGCCTGGTAAAAATACCTCCGTTAT 416
  K  A  N  S  G  F  N  R  L  S  G  G  P  G  K  N  T  S  V  I 140
CGGCGCAAACGACTCATCCGCCAGCTTTGAAAACACAGCTATAACGGACTTTGTCAGCGG 476
  G  A  N  D  S  S  A  S  F  E  N  T  A  I  T  D  F  V  S  G 160
CAAGGATAAATTGGATATTTCAGCCCTGCGCGCAGGCCACCCTCAACTCACCGTCACCCA 536
  K  D  K  L  D  I  S  A  L  R  A  G  H  P  Q  L  T  V  T  Q 180
GAAATATTATCCCAGTCATAACCTCACCTTGCTGCGTTTCGATTTCGATGGCGACAAAAA 596
  K  Y  Y  P  S  H  N  L  T  L  L  R  F  D  F  D  G  D  K  K 200
AGACGACTTCATGATGAGTATAAGTGGGCGAATCGTGCGTTCCGATTTCCATCCTTGACC 625
  D  D  F  M  M  S  I  S  G  R  I  V  R  S  D  F  H  P       218
TGAATGTGTTGACGCATGTATTGGCGTGCTCCGAGCGAAATTAGGTGTACTGCCCAAGTA 716
TTGGCTATACCTATAACTCATTCTTACAATGATGGTCGCCAGGGAGTTCACTAATGAAGT 776
TAAGGCTAATGATCGGCACACTGTGCGTTGCCTCGCTCGGTTTGGCCGGTTCGCGAGCA 836
AGGTGGTGCAACCGAATGAATACTCAGGGTTCCTCTCCAACTACAGGCAGCTCAAGGAAG 896
ACAAGTCACCGTCGGGGGCAGAGGTGATGCGCTGGGTTGGATCCCACAACTGGACTTGGA 950
GCCGCTACAACGGGGGGGTTCAATCGAG                                  978
```

b

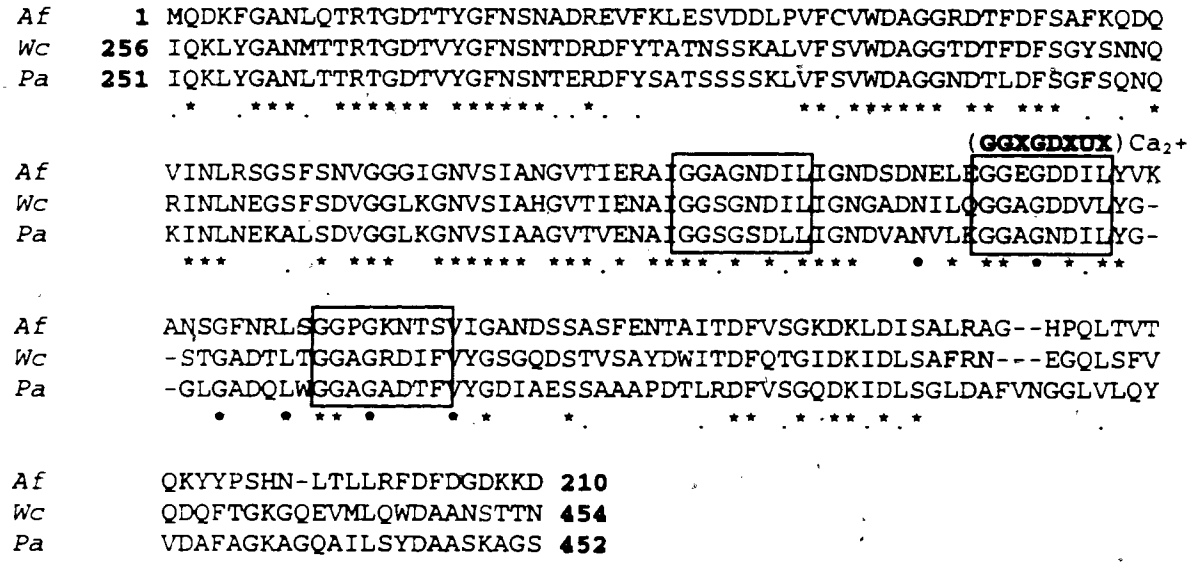
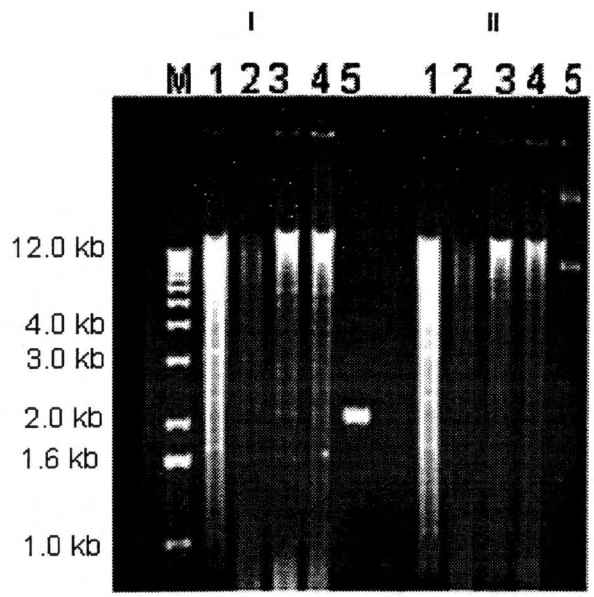

Af 1 MQDKFGANLQTRTGDTTYGFNSNADREVFKLESVDDLPVFCVWDAGGRDTFDFSAFKQDQ
Wc 256 IQKLYGANMTTRTGDTVYGFNSNTDRDFYTATNSSKALVFSVWDAGGTDTFDFSGYSNNQ
Pa 251 IQKLYGANLTTRTGDTVYGFNSNTERDFYSATSSSSKLVFSVWDAGGNDTLDFSGFSQNQ
(GGXGDXUX)Ca2+
Af VINLRSGSFSNVGGGIGNVSIANGVTIERAIGGAGNDILIGNDSDNELEGGEGDDILYVK
Wc RINLNEGSFSDVGGLKGNVSIAHGVTIENAIGGSGNDILIGNGADNILQGGAGDDVLYG-
Pa KINLNEKALSDVGGLKGNVSIAAGVTVENAIGGSGSDLLIGNDVANVLKGGAGNDILYG-
Af ANSGFNRLSGGPGKNTSVIGANDSSASFENTAITDFVSGKDKLDISALRAG--HPQLTVT
Wc -STGADTLTGGAGRDIFVYGSGQDSTVSAYDWITDFQTGIDKIDLSAFRN---EGQLSFV
Pa -GLGADQLWGGAGADTFVYGDIAESSAAAPDTLRDFVSGQDKIDLSGLDAFVNGGLVLQY
Af QKYYPSHN-LTLLRFDFDGDKKD 210
Wc QDQFTGKGQEVMLQWDAANSTTN 454
Pa VDAFAGKAGQAILSYDAASKAGS 452

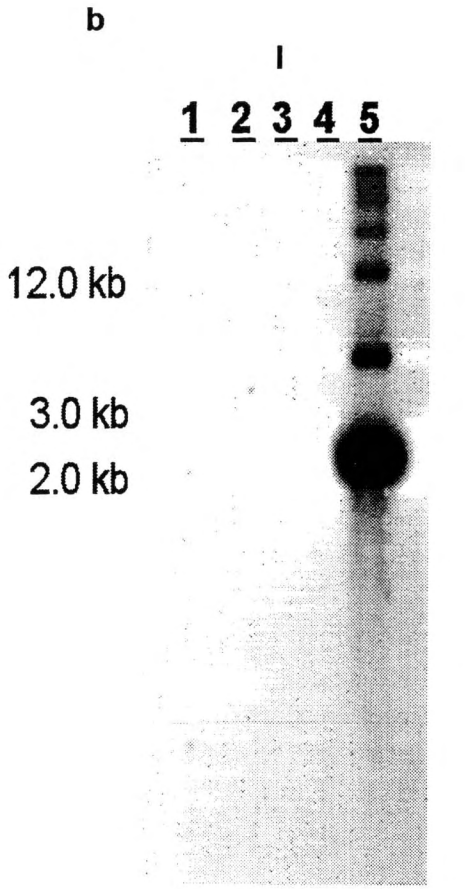
Figure 13. Southern analysis of two *A. fumigatus* strains and two bacterial strains' genomes.

(a) Ethidium bromide stained gel of an EcoRI genomic DNA digest of two wild-type *A. fumigatus* isolates and two *E. coli* strains. M = 1 kb ladder, lane 1 = *A. fumigatus* ATCC 13073, lane 2 = *A. fumigatus* ATCC 42202, lane 3 = DH5α, lane 4 = KW251, and lane 5 = 2.2 kb EcoRV/EcoRI fragment (section I of the gel) or the plasmid pYA1-2 (section II of the gel) as positive controls.

(b) Southern analysis of genomic DNA of *A. fumigatus* (ATCC 13073), DH5a, and KW251 (blot I and II) using the 2.2 kb EcoRI/EcoRV and a 4.8 kb EcoRI/EcoRV genomic fragments derived from pYA1-2 as a probes. M = 1 kb marker.

a

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I
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12.0 kb
3.0 kb
2.0 kb

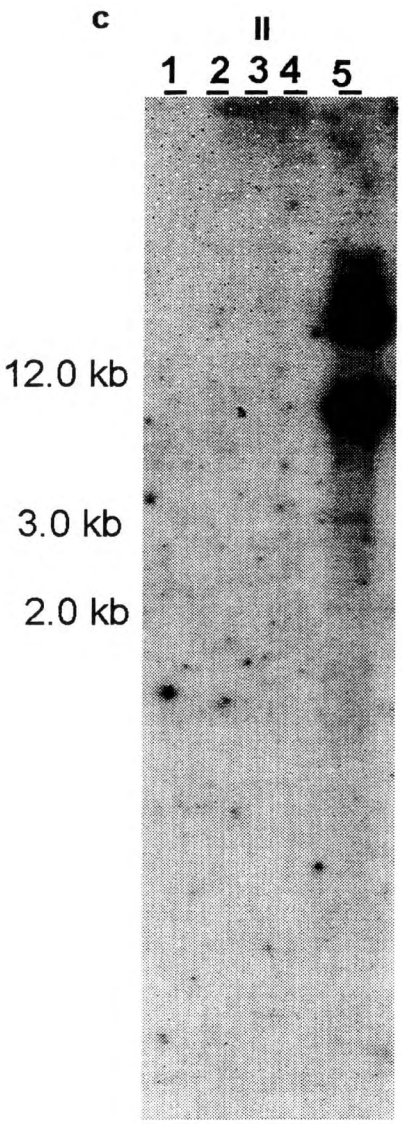
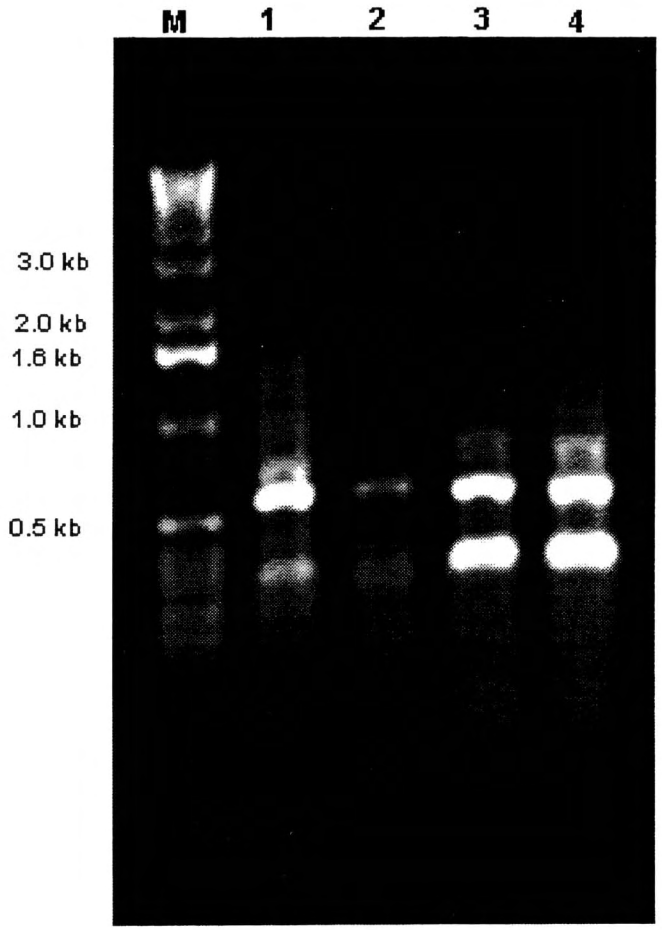

c
II
1 2 3 4 5
12.0 kb
3.0 kb
2.0 kb

Figure 14. PCR products obtained using primers designed from the 993 bp sequence (primer 1: 5'GGGACGCCGTGGTAGAGA3', primer 2: 5'GCTGCCTGTAGTTGGAGA3') obtained from the 2.2 kb EcoRI/EcoRV genomic fragment and using *A. fumigatus* (ATCC 13073 and 42202; lanes 1 and 2 respectively), DH5α (lane 3), and KW251 (lane 4) DNA as templates. The PCR primers are 500 bp a part. M = 1 kb marker.


M
1
2
3
4
3.0 kb
2.0 kb
1.6 kb
1.0 kb
0.5 kb

Section II

Molecular characterization of the *A. fumigatus* nitrite reductase gene (*niiA*).

a) Genomic sequence of the *A. fumigatus niiA* gene

The genomic sequence corresponding to the *A. fumigatus niiA* gene was obtained from the 6.5 kb EcoRI fragment (pYA2-3) subcloned in two parts (pYA21-3 and pYA22-3) using a combination of sequencing primers and nested deletions (Figure 15). Some of the *niiA-niaD* intergenic sequence was obtained from an adjacent 1.6 kb EcoRI fragment subcloned in pYA61 (Figure 15). Some of the entire *niiA-niaD* intergenic region, promoter and regulatory, sequence was obtained from the adjacent 1.6 kb EcoRI (subclone pYA61). A total of 5293 nucleotides were determined which included, the *niiA* coding region, and about 200 nucleotides of the 3' untranslated region (Figure 16).

b) The *niiA-niaD* intergenic region (promoter and regulatory regions)

The 1229 bp *niiA-niaD* intergenic region of *A. fumigatus* which includes the promoters of both genes contains consensus sequences typical of fungal gene regulatory regions. In length the *niiA-niaD* intergenic region of *A. fumigatus* (1.2 kb) is similar that of *A. nidulans* (Johnstone *et al.*, 1990, Punt *et al.*, 1995). The *A. fumigatus niiA* promoter sequence contains two putative TATA boxes (located at nucleotide positions -90 to -87 and -162 to –158 from *niiA* translation start point; Figure 16).

Figure 15. Sequence strategy used to obtain the entire genomic sequence of the *A. fumigatus niiA* gene (note: orientation is inverted compared to Fig. 3, 6 and 9 in Section I). The bold bar indicates the total region sequenced: (region A in pYA61, region B in pYA22-3 and region C in pYA21-3 (pYA22-3 and 21-3, the latter two were derived from the 6.5 kb EcoRI in pYA2-3. Numbered arrows represent primers, numbered bars represent nested deletions used for sequencing. The direction of transcription of the *niaD*, *niiA*, and *crnA* genes is indicated by arrows shown at the top of the figure.

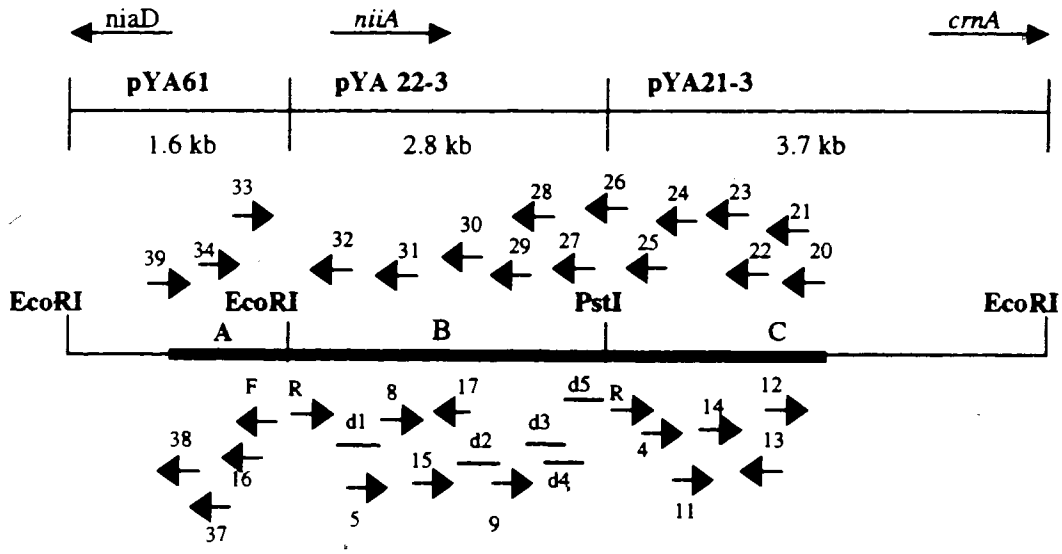

niaD
niiA
crnA
pYA61
pYA 22-3
pYA21-3
1.6 kb
2.8 kb
3.7 kb
EcoRI
EcoRI
PstI
EcoRI
A
B
C
F
R
d1
d2
d3
d4
d5
R

Figure 16. Complete nucleotide sequence of the *A. fumigatus niiA* gene. The initiation codons (**ATG**) of the *niiA* gene and (**CAT**) for the *niaD* gene which is transcribed from the opposite strand, are boxed. Promoter consensus sequences **TATA** and **CAAT** boxes as well as the putative **GATA** motifs for binding of the *areA* gene product, and the **TCCGCGGA** region for the *nirA* gene product, are noted in bold. Introns are shown in lower case. The deduced protein NIIA sequence is shown below the nucleotide sequence. The putative binding domains for FAD (AA residue 44 to 79) and NAD(P)H (AA residue 146 to 176) are underlined. The translation (**TAG**) stop codon (*) as well as a stop codon [***taa***] found in intron 8, are indicated.

```
CATTGCTCAGAGTACTACAGCTGATAATGTCGGTATAGAATGAAGGAAACACGGAGAAGC -1172
GAGCCAGCTTTTCTAGACTAGAAATCCCTCAAGGATAGGCCAGACAGAAGAAGGCAACCA -1112
TAGTCAGGATGGAATGAATGAGGAAAATGACGCATTCTGTTTACAAGTGAGGAAGCCAGT -1052
AGTCAGACACATTGTCTTGACTCAGATATTGCCATATTTGTCTGTATAAACAAATGGCCT  -992
CGGAGAAAGGCTAGAAGCAGGCCAGGGATATCTTCATGATGACGATCTCCCGCGGGGATC  -932
TCAAGGTAGTATTGTGTTGAAAAGCAGCTAGATAAGCCTTAAGATTACCACGGCATCTGC  -872
TCGGATCGCTGCGATCCCGATAGTGCAAATGGGAACTGGGTCTGGTATCCGCCATGGTAT  -812
GACGGTGAGCTTTCGCCTCTCATAGGAGCTCTCAGGCCAGTGTGGACTGCGGTCGAGGAA  -752
ATACAGCGCCTGCATCCTTGTTGGGAAGGAAACAATCTGAGCGCCCTGGAGCACCGTGGA  -692
ATTAGAGAGAATCATGAGACATTTCTTCAAAAATGAGGCCCATTCCTCCCATCCGCCACG  -632
GTGGGACGAAAGTAAGGGACGATCCCAGTAGCGGAGTCTGGCGGCTCTGATCTCGTTGTT  -572
GACTGTGCCACTTTCCCGAGGTCAGTGGCTGTGGTAAGAAATCATAGATAGAGGTCCCCG  -512
GTATCTCCATAGAAGCCAATCTGGAATTCAACGGATAGGAGGCGAAAGCAATCCTGCTTA  -452
TCTTGGTTCTCGGGATGGGCTTTCACTTGGCAGAGATAAGGCGTGACAGAGGGCTGGCTT  -392
ACGGAGTAGTCTCCGCGGAAATAGAAAAGGGAGATGATCCGCTATCGTCCGTATCAGTCC  -332
ACCCGAACGGTTACAAACATGGCTTGGTGCAGAGAGCAACCCTCTATCCCTGTTGCACAG  -272
TCACTCCATCATGGTGTTAAGTTGTATGGCTTGACTCTTCGCGAATATCGGCGATTAGGC  -212
TCGAACATGGGCGGATGCAATCGTAGCAGGGTTCGCCGATGACAATAAATTATTAGCAGA  -152
AATAAAATGCAACTGACTGGTTGCGTTTCGTCCATGCCAACTCAACGAAGAGCTGTAGAT   -92
TGTATATGAGCAGAGAGGTCCTCAGCCGTCCCCTAAAAGAGTTCATTCTGGTAATGAGCT   -32

                                +1
GTGGTCAATACTGGCGTTGAGACTTCGTCACGATGCCGTTGATTGATAACTCAAGAAGCA    27
                                 M  P  L  I  D  N  S  R  S        9
ACGATGCTGTCCAGAGCAGTATCTGCAACGGTATATCCCATACGACAATCATCGAGTCTG    87
N  D  A  V  Q  S  S  I  C  N  G  I  S  H  T  T  I  I  E  S      29
TCAGAGACCCAGATTATCGTCACAATGACCCGAACCGACGGCAGAAGATTGTAATCGTCG   147
V  R  D  P  D  Y  R  H  N  D  P  N  R  R  Q  K  I  V  I  V      49
                                                Putative FAD binding
GGTTGGGCATGGTTGCGATCTCATTCATgtatgttgccagcttggtggataaggaccgat   207
G  L  G  M  V  A  I  S  F  I                                    59
Domain (residue 44 to 79)
ctcgagaggggttgtctactaaccaccggtactatcaaatagTGAGAAAATCGTCAAACA   267
                                            E  K  I  V  K  Q     65
AGATGCAGAGAGACGCAAGTACGACATTGTTGTGATTGGGGAGGAGCCTCACATTGGCTA   327
  D  A  E  R  R  K  Y  D  I  V  V  I  G  E  E  P  H  I  G  Y    85
TAATCGCGTCGGTCTCTCCTCGTTTTTTGAACATCGCAAGATCGAGGATTTGTACCTCAA   387
  N  R  V  G  L  S  S  F  F  E  H  R  K  I  E  D  L  Y  L  N   105
TCCCAAGGAATGGgtaagtacttgacgatcaaggatttaggctttccatctagctgatac   447
  P  K  E  W                                                    109
taagcttctactcgtctacatagTACGGGTCATTCAAGGACCGGTCATTCGATTATCACC   507
                        Y  G  S  F  K  D  R  S  F  D  Y  H      121
TCAACACCAAGGTCACTGACATTTTCCCTGAACGCAAGACCGTCAGGACGTCAACTGGCG   567
L  N  T  K  V  T  D  I  F  P  E  R  K  T  V  R  T  S  T  G     141
ACGTTATCCCCTATGATATTCTAGTCCTCGCTACAGGCTCAGATGCAGTACTCCCCACAC   627
D  V  I  P  Y  D  I  L  V  L  A  T  G  S  D  A  V  L  P  T     161
           NAD(P)H binding domain(residue 146 to 176)
ACACTCCTGGCTATAATGCCAAGGGTATCTTCGTTTACAGAACAATTCAAGACCTGGAAC   687
H  T  P  G  Y  N  A  K  G  I  F  V  Y  R  T  I  Q  D  L  E     181
GTCTCATCGAGTTCGCTTCGAAACACAAGGGCGAGACCGCGGTCACTTGTAGGAGGGAGG   747
R  L  I  E  F  A  S  K  H  K  G  E  T  A  V  T  C  R  R  E     201
CTTGGTTCGGCTTGGAAGCAGCCAAAGCAATGAAAGATCTTCGAGGGTTTCGGAAGAGTC   807
A  W  F  G  L  E  A  A  K  A  M  K  D  L  R  G  F  R  K  S     221
AAACTCATTGCCCCCACCAAAGTGGGTTCTGGCACCGACAAGCTCCGACGGGCGATGCCG   867
Q  T  H  C  P  H  Q  S  G  F  W  H  R  Q  A  P  T  G  D  A     241
GCACAACTCGTAACTTCGGGAAGATCAAGAGAGCTAGGCCTGGACCGTATTACACCGAGA   907
G  T  T  R  N  F  G  K  I  K  R  A  R  P  G  P  Y  Y  T  E     261
AGCGGGTCCCCAAAAGATCCAAACGGGATGACCGACAACAAACGTCACCAGGCATCACCT   967
K  R  V  P  K  R  S  K  R  D  D  R  Q  Q  T  S  P  G  I  T     281
TTGAAGATGGGGAAGAGATTGACTGCTGCTGCATCTGTTTTGCTgtaagtcgctcttgaa  1027
F  E  D  G  E  E  I  D  C  C  C  I  C  F  A                    296
gagcgccatcagtacgctgctaaacttgcccttagATTGGAGTGCGACCCAGAGATGAG   1087
                                    I  G  V  R  P  R  D  E      304
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CTGGGGCCTCCTGCCGGCATTCAATGCGCTGGACGGGGAGGGTTTGTTATTAATGAAAGg  1147
 L  G  P  P  A  G  I  Q  C  A  G  R  G  G  F  V  I  N  E  S    324
tcagtagatattggctttttttttcggatatctatcgtgaactgactcgatactcgatgt  1207

tagTTTGCAAACTTCCATTCCCGATATCTATGCCATTGGCGAATGTGCTAGTTGGGAGAA  1267
     L  Q  T  S  I  P  D  I  Y  A  I  G  E  C  A  S  W  E  N    343

TCAGACGTTCGGAATAATAGCACCTGGGATTGAGATGGCCGACGTGCTTTCCTTCAACCT  1327
  Q  T  F  G  I  I  A  P  G  I  E  M  A  D  V  L  S  F  N  L    363
GACAAATCCCGATAAGGAGCCAAAGAGCTTCAAGCGACCCGATCTGAGCACAAAGCTCAA  1387
  T  N  P  D  K  E  P  K  S  F  K  R  P  D  L  S  T  K  L  K    383
GCTCCTTGGTGTTGACGTTGCCAGCTTTGGTGACTTCTTTGCCGACAGAGATGGGCCTAA  1447
  L  L  G  V  D  V  A  S  F  G  D  F  F  A  D  R  D  G  P  K    403
ATTCCTTCCGGGGCAACGGCCATCGATTGTCGATGGCTCTGTGGGGAACGCTGATGGTGA  1507
  F  L  P  G  Q  R  P  S  I  V  D  G  S  V  G  N  A  D  G  D    423
CAAAGAGCCAAGTGTCAAGGCATTGACATACAAGGACCCCTTCGCCGGAATCTACAAGAA  1567
  K  E  P  S  V  K  A  L  T  Y  K  D  P  F  A  G  I  Y  K  K    443
GTACTTGTTCACTATGGATGGGAAGTACTTGCTTGGAGGTATGATGATTGGCGACACCAA  1627
  Y  L  F  T  M  D  G  K  Y  L  L  G  G  M  M  I  G  D  T  K    463
GGACTACGTGAAGTTGAACCAGATGGTCAAGAGCCAAAAGCCTCTCGAAGTACCTCCCAG  1687
  D  Y  V  K  L  N  Q  M  V  K  S  Q  K  P  L  E  V  P  P  S    483
TCAGTTTATTCTTGGAGCGCAGAAGGAAGGCGAAGAGAATGCCGATGACTTgtaagtttt  1747
  Q  F  I  L  G  A  Q  K  E  G  E  E  N  A  D  D  L              500
gggctggcatgctggacaggactaggcaatgacacaaacattaacagAGACGACGACGCT  1807
                                                 D  D  D  A      504
CAGATCTGCTCGTGCCACAATGTCACAAAGGGGGACATCGTTGAGAACGTCAAGAGTGGT  1867
 Q  I  C  S  C  H  N  V  T  K  G  D  I  V  E  N  V  K  S  G    524
ACATGCAAGACTATTGGCGAGATTAAATCGTGCACGAAAGCCGGGAGTGGTTGTGGGGGC  1927
 T  C  K  T  I  G  E  I  K  S  C  T  K  A  G  S  G  C  G  G    544
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 C  M  P  L  V  Q  S  I  F  N  K  A  M  R  D  M  G  Q  E      563
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                                                    V  I        565
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V  C  M  H  I  P  Y  S  R  A  D  L  Y  T  V  I  A  V  K  R      585
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L  R  T  F  V  D  V  M  Q  A  V  G  R  K  P  D  S  L  G  C      605
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E  I  C  K  P  A  I  A  P  I  L  S  S  L  F  N  Q  H  I  M      625
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D  K  E  L  H  D  L  Q  E  T  N  D  R  F  L  A  N  I  Q  R      645
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N  G  T  F  S  V  V  P  R  V  P  G  G  E  I  T  A  D  K  L      665
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cgcccgtttaagattgagttttcttagctgacgtattctatcagAGCTGCGTCGGAACTA  2587
                                              S  C  V  G  T      728
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C  A  E  A  Q  N  K  D  *                                       776
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V  G  G  N  G  G  A  K  P  R  H  S  E  L  L  A  K  D  V  P      809
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P  E  K  V  I  P  I  I  D  R  Y  L  I  F  Y  I  R  T  A  D      829
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L  V  N  S  Y  F  C  E  W  T  E  T  V  R  N  P  K  R  R  K     889
ACTTTCAGCAGTTCGCCAACAGCGACGAGACTGTCGACACAGTTGAGGTCATCAAAGAAC  3187
Y  F  Q  Q  F  A  N  S  D  E  T  V  D  T  V  E  V  I  K  E     909

GCGAACAGCAGCGACCCACATACTGGCCCAAGGACTCGGCAGGCGAGGACTTCAAGGGTC  3247
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W  *                                                           1110
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CTATATAGTTGATCATGATTGGGAATATTGTGCATGCCACCCTTGGAAAGTCGCATATTC  3947
TGCTTCGTAGAGAGCTAAATCCTACAATAACAATACGATATTAGAAATGCTCATCCTAAA  4007
TGGTGATTGCTGT                                                 4020
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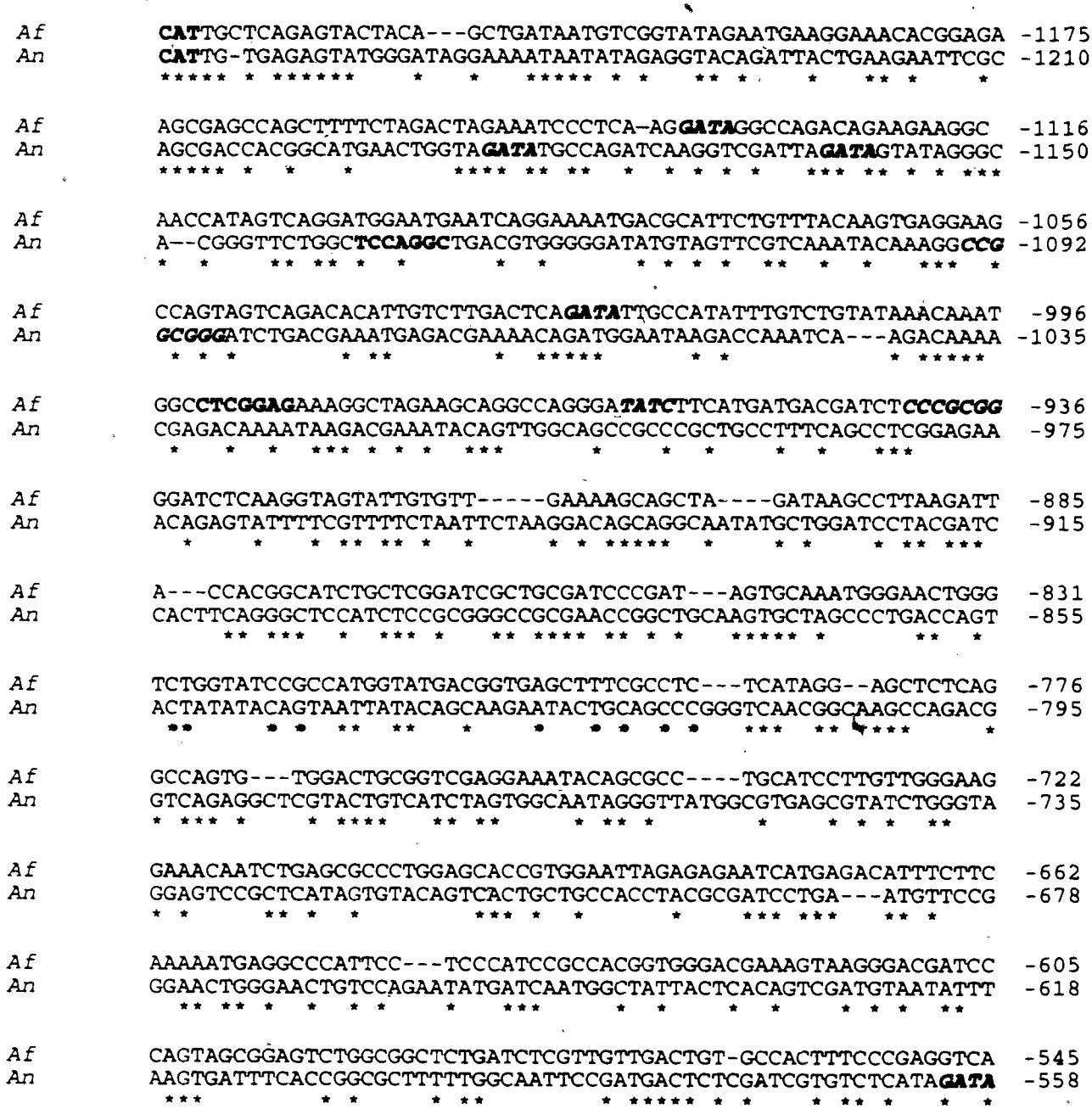
Figure 17. Pairwise alignment of the *niiA-niaD* intergenic promoter and regulatory region of *A. fumigatus* and *A. nidulans*. Special features detailed in legend of Fig. 16 are highlighted in bold. The promoter sequences share 42% sequence identity.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An ACAGAGTATTTTCGTTTTCTAATTCTAAGGACAGCAGGCAATATGCTGGATCCTACGATC -915
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Af TCTGGTATCCGCCATGGTATGACGGTGAGCTTTCGCCTC---TCATAGG--AGCTCTCAG -776
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An GGAACTGGGAACTGTCCAGAATATGATCAATGGCTATTACTCACAGTCGATGTAATATTT -618
Af CAGTAGCGGAGTCTGGCGGCTCTGATCTCGTTGTTGACTGT-GCCACTTTCCCGAGGTCA -545
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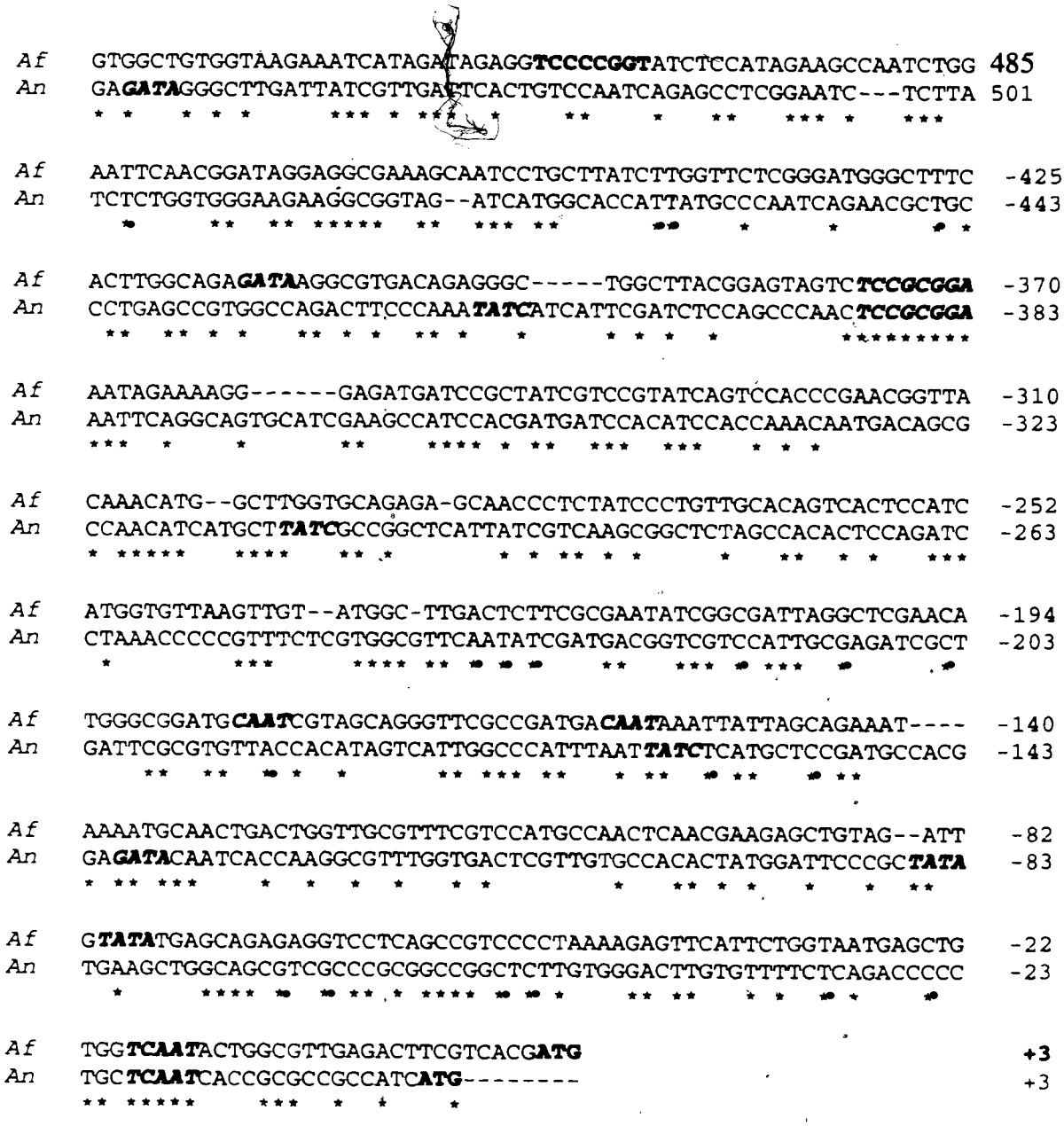
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An CCTGAGCCGTGGCCAGACTTCCCAAATATCATCATTCGATCTCCAGCCCAACTCCGCGGA -383
Af AATAGAAAAGG------GAGATGATCCGCTATCGTCCGTATCAGTCCACCCGAACGGTTA -310
An AATTCAGGCAGTGCATCGAAGCCATCCACGATGATCCACATCCACCAAACAATGACAGCG -323
Af CAAACATG--GCTTGGTGCAGAGA-GCAACCCTCTATCCCTGTTGCACAGTCACTCCATC -252
An CCAACATCATGCTTATCGCCGGCTCATTATCGTCAAGCGGCTCTAGCCACACTCCAGATC -263
Af ATGGTGTTAAGTTGT--ATGGC-TTGACTCTTCGCGAATATCGGCGATTAGGCTCGAACA -194
An CTAAACCCCCGTTTCTCGTGGCGTTCAATATCGATGACGGTCGTCCATTGCGAGATCGCT -203
Af TGGGCGGATGCAATCGTAGCAGGGTTCGCCGATGACAATAAATTATTAGCAGAAAT---- -140
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Af AAAATGCAACTGACTGGTTGCGTTTCGTCCATGCCAACTCAACGAAGAGCTGTAG--ATT -82
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Af GTATATGAGCAGAGAGGTCCTCAGCCGTCCCCTAAAAGAGTTCATTCTGGTAATGAGCTG -22
An TGAAGCTGGCAGCGTCGCCCGCGGCCGGCTCTTGTGGGACTTGTGTTTTCTCAGACCCCC -23
Af TGGTCAATACTGGCGTTGAGACTTCGTCACGATG +3
An TGCTCAATCACCGCGCCGCCATCATG-------- +3

Two putative CAAT boxes are located at nucleotide positions -194 to -191 and at -170 to -176 (Figure 16). Figure 17 shows pairwise alignment of the *A. fumigatus* and *A. nidulans* intergenic promoter and regulatory sequences which share 42% sequence identity. The same NIRA binding nucleotide sequences (**TCCGCGGA**, a perfect palindrome and CCCGCGGGG, incomplete palindrome), and AREA DNA binding sequences (GATA motifs) were found in the 5' regulatory regions.

c) The *niiA* gene sequence contains 8 small introns

The coding region is interrupted with eight small introns 42 to 74 bp long. The coding sequence of the *A. fumigatus* nitrite reductase translates to a putative protein sequence of 1110 amino acids when all of the eight introns are taken out (Figure 16). Observation in *A. nidualns niiA* gene suggested that intron 8 may at times retained as part of the coding sequence (Johnstone *et al.*, 1990). If this occurs in *A. fumigatus*, the open reading frame translates to a putative protein sequence of 776 amino acids because of inframe stop codon at the beginning of intron 8. The long open reading frame terminates with the stop codon TAG while the shorter one terminates with the stop codon TAA. The 3' untranslated region obtained (Figure 15) did not contain any consensus sequence corresponding to the polyadenylation signal found in most eukaryotes (AATAAA).

d) Putative protein sequence of the *niiA* gene

The *A. fumigatus niiA* amino acid sequence exhibits a high degree of homology (80% identity) (Figure 18a) to that of *A. nidulans*. Alignment of the *A. fumigatus* with *A. nidulans* NIIA protein sequence revealed several conserved motifs. The alignment

showed that a putative FAD domain of βαβ dinucleotide binding fold type was conserved in the same position (amino acid 44 to 79) and a NADPH domain (amino acids 146 to 176; Johnstone *et al.*, 1990) in both proteins (Figure 18b). Four cysteine residues identified in *A. nidulans* NIIA protein sequence, at positions 720, 726, 760, and 764, as metal-binding sites for iron-sulfur and siroheme (Johnstone *et al.*, 1990) were also found conserved in the *A. fumigatus* NIIA sequence at positions 725, 731, 765, 769. Multiple alignment of the NIIA protein sequences from *A. fumigatus*, *A. nidulans* (Johnstone *et al.*, 1990), *Neurospora crassa* (Exley *et al.*, 1993), and *E. coli* (Peakman *et al.*, 1990) showed significant sequence homology (Figure 19).

e) Codon usage of the *niiA* gene

The codon usage of the *A. fumigatus niiA* gene and those of *A. nidulans*, *N. crassa*, and *E coli* was determined. Table 2 summarizes the codon counts and percentages observed in the gene sequences examined. Determination of codon usage from these organisms provides information about codon preference and tRNA abundence.

f) Evidence for induction of the nitrate assimilation gene cluster (*crnA*, *niiA* and *niaD*) by nitrate.

Total RNA from *A. fumigatus* wildtype cells grown in minimal media containing either nitrate or ammonium as a sole nitrogen source was analyzed to check the inducibility of the nitrate assimilation genes in *A. fumigatus*, as found in *A. nidulans*. The mRNA levels of the three genes were visualized in Northern blots using probes corresponding to the *A. fumigatus niiA* gene (2.8 EcoRI/PstI kb cDNA EcoRI/EcoRV),

the *niaD* gene (1.6 kb EcoRI genomic fragment) and *crnA* gene (0.7 kb EcoRI genomic fragment; Figure 3). The *niiA*, *niaD*, and the *crnA* probes detected transcripts of 3.3 kb, 2.9 kb, and 1.8 kb respectively in total RNA from *A. fumigatus* grown on nitrate but not on ammonium (Figure 20). The Northern analysis indicates that the expression of the *niiA, niaD*, and *crnA* genes is induced by nitrate and almost completely repressed by ammonium, as found in *A. nidulans* (Cove, 1979).

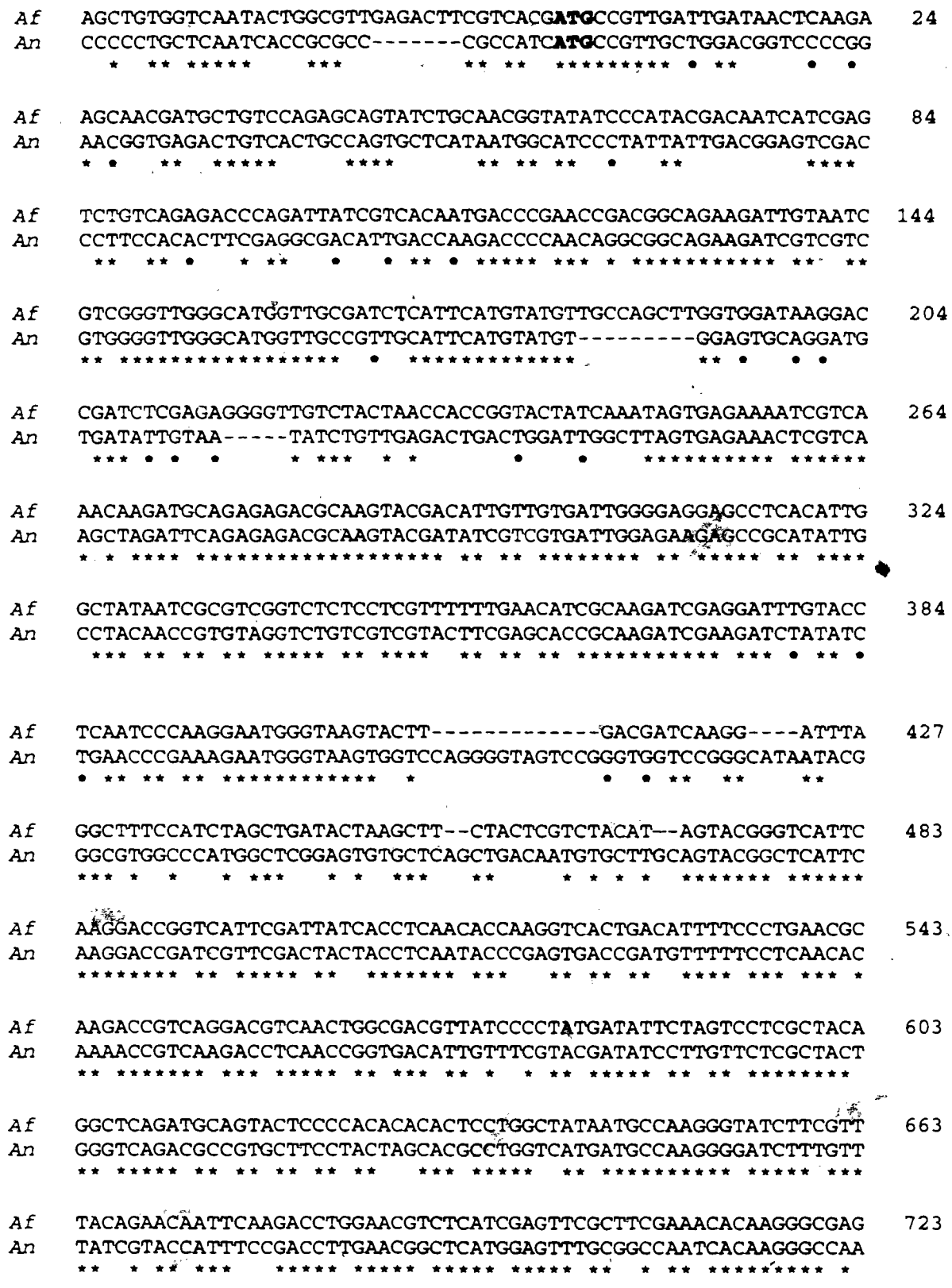
h) mapping of nitrate assimilation gene cluster to the largest chromosome of *A. fumigatus*.

Southern analysis of *A. fumigatus* intact chromosomes, separated by pulsed field gel electrophoresis, were probed with a 2.8 kb EcoRI/PstI *niiA* gene fragment. The *A. fumigatus* chromosomes migrated as four bands, and the *niiA* probe used detected the uppermost band. These results suggest that the nitrate gene cluster is located on one of the largest (approximately 5.3 Mb) chromosomes (Figure 21).

Figure 18. Nitrite reductase nucleotide sequences and proteins pairwise alignments:

(a) *niiA* genes, starting from –36 (Figure 17a). The (*) denotes identical nucleotides. Dashes were inserted to maximize the sequence homology.

(b) Nitrite reductase protein sequences. The (*) denotes identical amino acids and the (·) denotes conserved amino acids. Dashes were inserted to maximize the sequence homology.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      ****    * *   ********** *  ** ** ***    ******** **** **

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An   CGGTTTGGAG-GTGCTCCAC-GAGAAACGGGTGGCTA--AGATTCATACTG-ATGAC-GA
      **  ****  **  * *** ***** *****  * *  **** ** ** * ***** **

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     ******  ** **  ** **   *** ** ** **  * *** **** ** ***** ***

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An   TGTTTTGCTGTATGCTTCCAAGACTTTCATCCTGCTTGTATGGTGCTGACTTAACCAT--
     ************ *   *              *     *** * **** *  *  ** *

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An   AGATTGGCATTCGACCGCGAGACGAGCTGGGAGGAAGCACCGGTATTCAATGCGCAAAGC
     *******  * *****  **** ********         **** **********   *

Af   GGGGAGGGTTTGTTATTAATGAAAGGTCAGTAGATATTGGCTTTTTTTTTCGGATATCTA   1202
An   GTGGCGGCTTCGTCATTGACGAAAG----------------------------------
     * ** ** ** ** *** * *****

Af   TCGTGAACTGACTCGATACTCGATGTTAGTTTGCAAACTTGCATTCCCGATATCTATGCC   1262
An   ------------------------------CTTGCGAACATCGGTTAATGACATTTACGCT
                                    **** *** **  **   ** ** ** **

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An   ATGGCCGATGTGCTATCATTTAACTTGACAAACCCGGACAAGGAACCGAAGAGATTCAAC
     ******** ***** ** ** *** ******* ** ** ***** ** ***** *****

Af   CGACCCGATCTGAGCACAAAGCTCAAGCTCCTTGGTGTTGACGTTGCCAGCTTTGGTGAC   1442
An   AGGCCGGACCTAAGCACCAAACTCAAGCTGTTAGGAGTCGATGTTGCCAGTTTTGGTGAC
      * ** ** ** ***** ** ********  * ** ** ** ******** *********

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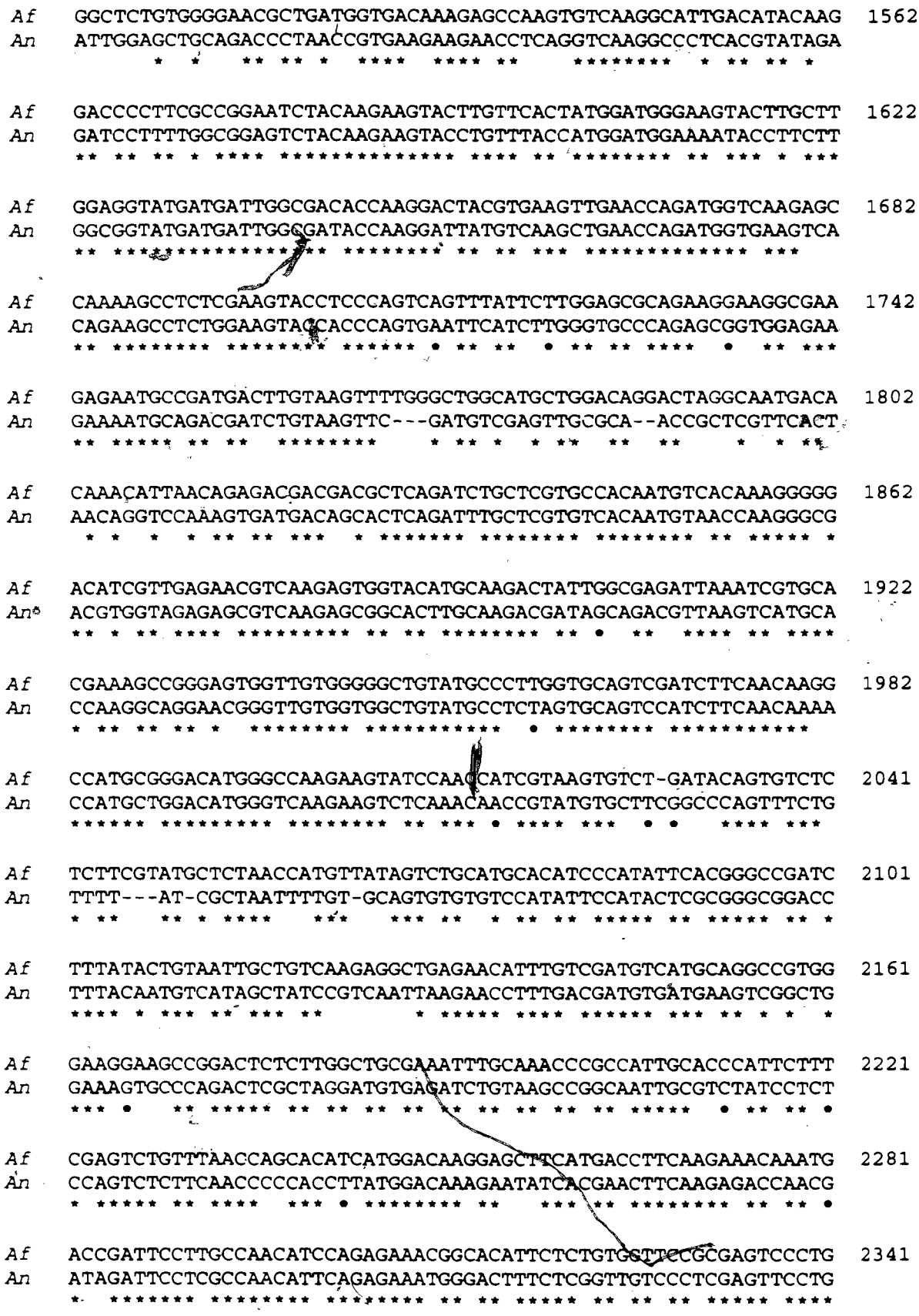
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Af CCATGCGGGACATGGGCCAAGAAGTATCCAACCATCGTAAGTGTCT-GATACAGTGTCTC 2041
An CCATGCTGGACATGGGTCAAGAAGTCTCAAACAACCGTATGTGCTTCGGCCCAGTTTCTG
Af TCTTCGTATGCTCTAACCATGTTATAGTCTGCATGCACATCCCATATTCACGGGCCGATC 2101
An TTTT---AT-CGCTAATTTTGT-GCAGTGTGTGTCCATATTCCATACTCGCGGGCGGACC
Af TTTATACTGTAATTGCTGTCAAGAGGCTGAGAACATTTGTCGATGTCATGCAGGCCGTGG 2161
An TTTACAATGTCATAGCTATCCGTCAATTAAGAACCTTTGACGATGTGATGAAGTCGGCTG
Af GAAGGAAGCCGGACTCTCTTGGCTGCGAAATTTGCAAACCCGCCATTGCACCCATTCTTT 2221
An GAAAGTGCCCAGACTCGCTAGGATGTGAGATCTGTAAGCCGGCAATTGCGTCTATCCTCT
Af CGAGTCTGTTTAACCAGCACATCATGGACAAGGAGCTTCATGACCTTCAAGAAACAAATG 2281
An CCAGTCTCTTCAACCCCCACCTTATGGACAAAGAATATCACGAACTTCAAGAGACCAACG
Af ACCGATTCCTTGCCAACATCCAGAGAAACGGCACATTCTCTGTGGTTCCGCGAGTCCCTG 2341
An ATAGATTCCTCGCCAACATTCAGAGAAATGGGACTTTCTCGGTTGTCCCTCGAGTTCCTG

```
Af   GTGGTGAGATCACTGCAGATAAACTGATCACCATTGGTCAAGTGGCGAAAAAATACAACC  2401
An   GAGGTGAAATCACAGCCGACAAGTTGATTGCAATTGGGCAGGTAGCCAAGAAATACAATC
     * ***** ***** ** ** **  ****  * ***** ** ** ** ** ******** *

Af   TTTACTGCAAGATTACAGGAGGTCAGCGTATTGACTTGTTTGGTGCCAAGAAGCAAGATC  2461
An   TTTACTGCAAGATCACAGGTGGTCAGCGTATCGATATGTTTGGTGCCAGGAAGCAGGATC
     ************** ***** ********** **  ************ ****** ****

Af   TTCTCGACATCTGGACGGAGCTCGTGGACGCCGGAATGGAGAGTGGTCATGCTTATGCCA  2521
An   TACTCGATATTTGGACTGAGCTCGTCGATGCCGGTATGGAGAGTGGCCATCGGTACGCCA
     * ***** ** ***** ******** ** ***** ********** ***   ** ****

Af   AGTCGCTCCGAACAATCAAGGTACTGCCGCCCGTTTAAGATTGAGTTTTCTTAGCTGACG  2581
An   AGTCACTCCGAACTGTTAAGGTGA-GATTTATTCTTAAGTCAATGCAAACCGAGTTAACG
     **** ********  * *****  *       *****     *   * ** * ***

Af   TATTCTATCAGAGCTGCGTCGGAACTACATGGTGCCGCTTTGGTATCGGCGACAGTGTTG  2641
An   GAAT---TCAGAGTTGTGTTGGAACAACCTGGTGCCGATTCGGCGTCGGAGACAGCGTTG
      * *   ****** ** ** ***** ** ******** ** **  **** ***** ****

Af   GAATGGCGATCCGACTGGAGGAGCGCTACAAGAGTATTCGGTCACCCCATAAGCTCAAGG  2701
An   GAATGGCTATCCGCTTGGAGCAACGGTATAAGAGTATCCGAGCTCCACACAAGTTCAAGG
     ******* *****  *****  * ** ** ******** **  * ** ** *** ******

Af   GTGCGGTATCTGGTTGTGTCAGAGAGTGCGCCGAAGCTCAGAACAAGGAGTAAGCACAAA  2761
Af   GTGCTGTCTCTGGCTGTGTCCGAGAGTGTGCCGAAGCTCAAAACAAGGAGTGAGTAACGT
     **** ** ***** ****** ******* ********** ********** ** *

Af   ATTCATTCTTAGACTATGAATGAACAAGCAGTTAATTTCCGATCAGTTTCGGATTGATTG  2821
An   ATCACTTTTTGG--------TAAAAGCGCCGTTAACGT--GAATAGCTTTGGTCTTATTG
     **   ** ** *        * **   ** ***** *  **  ** ** ** ** * ****

Af   CAACCGAGAAGGGATATAACATTTTCGTTGGTGGCAATGGAGGTGCCAAACCCCGGCATT  2881
An   CTACCGAGAAGGGATTCAATATCTTCGTTGGTGGCAACGGAGGTGCCAAACCCCGTCATT
     * ************** ** ** ************** ***************** ****

Af   CGGAATTGCTTGCGAAAGATGTGCCTCCTGAGAAAGTCATTCCTATCATTGATCGATATC  2941
An   CAGAGTTACTTGCCAAGGATGTACCACCTGAGGAGGTGATTCCGATCCTGGATCGCTACG
     * ** ** ***** ** ***** ** ****** * ** ***** *** * ***** **

Af   TCATATTCTATATTAGAACGGCAGACAAACTTCAGCGGACTGCCCGGTGGATCGAGAACC  3001
An   TGATCTTCTACATCAGAACTGCAGACAAACTCCAGCGAACGGCGAGATGGCTCGAGAGCC
     * ** ***** ** ***** ********** ***** ** **  * *** ****** **

Af   TCCCCGGCGGTATCAACTATCTGCGAGAGGTGATCATCGACGACAAGTTGGGGATTGGCG  3061
An   TTCCGGGCGGCATTGAATACCTCAAGGACGTTGTTCTCAATGATAAACTTGGAATAGCAG
     * ** ***** **  * ** **    ** **  *  ** * ** **  * ** ** *  *

Af   CTGAGATGGAACAGCAGATGGAAGAACTGGTGAACAGCTACTTTTGTGAGTGGACCGAGA  3121
An   CAGAGATGGAGCGTCAAATGCAGGAGCTGGTTGACAGCTACTTCTGCGAATGGACCGAGA
     * ******** *  ** *** * ** *****  ********** ** ** **********
```

```
Af   CAGTCCGCAATCCTAAGCGACGCAAGTACTTTCAGCAGTTCGCCAACAGCGACGAGACTG  3181
An   CAGTCAGAAATCCCAAACGTCGCAAGTACTTCCAACAATTCGCCAACACTGACGAGACGG
     ***** * ***** ** ** ********** ** ** **********  ******** *

Af   TCGACACAGTTGAGGTCATCAAAGAACGCGAACAGCAGCGACCCACATACTGGCCCAAGG  3241
An   TCGAGAACGTGGAAATTGTTAAGGAGCGCGAGCAAGTGCGCCCGACTTACTGGCCCAAGG
     **** *  ** **  *  * ** ** ***** ** * ***** ** ** ************

Af   ACTCGGCAGGCGAGGACTTCAAGGGTCACCAGTGGTCGTCTCTATCATGGCAGCCTGTCA  3301
An   ACGGAGCCAACGAAGACTTCAAGGGTCACCAATGGTCCAGCCTCTCGTGGCAGCCAGTTA
     **   **   *** ****************** *****    ** ** ******** ** *

Af   TCGAAGCCAACTACTTTTCAGATGAGCACCCGCAAATCTCCTCGGCCAACATCAAGCGCG  3361
An   TCAAGGCTGACTACTTCTCCGACGGCCCACCCGCAATCTCGTCCGCCAATATCAAGCGCG
     ** * **  ******* ** ** * *  **   ****** ** ***** **********

Af   GGGATACTCAGTTGGCCATCTTCAAGGTCAAGGGCAAGTACTACGCCACACAACAGATGT  3421
An   GTGATACCCAATTGGCCATTTTCAAGGTCAAGGGCAAGTACTACGCTACACAACAAATGT
     * ***** ** ******** ************************** ******** ****

Af   GCCCGCACAAACGCGCCTTCGTGCTATCTGATGGCTTGATTGGCGATGACGATGCGGGTA  3481
An   GCCCTCACAAGCGAACCTTTGTCTTGTCCGACGGTCTGATTGGCGACGACGACAACGGCA
     ****-***** **  **** **  * ** ** **  ********** *****    ** *

Af   AATATTGGGTCTGCTGCCCTTACCACAAACGCAACTTCGACAACTTCGAGCTCAACGGTG  3541
An   AATACTGGGTATCGTGTCCGTACCACAAGCGG---------AACTTCGAACTCAACGGCG
     **** ***** ** ** ** ******** **          ******** ******** *

Af   AGCAGGCAGGGCGTTGCACGAATGACGAGAGCATGAACATTGCGACCTTCCCGGTTGAGG  3601
An   AGCAGGCTGGCCGTTGCCAAAACGATGAGGCGATGAATATTGCCACATTCCCAGTTGAGG
     ******* ** ******   ** ** ***   ***** ***** ** ***** *******

Af   AACGCGACGACGGCTGGGTGTATATGAAGCTGCCTCCCGTTGAGGAACTAGATTCCGTTT  3661
An   AGCGGGAAGATGGCTGGATTTACATGAAACTTCCACCAGTTGAGGAGCTGGATTCCGTTC
     * ** ** ** ****** * ** ***** ** ** ** ******** ** *********

Af   TGGGAACGGAGAAATGGAAGGTGAGGAAAGGGGAAGCACCAGATCCGTTCCAGAAGGTCG  3721
An   TTGGTACGGAAAAGTGGAAGGTGAAGAAGGGTGAAGCTGTGGACCCGTTTGAGGCGTATG
     * ** ***** ** ********** *** ** *****    ** *****  **  *   *

Af   ACAAGAGGTACAAGGGCATGAGGGGCAAGAAGGCGTGTGATATCAGTAC-GAAAGCCCCG  3780
An   ACAAGAAGTACAGCGGGATGAAAGGGAAGAGAGCCGGCGCCAAGGGAATTGAGGGCAGCA
     ****** *****  ** ****  ** ****  **  ▬  •  • •  **  **  •

Af   ACCACACAGG-----CGGCAAACATCATCGATTGGTAGCACGACTTTGGCATACAGATGA  3835
An   AGCCCACTCGGTCTCCTTCAAACACAATAGACTGGTAGACTGACGAGGATACGTTT-TGC
     * * ***  *     *  ******  ** ** ******   ***   *  *      **

Af   GGCGTACTGGTTATCTTTTAACATGGGTTCCTTATTTACTTGCTGATGTTTACATGTATT  3895
An   GATGTGATATTAGTATGGTGGACATGCTTATTGGTTTGCATGGCG-TTTTTCTATTCAGG
     •  **  *  •  * *  •      • **  •  *** • **  * • ***  **  •

Af   ACGAGTTGCACAGATATCCTTGTCCTTAGTATACTAT----ATAGTTGATCATGATTGGG  3951
An   CGGTTCTATGCATTATACCTAGTGTTAAACAATCTATGATTATACTATACTCGAATCGGT
       *   •   **     *** **  • •  •  ****    *** •  *     ** **
```

```
Af   AATATTGTGCATGCCACCCTTGGAAAGTCGCATATTCTGCTTCGTAGAGAGCTAAATCCT  4011
An   AACA--GTCCATAGAACGCTGCCTACATAAGTTGAATTGCCTCGCGA--------------
     ** *  ** ***   ** **    *  *    *    *** ***

Af   ACAATAACAATACGATATTAGAAATGCTCATCCTAAATGGTGATTGCTGT            4061
An   -------------------------------------------------
```

b

```
Af 1 MPLIDNSRSNDAVQSSICNGISHTTIIESVRDPDYRHNDPNRRQKIVIVGLGMVAISFIE
An 1 MPLLDGPRNGETVTASAHNGIPIIDGVDPSTLRGDIDQDPNRRQKIVVVGLGMVAVAFIE
     ***.*. *  ..* .*  ***                 *********.*******..***

Af   KIVKQDAERRKYDIVVIGEEPHIGYNRVGLSSFFEHRKIEDLYLNPKEWYGSFKDRSFDY
An   KLVKLDSERRKYDIVVIGEEPHIAYNRVGLSSYFEHRKIEDLYLNPKEWYGSFKDRSFDY
     *.** *.**************** ********.***************************

Af   HLNTKVTDIFPERKTVRTSTGDVIPYDILVLATGSDAVLPTHTPGYNAKGIFVYRTIQDL
An   YLNTRVTDVFPQHKTVKTSTGDIVSYDILVLATGSDAVLPTSTPGHDAKGIFVYRTISDL
      ***.***.**..***.*****.. **************** *** .********** **

Af   ERLIEFASKHKGETAVTCRREAWFGLEAAKAMKDLRGFRKSQTHCPHQSGFWHRQAPTGD
An   ERLMEFAANHKGQTGVTVG-GGLLGLEAAKAMTDLEDFG--SVKLIDRNKWVLARQLDGD
     ***.***. ***.* **       ******** **  *                    **

Af   AGTTRNFGKIKRARPGPYYTEKRVPKRSKRDDRQQTSPGITFEDGEEIDCCCICFAIGVR
An   AGS--LVTKKIRDLGLEVLHEKRVAKIHTDDDNN--VTGILFEDGQELDCCCICFAIGIR
     **.     *  *        **** *    **      **.****.*.**********.*

Af   PRDELGPPAGIQCAGRGGFVINESLQTSIPDIYAIGECASWENQTFGIIAPGIEMADVLS
An   PRDELGGSTGIQCAKRGGFVIDESLRTSVNDIYAIGECASWENQTFGIIAPGIEMADVLS
     ******   ***** ******.***.**. ******************************

Af   FNLTNPDKEPKSFKRPDLSTKLKLLGVDVASFGDFFADRDGPKFLPGQRPSIVDGSVGNA
An   FNLTNPDKEPKRFNRPDLSTKLKLLGVDVASFGDFFADRDGPKFLPGQRP--SAESIGAA
     *********** *.************************************     *.* *

Af   DGDKE--PSVKALTYKDPFAGIYKKYLFTMDGKYLLGGMMIGDTKDYVKLNQMVKSQKPL
An   DPNREEEPQVKALTYRDPFGGVYKKYLFTMDGKYLLGGMMIGDTKDYVKLNQMVKSQKPL
     * ..*  * ******.*** *.**************************************

Af   EVPPSQFILGAQKEGEENADDLDDDAQICSCHNVTKGDIVENVKSGTCKTIGEIKSCTKA
An   EVPPSEFILGAQSGGEENADDLDDSTQICSCHNVTKGDVVESVKSGTCKTIADVKSCTKA
     *****.******  **********..************.**.*********...******

Af   GSGCGGCMPLVQSIFNKAMRDMGQEVI--VCMHIPYSRADLYTVIAVKRLRTFVDVMQAV
An   GTGCGGCMPLVQSIFNKTMLDMGQEVSNNLCVHIPYSRADLYNVIAIRQLRTFDDVMKSA
     *.***************.* ******   .*.**********.***...**** ***.. 

Af   GRKPDSLGCEICKPAIAPILSSLFNQHIMDKELHDLQETNDRFLANIQRNGTFSVVPRVP
An   GKCPDSLGCEICKPAIASILSSLFNPHLMDKEYHELQETNDRFLANIQRNGTFSVVPRVP
     *. ************** ******* *.**** *.*************************

Af   GGEITADKLITIGQVAKKYNLYCKITGGQRIDLFGAKKQDLLDIWTELVDAGMESGHAYA
An   GGEITADKLIAIGQVAKKYNLYCKITGGQRIDMFGARKQDLLDIWTELVDAGMESGHRYA
     **********.*********************.***.******************** **

Af   KSLRTIKSCVGTTWCRFGIGDSVGMAIRLEERYKSIRSPHKLKGAVSGCVRECAEAQNKD
An   KSLRTVKSCVGTTWCRFGVGDSVGMAIRLEQRYKSIRAPHKFKGAVSGCVRECAEAQNKD
     *****.************.***********.******.*** ******************
```

```
Af   FGLIATEKGYNIFVGGNGGAKPRHSELLAKDVPPEKVIPIIDRYLIFYIRTADKLQRTAR
An   FGLIATEKGFNIFVGGNGGAKPRHSELLAKDVPPEEVIPILDRYVIFYIRTADKLQRTAR
     ********* ***************************** **** *** ***************

Af   WIENLPGGINYLREVIIDDKLGIGAEMEQQMEELVNSYFCEWTETVRNPKRRKYFQQFAN
An   WLESLPGGIEYLKDVVLNDKLGIAAEMERQMQELVDSYFCEWTETVRNPKRRKYFQQFAN
     *.* ***** **..*.. ***** **** ** *** ************************

Af   SDETVDTVEVIKEREQQRPTYWPKDSAGEDFKGHQWSSLSWQPVIEANYFSDEHPQISSA
An   TDETVENVEIVKEREQVRPTYWPKDGANEDFKGHQWSSLSWQPVIKADYFSDGPPAISSA
     .**** .** ..***** ******** *.***************** * **** * ****

Af   NIKRGDTQLAIFKVKGKYYATQQMCPHKRAFVLSDGLIGDDDAGKYWVSCPYHKRNFDNF
An   NIKRGDTQLAIFKVKGKYYATQQMCPHKRTFVLSDGLIGDDDNGKYWVSCPYHKR---NF
     ******************************.************ ************   **

Af   ELNGEQAGRCTNDESMNIATFPVEERDDGWVYMKLPPVEELDSVLGTEKWKVRKGEAPDP
An   ELNGEQAGRCQNDEAMNIATFPVEEREDGWIYMKLPPVEELDSVLGTEKWKVKKGEAVDP
     ********** *** ********** *** ********************* **** **

Af   FQKVDKRYKGMRGK--KACDISTKAPTTQAANIIDW 1110
An   FEAYDKKYSGMKGKRAGAKGIEGSKPTRSPSNTIDW 1104
     *.  **.* **.**   *  *   **   .* ***
```

Figure 19. Multiple alignment of the *A. fumigatus* (*Af*), *A. nidulans* (*An*), *N. crassa* (*Nc*), and *E. coli* (*Ec*) nitrite reductase protein sequences. The (*) denotes identical amino acids and the (·) denotes conserved amino acids. Dashes were inserted to maximize the sequence homology. The four cysteine residues implicated as metal-binding sites are boxed.

```
Af  MPLIDNSRSNDAVQSSICNGISHTTIIESVRDPDYRHNDPNRRQKIVIVGLGMVAISFIE
An  MPLLDGPRNGETVTASAHNGIPIIDGVDPSTLRGDIDQDPNRRQKIVVVGLGMVAVAFIE
Nc  ---------------MANTSLDMASSTSPSPSP--ESTTTPRK-RIVVVGLGMVGIAFIE
Ec  ------------------------------------------MSKVRLAIIGNGMVGHRFIE
                                                 . .. ..* *** *** 

Af  KIVKQDAERRKYDIVVIGEEPHIGYNRVGLSSFFEHRKIEDLYLNPKEWYGSFKDRS-FD
An  KLVKLDSERRKYDIVVIGEEPHIAYNRVGLSSYFEHRKIEDLYLNPKEWYGSFKDRS-FD
Nc  KLIKLDTQR-QYEIVVIGEEPHVAYNRVGLTSFFSHREVEQLYLNPLEWYKQHLQTSSLT
Ec  DLLDK-SDAANFDITVFCEEPRIAYDRVHLSSYFSHHTAEELSLVREGFYEKHGIKV---
     .. .  ..* *  *** .. * ** * .*.* *.  * * *     *

Af  YHLNTKVTDIFPERKTVRTSTG------DVIPYDILVLATGSDAVLPTHTPGYNAKGIFV
An  YYLNTRVTDVFPQHKTVKTSTG------DIVSYDILVLATGSDAVLPTSTPGHDAKGIFV
Nc  HHLSTAALSLSPATKSLTISPPPSTPSLTTLPYDHLILATGSSALLPTSTPGHDASGVFV
Ec  -LVGERAITINRQEKVIHSSAG------RTVFYDKLIMATGSYPWIPPIK-GSDTQDCFV
     .  .  .  * .  *  .  ** *..**** .*  *  .  **

Af  YRTIQDLERLIEFASK--HKGETAVTCRREAWFGLEAAKAMKDLRGFRKSQTHCPHQSGF
An  YRTISDLERLMEFAAN--HKGQTGVTVG-GGLLGLEAAKAMTDLEDFGSVKLIDRNK---
Nc  YRNIADLQSLITWSSDTQIKGSTGVVVG-GGLLGLEAAKALMDLQVFGRVVVIERNG---
Ec  YRTIEDLNAIESCARR---S-KRGAVVG-GGLLGLEAAGALKNLGIETHVIEFAPML---
    .** * **  .  .  .  .  ***** *.  *

Af  WHRQAPTGDAGTTRNFGKIKRARPGPYYTEKRVPKRSKRDDRQQ------TSPGITFEDG
An  -WVLARQLDGDAGSLVTKKIRDLGLEVLHEKRVAKIHTDDDNN---------VTGILFEDG
Nc  -WVLSRQVDGEAGALVLEGVRGLGVEVLTRKRVKEVECDESKDEGEKEKKRVKGIRFEDG
Ec  ---MAEQLDQMGGEQLRRKIESMGVRVHTSKNTLEIVQEGVEAR--------KTMRFADG
       .  •                    •                      . • **

Af  EEIDCCCICFAIGVRPRDELGPPAGIQCAGRGG--FVINESLQTSIPDIYAIGECASWEN
An  QELDCCCICFAIGIRPRDELGGSTGIQCAKRGG--FVIDESLRTSVNDIYAIGECASWEN
Nc  EYLACSTICFAIGIKARDELAREAGITCAERGGGGIVVDDSLQTSAPDVYAIGECASWKG
Ec  SELEVDFIVFSTGIRPRDKLATQCGLDVAPRGG--IVINDSCQTSDPDIYAIGECASWNN
      .   * *. *.. ** *   .*.  * *** .  *. .* .** *.********* .

Af  QTFGIIAPGIEMADVLSFNLTNPDKE-PKSFKRPDLSTKLKLLGVDVASFGDFFADRDGP
An  QTFGIIAPGIEMADVLSFNLTNPDKE-PKRFNRPDLSTKLKLLGVDVASFGDFFADRDGP
Nc  QTFGLIGPGVEMADVLAFNFSQAHLHTPRVFKRPDLSTKLKLLGVEVASFGDFFADRDGP
Ec  RVFGLVAPGYKMAQVAVDHILGSE----NAFEGADLSAKLKLLGVDVGGIGDAHGRTPG-
    . **.. **  **  •              •   ***.*******.•   ••      • .

Af  KFLPGQRPSIVDGSVGNADGDKEPSVKALTYKDPFAGIYKKYLFTMDGKYLLGGMMIGDT
An  KFLPGQRPSAESIGAADPNREEEPQVKALTYRDPFGGVYKKYLFTMDGKYLLGGMMIGDT
Nc  KELPPKLRRELKKSGG------KAEVKALTYKDPFLSVYKKYIFTSDGKYLLGGMMIGDT
Ec  -------------------------ARSYVYLDESKEIYKRLIVSEDNKTLLGAVLVGDT
                             .. * *   .**. . . *.* *** ...***

Af  KDYVKLNQMVKSQKPLEVPPSQFILGAQKEGEEN----ADDLDDDAQICSCHNVTKGDIV
An  KDYVKLNQMVKSQKPLEVPPSEFILGAQSGGEEN----ADDLDDSTQICSCHNVTKGDVV
Nc  TDYVRLVPLVKTHKELDVPPSQLILGAKKSGDDN---GDDDLPDDTQICSCHNVTKADLV
Ec  SDYGNLLQLVLNAIELPENPDSLILPAHSGSGKPSIG-VDKLPDSAQICSCFDVTKGDLI
     ** • .*    •  •  ** *.          • • * .***** *** *..

Af  ENVKSGTCKTIGEIKSCTKAGSGCGGCMPLVQSIFNKAMRDMGQEVI--VCMHIPYSR-A
An  ESVKSGTCKTIADVKSCTKAGTGCGGCMPLVQSIFNKTMLDMGQEVSNNLCVHIPYSR-A
Nc  APLKSGECTSLGDLKSCTKAGTGCGGCMPLVTSIFNRTMASLGTEVKNNLCPHFPEYSRA
Ec  AAINKG-CHTVAALKAETKAGTGCGGCIPLVTQVLNAELAKQGIEVNNNLCEHEAYSR-Q
     .  * * ..  .*. **** ***** *** .  *  .   * **   .* *
```

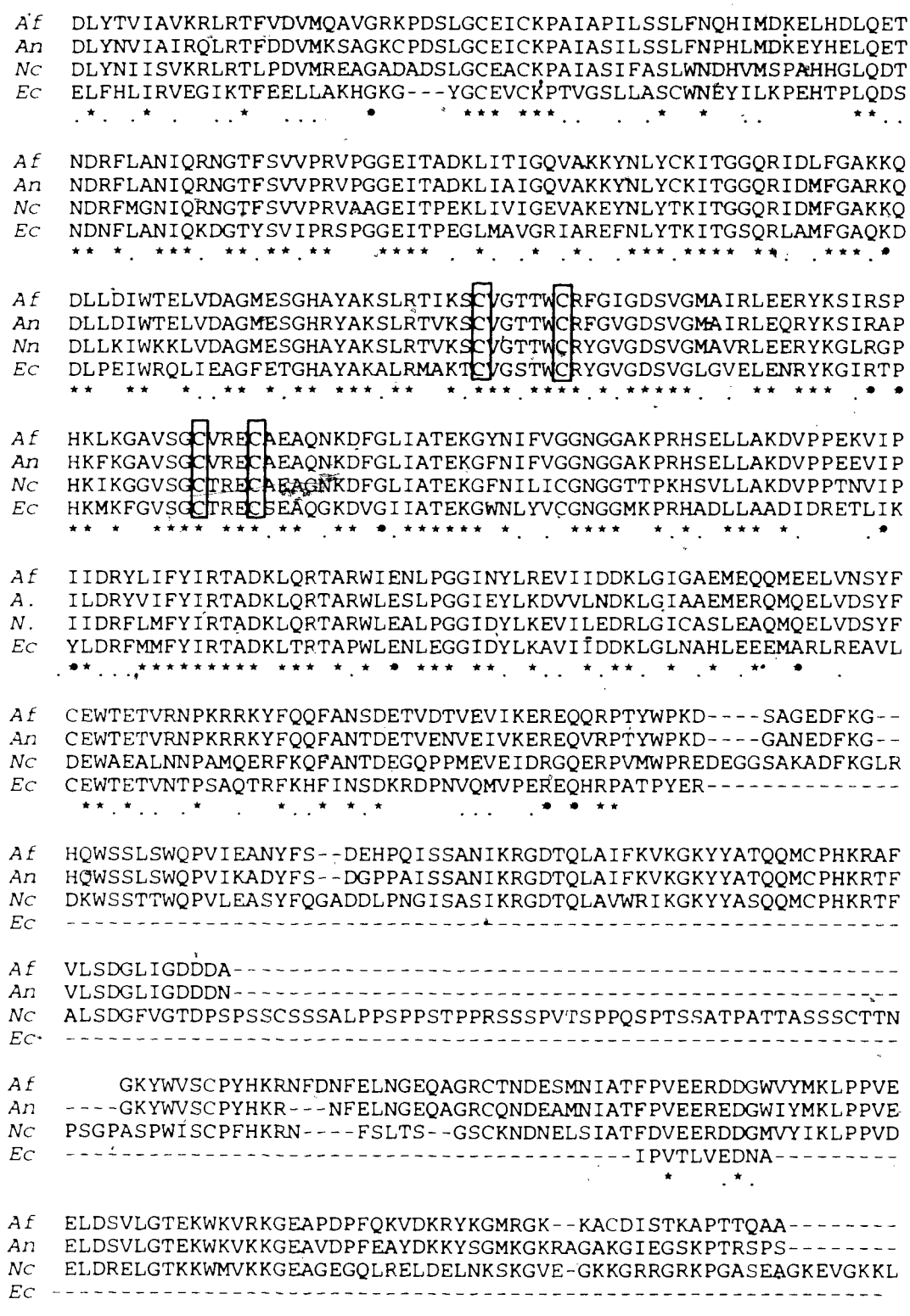

Af DLYTVIAVKRLRTFVDVMQAVGRKPDSLGCEICKPAIAPILSSLFNQHIMDKELHDLQET
An DLYNVIAIRQLRTFDDVMKSAGKCPDSLGCEICKPAIASILSSLFNPHLMDKEYHELQET
Nc DLYNIISVKRLRTLPDVMREAGADADSLGCEACKPAIASIFASLWNDHVMSPAHHGLQDT
Ec ELFHLIRVEGIKTFEELLAKHGKG---YGCEVCKPTVGSLLASCWNEYILKPEHTPLQDS
Af NDRFLANIQRNGTFSVVPRVPGGEITADKLITIGQVAKKYNLYCKITGGQRIDLFGAKKQ
An NDRFLANIQRNGTFSVVPRVPGGEITADKLIAIGQVAKKYNLYCKITGGQRIDMFGARKQ
Nc NDRFMGNIQRNGTFSVVPRVAAGEITPEKLIVIGEVAKEYNLYTKITGGQRIDMFGAKKQ
Ec NDNFLANIQKDGTYSVIPRSPGGEITPEGLMAVGRIAREFNLYTKITGSQRLAMFGAQKD
Af DLLDIWTELVDAGMESGHAYAKSLRTIKSCVGTTWCRFGIGDSVGMAIRLEERYKSIRSP
An DLLDIWTELVDAGMESGHRYAKSLRTVKSCVGTTWCRFGVGDSVGMAIRLEQRYKSIRAP
Nn DLLKIWKKLVDAGMESGHAYAKSLRTVKSCVGTTWCRYGVGDSVGMAVRLEERYKGLRGP
Ec DLPEIWRQLIEAGFETGHAYAKALRMAKTCVGSTWCRYGVGDSVGLGVELENRYKGIRTP
Af HKLKGAVSGCVRECAEAQNKDFGLIATEKGYNIFVGGNGGAKPRHSELLAKDVPPEKVIP
An HKFKGAVSGCVRECAEAQNKDFGLIATEKGFNIFVGGNGGAKPRHSELLAKDVPPEEVIP
Nc HKIKGGVSGCTRECAEAGNKDFGLIATEKGFNILICGNGGTTPKHSVLLAKDVPPTNVIP
Ec HKMKFGVSGCTRECSEAQGKDVGIIATEKGWNLYVCGNGGMKPRHADLLAADIDRETLIK
Af IIDRYLIFYIRTADKLQRTARWIENLPGGINYLREVIIDDKLGIGAEMEQQMEELVNSYF
A. ILDRYVIFYIRTADKLQRTARWLESLPGGIEYLKDVVLNDKLGIAAEMERQMQELVDSYF
N. IIDRFLMFYIRTADKLQRTARWLEALPGGIDYLKEVILEDRLGICASLEAQMQELVDSYF
Ec YLDRFMMFYIRTADKLTRTAPWLENLEGGIDYLKAVIIDDKLGLNAHLEEEMARLREAVL
Af CEWTETVRNPKRRKYFQQFANSDETVDTVEVIKEREQQRPTYWPKD----SAGEDFKG--
An CEWTETVRNPKRRKYFQQFANTDETVENVEIVKEREQVRPTYWPKD----GANEDFKG--
Nc DEWAEALNNPAMQERFKQFANTDEGQPPMEVEIDRGQERPVMWPREDEGGSAKADFKGLR
Ec CEWTETVNTPSAQTRFKHFINSDKRDPNVQMVPEREQHRPATPYER--------------
Af HQWSSLSWQPVIEANYFS--DEHPQISSANIKRGDTQLAIFKVKGKYYATQQMCPHKRAF
An HQWSSLSWQPVIKADYFS--DGPPAISSANIKRGDTQLAIFKVKGKYYATQQMCPHKRTF
Nc DKWSSTTWQPVLEASYFQGADDLPNGISASIKRGDTQLAVWRIKGKYYASQQMCPHKRTF
Ec ----------
Af VLSDGLIGDDDA----------
An VLSDGLIGDDDN----------
Nc ALSDGFVGTDPSPSSCSSSALPPSPPSTPPRSSSPVTSPPQSPTSSATPATTASSSCTTN
Ec ----------
Af GKYWVSCPYHKRNFDNFELNGEQAGRCTNDESMNIATFPVEERDDGWVYMKLPPVE
An ----GKYWVSCPYHKR---NFELNGEQAGRCQNDEAMNIATFPVEEREDGWIYMKLPPVE
Nc PSGPASPWISCPFHKRN----FSLTS--GSCKNDNELSIATFDVEERDDGMVYIKLPPVD
Ec ----------IPVTLVEDNA----------
Af ELDSVLGTEKWKVRKGEAPDPFQKVDKRYKGMRGK--KACDISTKAPTTQAA--------
An ELDSVLGTEKWKVKKGEAVDPFEAYDKKYSGMKGKRAGAKGIEGSKPTRSPS--------
Nc ELDRELGTKKWMVKKGEAGEGQLRELDELNKSKGVE-GKKGRRGRKPGASEAGKEVGKKL
Ec ----------

Table 1. Codon usage in the nitrite reductase gene sequence from *A. fumigatus* (1111 amino acids), *A. nidulans* (1105 amino acids), and *N. crassa* (1177 amino acids), and *E. coli* (840 amino acids). The count and percentage for each codon used is shown.

Codon	*A. fumigatus*		*A. nidulans*		*N. crassa*		*E. coli*	
	Count	%	Count	%	Count	%	Count	%
TTT-Phe	16	1.44	16	1.44	12	1.01	12	1.42
TTC-Phe	30	2.7	27	2.44	24	2.03	14	1.66
TTA-Leu	1	0.09	5	0.45	2	0.16	3	0.35
TTG-Leu	17	1.53	10	0.9	29	2.46	1	0.11
TCT-Ser	7	0.63	5	0.45	17	1.44	9	1.07
TCC-Ser	8	0.72	10	0.9	29	2.46	11	1.3
TCA-Ser	11	0.99	11	0.99	13	1.1	1	0.11
TCG-Ser	12	1.08	13	1.17	12	1.01	6	0.71
TAT-Tyr	16	1.44	7	0.63	6	0.5	7	0.83
TAC-Tyr	21	1.89	29	2.62	21	1.78	17	2.02
TAA-***	0	0	0	0	0	0	1	0.11
TAG-***	1	0.09	1	0.09	0	0	0	0
TGT-Cys	8	0.72	14	1.26	10	0.84	8	0.95
TGC-Cys	20	1.8	11	0.99	16	1.35	13	1.54
TGA-***	0	0	0	0	1	0.08	0	0
TGG-Trp	15	1.35	14	1.26	16	1.35	8	0.95
CTT-Leu	16	1.44	21	1.9	6	0.5	5	0.59
CTC-Leu	16	1.44	23	2.08	41	3.48	11	1.3
CTA-Leu	4	0.36	7	0.63	8	0.67	0	0
CTG-Leu	11	0.99	16	1.44	20	1.69	57	6.78
CCT-Pro	18	1.62	16	1.44	17	1.44	3	0.35
CCC-Pro	20	1.8	9	0.81	22	1.86	0	0
CCA-Pro	7	0.63	11	0.99	13	1.1	4	0.47
CCG-Pro	12	1.08	15	1.35	18	1.52	24	2.85
CAT-His	7	0.63	7	0.63	8	0.67	7	0.83
CAC-His	14	1.26	11	0.99	12	1.01	15	1.78
CAA-Gln	17	1.53	19	1.71	16	1.35	2	0.23
CAG-Gln	27	2.43	18	1.62	16	1.35	22	2.61
CGT-Arg	5	0.45	10	0.9	3	0.25	25	2.97
CGC-Arg	13	1.17	9	0.81	22	1.86	21	2.5
CGA-Arg	14	1.26	14	1.26	4	0.33	0	0
CGG-Arg	13	1.17	10	0.9	7	0.59	0	0
ATT-Ile	37	3.33	36	3.25	15	1.27	18	2.14
ATC-Ile	40	3.6	26	2.35	34	2.88	38	4.52
ATA-Ile	5	0.45	5	0.45	5	0.42	0	0
ATG-Met	22	1.98	23	2.08	23	1.95	19	2.26
ACT-Thr	15	1.35	17	1.53	9	0.76	11	1.3
ACC-Thr	11	0.99	21	1.9	35	2.97	25	2.97
ACA-Thr	21	1.89	12	1.08	12	1.01	1	0.11
ACG-Thr	11	0.99	7	0.63	18	1.52	7	0.83
AAT-Asn	13	1.17	15	1.35	6	0.5	3	0.35
AAC-Asn	28	2.52	28	2.53	24	2.03	30	3.57
AAA-Lys	26	2.34	19	1.71	22	1.86	39	4.64
AAG-Lys	61	5.49	64	5.79	60	5.09	9	1.07
AGT-Ser	14	1.26	8	0.72	8	0.67	2	0.23
AGC-Ser	10	0.9	15	1.35	17	1.44	16	1.9
AGA-Arg	12	1.08	13	1.17	7	0.59	1	0.11
AGG-Arg	9	0.81	3	0.27	16	1.35	0	0
GTT-Val	16	1.44	23	2.08	19	1.61	18	2.14
GTC-Val	30	2.7	27	2.44	26	2.2	15	1.78
GTA-Val	5	0.45	7	0.63	9	0.76	10	1.19

GTG-Val	16	1.44	21	1.9	25	2.12	13	1.54
GCT-Ala	15	1.35	20	1.8	13	1.1	10	1.19
GCC-Ala	29	2.61	32	2.89	36	3.05	20	2.38
GCA-Ala	14	1.26	12	1.08	8	0.67	15	1.78
GCG-Ala	11	0.99	6	0.54	28	2.37	24	2.85
GAT-Asp	37	3.33	33	2.98	19	1.61	21	2.5
GAC-Asp	44	3.96	52	4.7	51	4.33	26	3.09
GAA-Glu	26	2.34	30	2.71	36	3.05	53	6.3
GAG-Glu	43	3.87	41	3.71	39	3.31	10	1.19
GGT-Gly	24	2.16	34	3.07	14	1.18	36	4.28
GGC-Gly	31	2.79	35	3.16	37	3.14	39	4.64
GGA-Gly	17	1.53	23	2.08	20	1.69	1	0.11
GGG-Gly	21	1.89	8	0.72	45	3.82	3	0.11
Total	1111	99.99	1105	99.7	1177	99.56	840	99.7

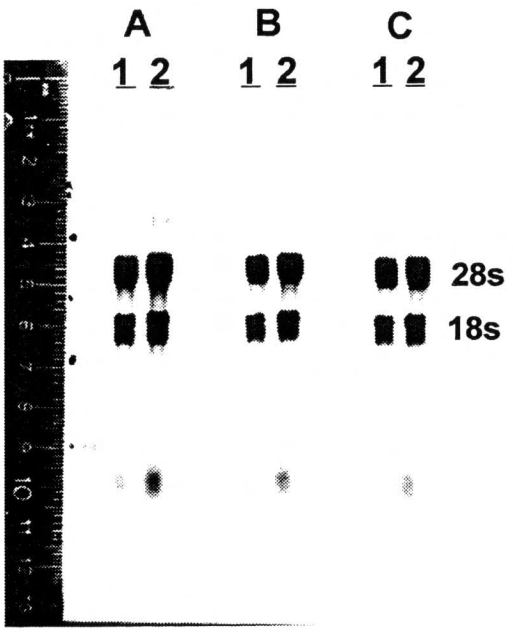
Figure 20. Evidence for coordinate induction of the nitrate assimilation gene cluster in nitrate Vs. ammonium grown cultures of *A. fumigatus*. Total RNA was blotted onto a nylon membrane from wild-type *A. fumigatus* cells grown on minimal media either containing nitrate (inducer) or ammonium (repressor) as the sole nitrogen source. In all figures (20a-c), represents total RNA (20μg of RNA/lane) from nitrate-induced cells, and ammonium-repressed cells.

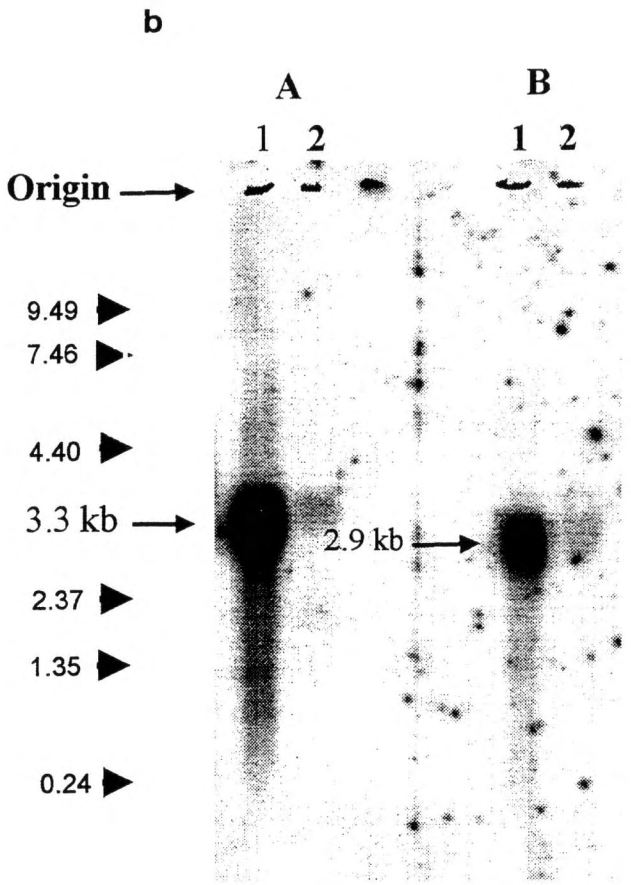
(a) Total RNA from nitrate-grown (1) or ammonium-grown (2) cells, as loaded onto formaldehyde denaturing agarose gels in triplicates for Northern blot analysis with three probes (A-C).

(b) Northern blots probed either with (A) *niiA* gene DNA (2.8 EcoRI/PstI fragment; Fig. 3), or (B) with *A. fumigatus niaD* DNA (1.6 kb EcoRI fragment; Fig. 3).

(c) Northern blot probed with (C) *crnA* gene DNA (0.7 kb EcoRI fragment; Fig. 3).

a

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A
B
1
2
1
2
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4.40
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2.9 kb
2.37
1.35
0.24

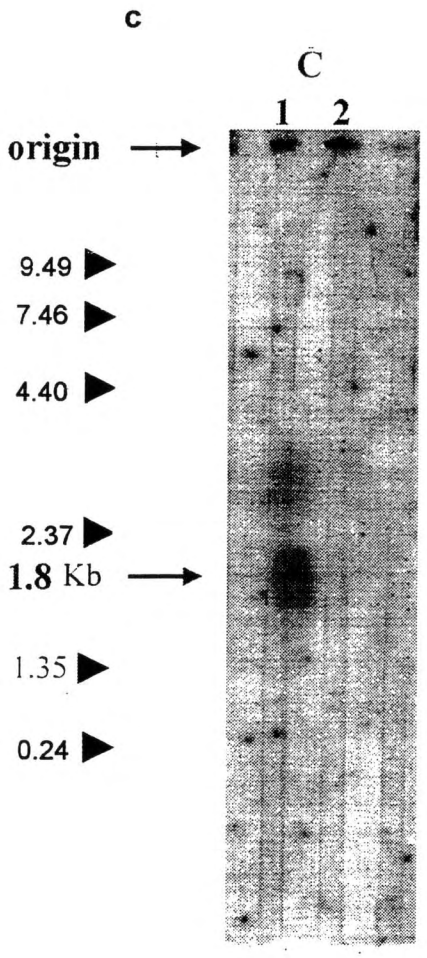
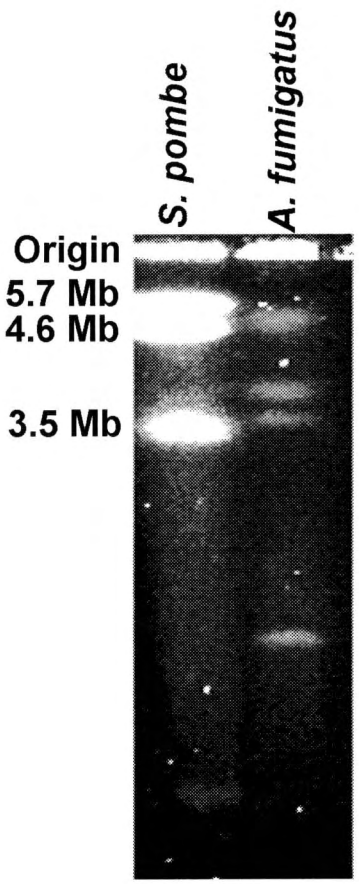

c
C
1 2
origin
9.49
7.46
4.40
2.37
1.8 Kb
1.35
0.24

Figure 21. Pulsed field gel analyis of *A. fumigatus* chromosomes:

(a) Ethidium bromide stained pulsed field gel of the intact chromosomes from *A. fumigatus* and the *S. pombe* chromosomes as a marker.

(b) Southern blot analysis of wild-type *A. fumigatus* chromosomes with *niiA* 2.8 kb EcoRI/PstI genomic fragment. The probe detected one of the top bands estimated to be approximately 5.3 Mb.

a



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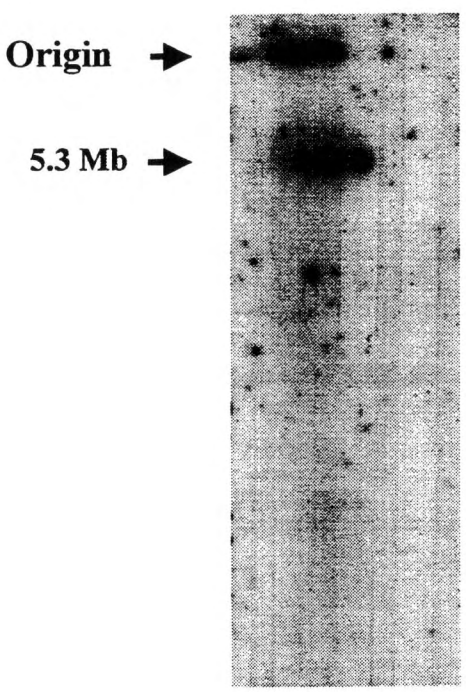


Section III

Gene disruption of the *niiA* gene and complementation by *niiA* clones of *A. fumigatus* and *A. nidulans*.

a) Disruption of the *niiA* gene

To obtain *A. fumigatus* strains lacking the nitrite reductase by gene disruption (*niiA*$^-$), a plasmid containing an internal fragment of the cloned niiA gene and the prokaryotic phosphotransferase gene (*hph*; hygromycin resistance gene) was constructed (pYA10), Fig. 22 for details, see Methods Section).

Using the transformation procedures outlined in the material and methods, this plasmid was transformed into wild-type *A. fumigatus* protoplasts, by selection for hygromycin resistance. After incubation of the transformed protoplasts for 7 days at 30° C, *A. fumigatus* hyg^r transformant colonies were clearly visible on the selection plates. Eighteen colonies were randomly picked (from a total of about 500), and transferred onto test plates containing either nitrate, nitrite, or ammonium plus 250 µg/ml hygromycin B, and grown for 5 days. Two of the eighteen *A. fumigatus* hyg^r transformants tested (T3 and T6) tested showed poor growth compared to wildtype on minimal medium with nitrate as the sole nitrogen source suggesting that the *niiA* gene had been disrupted. The transformants T3 and T6 were further tested on liquid minimal media (2% glucose, salt solution and trace elements with 250 µg /ml hygromycin B). Both transformants T3 and T6 showed no growth in nitrate minimal liquid culture medium after 3 days of incubation (28° C with shaking at 200 rpm) and presumably were *niiA*$^-$, in contrast to wild type

(however, weak growth was found after 72 hrs of incubation at 28°C). The above transformation experiment (with pYA10) resulted in about 10 hyg-resistant transformants /μg of DNA.

b) Molecular analysis of the *niiA*⁻ transformant (T6)

The transformant T6 was chosen for further DNA analysis. A Southern blot containing DNA of the wild-type *A. fumigatus* strain (ATCC 13073) and of T6 digested with EcoRI and several other enzymes [EcoRI/HindIII, SalI, EcoRI/SalI, and EcoRI/PstI] (Figure 23a) was probed with the 1.9 kb HindIII/PstI genomic fragment corresponding to the N- terminus of the *A. fumigatus niiA* gene (Figure 22). As expected, from the sequence and restriction map of the *niiA*, the probe detected a single band in all digests of wild-type DNA: namely bands of 6.5 kb for EcoRI, 5 kb for EcoRI/HindIII, >8 kb for SalI, 3.6 kb for SalI/EcoRI, and 2.7 kb for EcoRI/PstI (in lanes 1, 3, 5, 7, and 9, respectively; Figure 23b). In contrast, the *niiA* gene probe detected two bands in lanes containing DNA of the transformant T6 which contained the disrupted plasmid and therefore the 5' fragment of *niiA* (7.4 and 6.4 kb for EcoRI fragments, 7.3 and 5 kb for EcoRI/HindIII fragments, 8 kb and 2.7 kb for SalI fragments, 6.2 kb and 2.9 kb for SalI/EcoRI fragments, and 2.7 kb and 2 kb for EcoRI/PstI in lane 2, 4, 6, 8, and 10 (Figure 23b). These hybridization results clearly indicated that the plasmid pYA10 was integrated within the *A. fumigatus niiA* gene and hence the *niiA* gene was disrupted and non-functional (see below, Figure 25).

In addition, DNA from *niiA*-disruption transformant (T6) was digested as above

and also BamHI (Figure 24a) and was analyzed using two probes; the Bluescript® plasmid (Figure 24b) and DNA of the hygromycin B resistance gene (Figure 24c). The Bluescript® probe detected a single band in each DNA lane: a 7.4 kb EcoRI fragment, a 6.6 kb EcoRI/HindIII fragment, a 5.2 EcoRI/PstI fragment, a 8 kb SalI fragment, and a 3.7 kb BamHI fragment in lanes 1-5, respectively, suggesting that a single copy of the plasmid was integrated in the genome (Figure 24b). The hygromycin B resistance gene probe detected the following bands: 7.4 kb and 6.3 kb EcoRI fragments, 6.5 kb and 0.7 kb EcoRI/HindIII fragments, 4.7 kb and 0.5 kb EcoRI/PstI, a 2.4 kb SalI fragment, and 8 kb BamHI fragment in lanes 1-5 respectively (Figure 24c). All in all the signals detected from the transformant T6 DNA by the three probes suggest that the transformation plasmid pYA10 was specifically integrated within the *niiA* gene (Figure 25).

c) Mitotic stability of the disrupting insert (pYA10) in the transformant T6

In order to determine the mitotic stability of the *niiA*⁻ transformant (T6), cells derived from a single T6 protoplast were inoculated into non-selective medium (not containing hygromycin B) and incubated at 28° C for 3 to 5 days. After full vegetative growth, conidia were mass inoculated into selective media (containing 250 µg /ml hygromycin B) and incubated at 28° C for 3 to 5 days. The *A. fumigatus niiA* mutant (transformant T6) grew well on the selective media suggesting that the T6 transformant was mitotically stable. The original transformant T6 (disrupted for *niiA*) was also re-tested on minimal plates containing nitrate or ammonium as a N-source. In contrast to the wildtype, it showed poor growth on nitrate.

Figure 22. Schematic diagram showing the construction of the *niiA*-disruption plasmid: pYA10. The plasmid pYA22-3 contains a 2.8 kb EcoRI genomic fragment (open line) of the *A. fumigatus niiA* gene, which includes part of the promoter and the first third of the coding sequence, and the thin line represents the Bluescript® vector. The solid black line in plasmid pID21 (Tang *et al.*, 1992) represents a 2.4 kb SalI/HindIII fragment consisting of the bacterial hygromycin B resistance gene (*hph*) flanked by the *A. nidulans trpC* promoter and terminator sequences, and the thin line represents pUC18 vector (see materials and methods). Restriction sites: E= EcoRI, P= PstI, S= SalI, and B= BamHI.

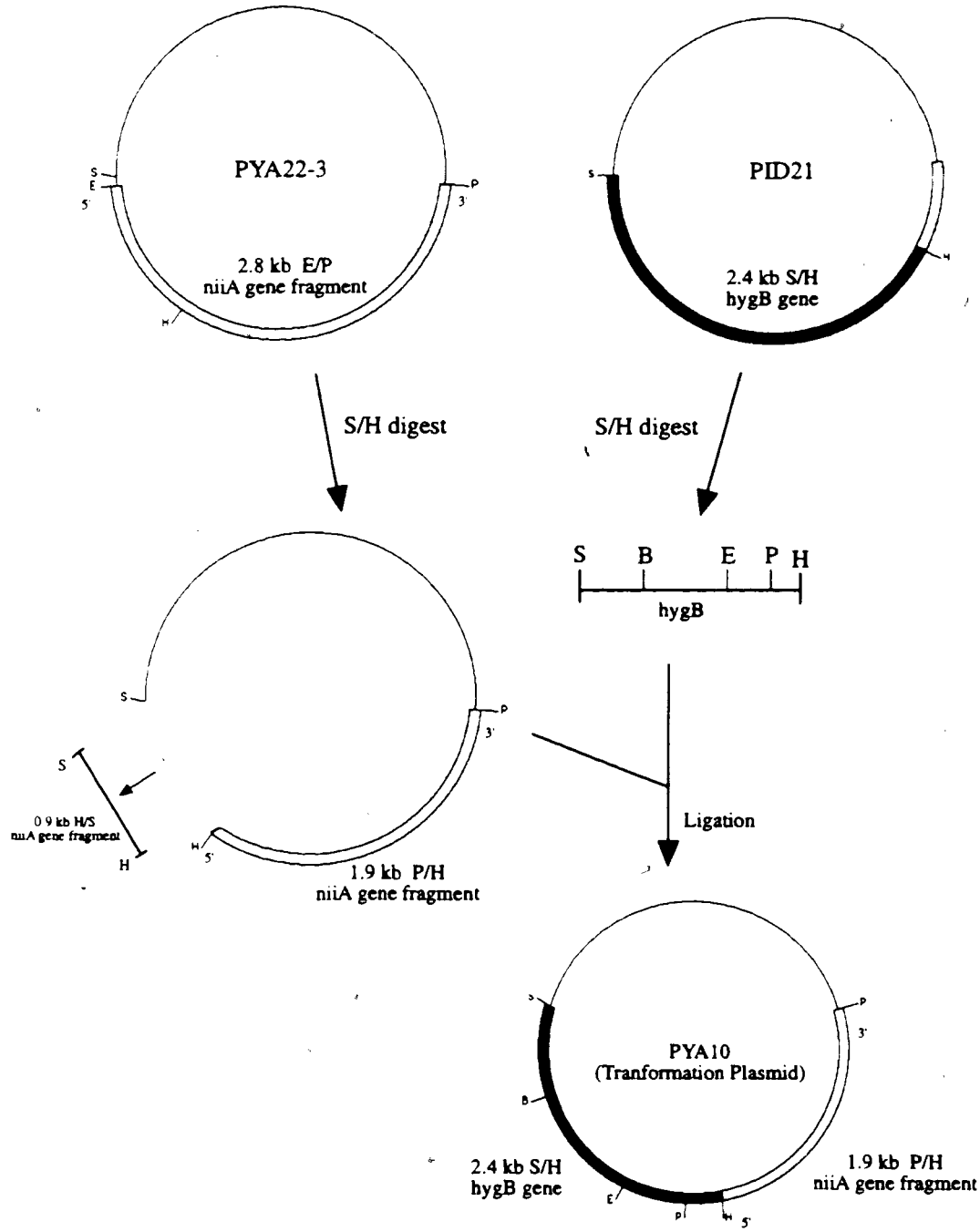
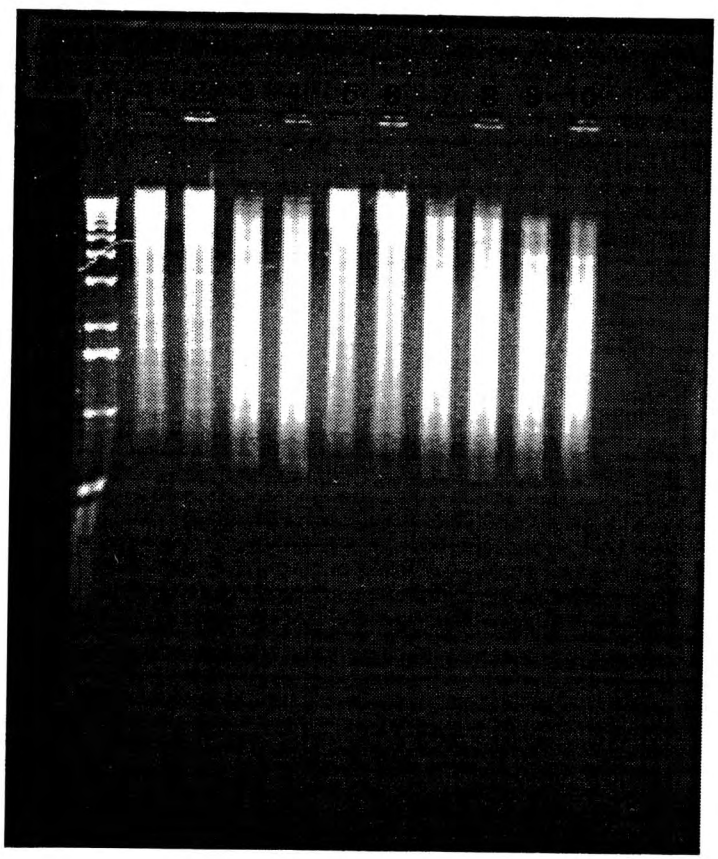

PYA22-3
2.8 kb E/P
niiA gene fragment
PID21
2.4 kb S/H
hygB gene
S/H digest
S/H digest
S B E P H
hygB
0.9 kb H/S
niiA gene fragment
1.9 kb P/H
niiA gene fragment
Ligation
PYA10
(Tranformation Plasmid)
2.4 kb S/H
hygB gene
1.9 kb P/H
niiA gene fragment

Figure 23. Analysis of the restriction pattern at the *niiA* locus in putative *niiA*-disruption transformants and wildtype, using *niiA* DNA as a probe.

(a) Ethidium bromide stained gel containing genomic DNA from *A. fumigatus* wildtype and transformant T6, digested with a number of enzymes. Lanes 1, 3,5,7, and 9 contain wildtype DNA and lanes 2, 4, 6, 8, and 10 T6 DNA. Both types of DNA were digested with EcoRI (lanes 1, 2), EcoRI/HindIII (lanes 3, 4), SalI (lanes 5, 6), EcoRI/SalI (lanes 7, 8), and EcoRI/PstI (lanes 9 and 10).

(b) Southern analysis of *A. fumigatus* wildtype and T6 DNA, using the 1.9 kb HindIII/PstI *niiA* genomic fragment as a probe. M = 1 kb marker.

a



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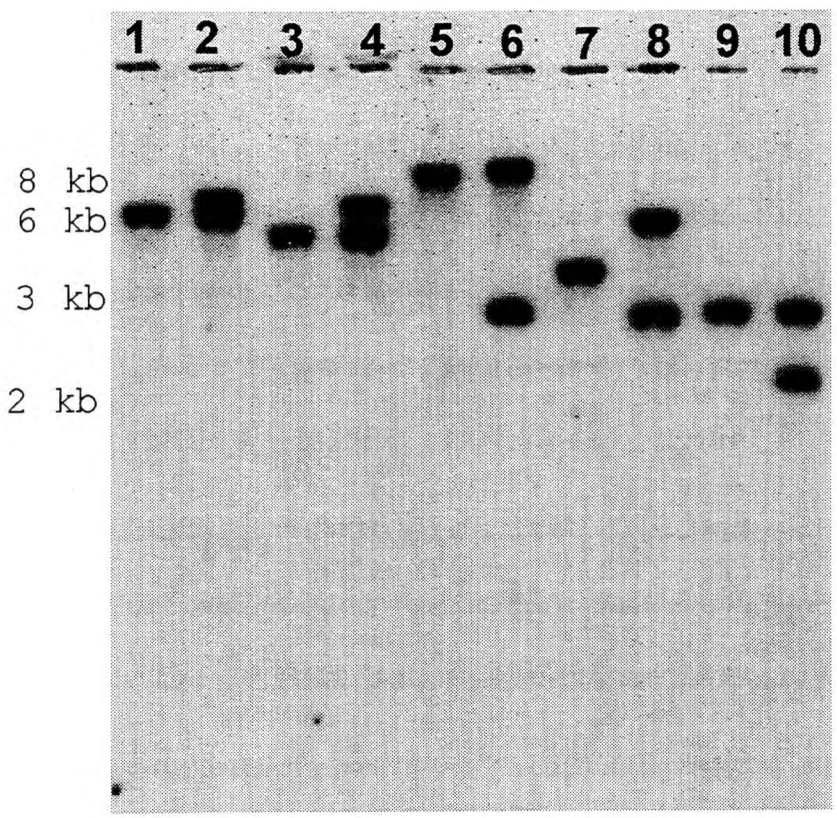


d) Complementation of the *niiA*⁻ phenotype by *niiA*⁺ cloned DNA

Protoplasts from the *A. fumigatus niiA*⁻ mutant (disruptant **T6**) were transformed with linearized *niiA* DNA (in pYA2-3; Fig. 3) and were plated on *niiA*⁺ selecting media. Some of the many colonies appearing on plates containing media selective for the *niiA*⁺ transformants were randomly picked, purified, and inoculated into non-selective media. After 3 to 5 days of incubation at 28° C, conidia from four colonies were transferred onto selective media [minimal liquid and solid media containing purified (OXOID) agar with nitrate as the sole nitrogen source] and were incubated at 28° C for 3 to 5 days. The four isolates grew as well as the wildtype on such agar media. Corresponding results were obtained in even shorter time for tests in liquid media. Table 1 shows the growth scores in liquid minimal nitrate medium recorded for four rescued colonies (A-D), T6 (*niiA*⁻), and wildtype (*niiA*⁺). Similar results were obtained when protoplasts of T6 were transformed with the plasmid *pniiA* containing the *niiA* gene

from *A. nidulans* (5 µg of circular plasmid DNA; Table 2, colonies E-H). These results demonstrate that transformation with both the *A. fumigatus niiA* gene sequence (pYA2-3) and the *A. nidulans niiA* DNA (p*niiA* plasmid) rescued the *A. fumigatus niiA* mutant.

To test the untransformed (*niiA*⁻) T6 control (0 DNA) for rare revertants and additive growth, protoplasts were plated on selective medium containing either 5 mM nitrate or onto non-selective medium with 10 mM ammonium. On the latter medium T6 protoplasts formed a lawn of cells and conidia from five different spots on the plate were again transferred to non-selective medium. After 5 days of secondary growth, the five *niiA*⁻ T6 colonies were tested for reversion and therefore growth in selective liquid media

containing 10 mM nitrate as the nitrogen source. No growth was observed during the first 48 hours (but slight growth was seen by 72 hrs as is typical for the T6 strain; Table 2, colonies A-E). Similarly, small and large colonies were picked from the original (hyg^r-selective) transformation plates containing $NH4^+$ (as the sole nitrogen source) and were transferred to non-selective MM medium with five small and five large colonies, these secondary colonies were tested for growth on nitrate minimal liquid media. The five large colonies showed growth identical to that of wild-type as shown on the original selective plates(Table 2, colonies F-J). However, the five small colonies showed reduced growth (Table 2, colonies K-O).

e) Chlorate toxicity assay

In order to assess the chlorate toxicity *A. fumigatus*, wild-type *A. fumigatus* conidia were inoculated onto three sets of minimal media plates containing one of three nitrogen sources: ammonium, urea, or L-arginine. In addition, conidia were inoculated into agar plates containing these minimal media but supplemented with potassium chlorate of different concentrations (Table 3). Plates were incubated at 28° C for 5 days, and were scored for growth by measuring the radial diameter (Table 5). In presence of chlorate concentration at 0.0, 100, 200, and 400mM, the radial diameter decreasing with increasing chlorate, especially if the N-source was arginine while growth was less reduced in MM plus ammonium or urea as nitrogen source (see Discussion).

Table 2. Test scores of growth for 1-3 days in liquid minimal medium containing 10 mM nitrate as the sole nitrogen source for the *A. fumigatus* T6 *niiA*⁻ disruptant transformed with *niiA*⁺ DNA, and controls. Control strains were wildtype (Wt; *niiA*⁺) and 4 isolates were tested each from T6 transformed with either *A. fumigatus niiA*⁺ (pYA2-3; isolates A-D) or with *A. nidulans niiA*⁺ (p*niiA*; isolates E-H),and T6 (original disruption; *niiA*⁻). The numerical scores indicate relative growth, scored visually, (3 strongest, 0.5 weakest, 0 no growth). d = T6 *niiA*⁻ disruptant.

	Incubation time in hours (nitrate MM)		
Colony Id	24	48	72
Wt (*niiA*⁺)	1	2	3
T6 (d niiA⁻)	**0**	**0**	**0.5**
A-D (d +*Af niiA*⁺)	1	2	3
E-H (d + An *niiA*⁺)	1	2	3

Table 3. Growth test scores in liquid nitrate minimal media of T6 control cells (0 DNA) from colonies grown on minimal media plates, either containing 5 mM nitrate or 10 mM ammonium as the sole nitrogen source. Colonies A-E were picked from plates containing 10 mM ammonium. Colonies F-J large and K-O small colonies selected from plates containing 5 mM nitrate. Wt = wild-type, T6 = *A. fumigatus niiA* disruption mutant. The numerical scores indicate relative growth, scored visually, (3 strongest, 0.5 weakest, 0 no growth).

	Incubation time in hrs (nitrate MM)		
Colonies	24	48	72
Wt	1	2	3
T6	**0**	**0**	**0.5**
A-E	0	0	0.5
F-J (large)	1	2	3
K-O (small)	0.4	0.8	1.2

Table 4. Shows the effect of three different chlorate concentrations on growth of wild-type *A. fumigatus* cells using three different nitrogen sources. The effect of growth was assessed by measuring the radial growth diameter.

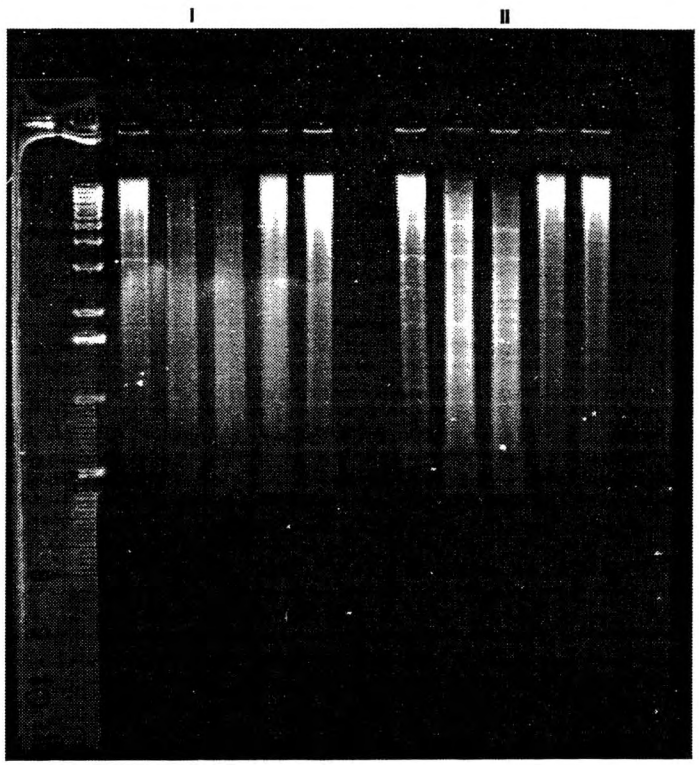
	Radial Growth in cm.			
Chlorate Conc. (mM) / Nitrogen Source	0	100	200	400
10 mM ammonium tartrate	4.0	2.0	1.2	0.6
10 mM urea	4.0	1.4	0.8	0.3
50 mM L-arginine	3.0	0	0	0

Figure 24 Southern analysis of the putative *niiA* disrupted transformant (T6; similar to Fig. 23 but with different digests and probes).

(a) Ethidium bromide stained gel containing T6 DNA digested with a number of restriction enzymes. Lane 1 = EcoRI, lane2 = EcoRI/HindIII, lane3 = EcoRI/PstI, lane 4 = SalI, and lane 5 = BamHI. Section I and II are the same DNA digest.

(b) Southern analysis of T6 DNA using the Bluescript® as a probe (section I); this probe detected a single band in lanes 1-5 indicating a single copy integration of pYA10.

a



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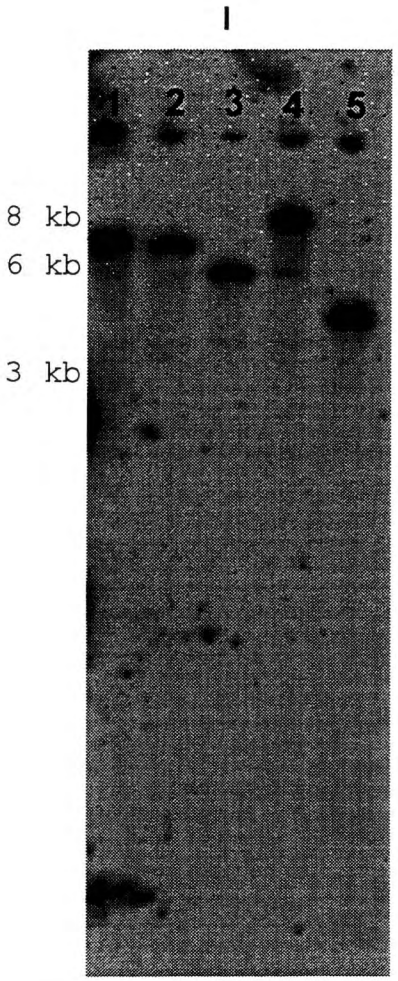

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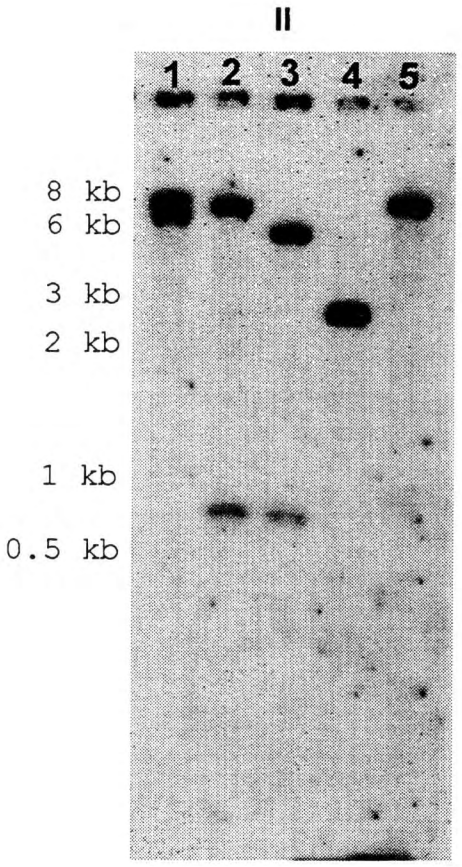
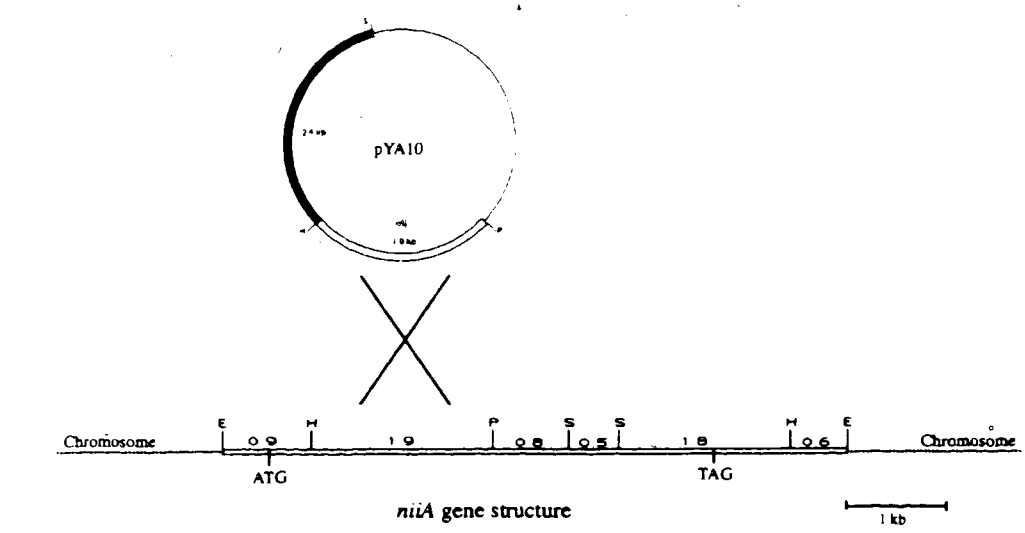
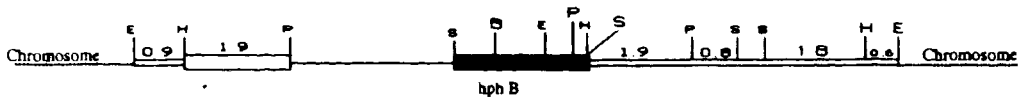


Figure 25. Restriction map of the *A. fumigatus niiA* gene region before and after insertion of the disruption construct (pYA10) which contains part of *niiA* gene (open region), the Bluescript® (thin line), the hygromycin gene (solid black bar representing the 2.4 kb SalI/HindIII fragment of the *hph* gene, flanked with the *A. nidulans* promoter and terminator). Doted lines represent *A. fumigatus* chromosomal DNA flanking the *niiA* gene. Numbers indicate fragment sizes in kb. B = BamHI, E = EcoRI, H = HindIII, P = PstI, and S = SalI.



niiA gene structure



Disrupted *niiA* gene

DISCUSSION

Section I

Cloning and characterization of the nitrate assimilation gene cluster (*crnA-niiA-niaD*) of *A. fumigatus*

A genomic clone (λYA4-3) contains most of the *Aspergillus fumigatus* nitrate assimilation gene cluster

Sequence data determined in this study indicates that the *A. fumigatus* 15.5 EcoRI genomic insert of λYA4-3 contains most of the nitrate assimilation gene cluster (*crnA-niiA-niaD*) over an 8.8 kb EcoRI fragment. The *A. fumigatus* nitrate assimilation gene cluster is highly conserved with respect to that of *A. nidulans* (Johnstone *et al.*, 1990).

The plasmid pYA2-3 contains the entire sequence of the *niiA* gene

The genomic sequences derived from the ends as well as internal regions the 6.5 kb EcoRI genomic fragment suggested that this fragment contains sequences corresponding to the *A. fumigatus niiA* gene. As in *A. nidulans* and *A. niger*, the *A. fumigatus* nitrite reductase gene and a nitrate transporter gene are physically linked, and are transcribed in the same direction. The nitrate reductase (*niaD*) gene is also linked to this unit and in all cases (*A. nidulans*, *A. niger*, *A. parasiticus*) the two are transcribed in the opposite direction (Johnstone *et al.*, 1990, Unkles *et al.*, 1992, Chang *et al.*, 1996). Considering the high homology found not only for the *A. fumigatus niiA* gene but also for most of the intergenic promoter and regulatory region as well as the coding region of the *niaD* gene. It seems well justified to conclude that the *A. fumigatus niiA* and *niaD* genes transcribed in opposite direction (see Section II Discussion).

The genomic library clone contains part of the *A. fumigatus crnA* gene

It is clear from the high degree of sequence homology observed that the genomic sequences obtained from regions 6, 7, and 8 must correspond to the *A. fumigatus crnA* gene. The combined genomic sequence obtained from regions 6 and 7 contains two small introns. The *A. fumigatus* coding sequence obtained in this study corresponds to transmembrane domains II, III, IV, V, VI, VII, and VIII of *A. nidulans* CRNA which has 12 transmembrane domains (I-XII) (Unkles *et al.*, 1991, 1995). It also shares significant homology (40% identity) with the high-affinity nitrate transporters *nar-3* from *Chlamydomonas reinhardtii* and *BCHI* from *Hordeum vulgare* (Quesada *et al.*, 1994, Trueman *et al.*, 1996).

The partial putative *A. fumigatus* CRNA sequence contained the motifs: D/NRXGRR/K and IXXRXXGXXXC found conserved in a group of transporters known as the major facilitator superfamily (MFS). The MFS encompasses a diverse collection of transporters from prokaryotes and eukaryotes which include the mammalian passive glucose transporters, antibiotic efflux pumps, and a number of bacterial proton-dependent sugar transporters (Marger and Saier, 1993).

The plasmid pYA61 contains part of the *A. fumigatus niaD* gene.

Based on homology to the *A. niger niaD* gene sequence (Unkles, 1992), the genomic sequences obtained from pYA61 were found to correspond to the *A. fumigatus niaD* gene. The structural gene for the nitrate reductase has been cloned and characterized from a number of organisms representing prokaryotes, eukaryotes, plants, and algae (Kinghorn and Campbell, 1989). Gene sequences and their putative amino acid sequences from different organisms were aligned so that sequence similarities could be inferred. When aligned, the

deduced amino acid sequence from region 1 (far N-terminus) showed extensive homology with the corresponding regions for the nitrate reductase protein sequences from *A. parasiticus* (Chang *et al.*, 1996), *A. niger* (Unkles, 1991), *Ustilago maydis* (Banks *et al.*, 1993), *Neurospora crassa* (Exley *et al.*, 1993), *Hordeum vulgare* (Schnorr *et al.*, 1991), *Arabidopsis thaliana* (Crawford *et al.*, 1988). One stretch of 1 to 13 amino acid consecutive matches among all the sequences was seen. The region aligned falls within the MoCo-binding domain, which contains a cysteine residue identified in *A. nidulans* NIAD (cysteine residue 150) as a critical residue for binding the molybdenum atom. Replacement of this cysteine residue with an alanine leads to a non-functional NIAD (Grade *et* al., 1995). The significant sequence homology among plant and fungal nitrate reductase sequences suggests that these genes may have originated from a common ancestral gene (Campbell and Kinghorn, 1990). In addition, genomic sequence corresponding to the N-terminus was also obtained. This genomic sequence is found to contain two introns. Alignment of the *A. fumigatus* far N-terminus with that of *A. parasiticus* and *A. niger* NIAD showed some homology but not as extensive as that shared among the same region from *A. parasiticus*, *A. niger*, and *A. nidulans*. This could be due to the fact that the *A. fumigatus niaD* N-terminus was sequenced only once.

The sequence up-stream of the *A. fumigatus niaD* was found to be 40% identical to corresponding regions from other *A. parasiticus* and *A. nidulans* sequences (Chang *et al.*, 1996, Johnstone *et al.*, 1990). In addition, the four small introns identified in the partial *niaD* gene sequence obtained in this study were found to occur at the same position as introns 1,2, 3 and 4 of both *A. nidulans* and *A. niger*; which means that the niaD gene sequences are highly conserved.

Genetic and molecular studies have shown that the enzyme nitrate reductase may regulate its own synthesis as well as the synthesis of the enzyme nitrite reductase (*niiA*) and nitrate transporter (*crnA*) genes (Hawker *et al.*, 1992, Cove, 1979, Tomsett and Garrèt, 1980). However, the exact mechanism of such regulation has not yet been resolved. Some studies suggested that the nitrate reductase enzyme may interact directly with NIRA protein to exert its regulatory effect by preventing these regulatory proteins from entering the nucleus and as a result prevent the expression of the nitrate assimilation gene cluster. In presence of nitrate, the NIRA proteins dissociate from the nitrate reductase and are free to enter the nucleus and to turn on the transcription of the nitrate assimilation genes (Maloy and Stewart, 1993). Other studies suggested that the nitrate reductase may not directly interact with NIRA protein but that it may have indirect metabolic involvement (Maloy, 1993).

To gain further insights into the *A. fumigatus* NIAD structure, function, evolution, and its regulation, the rest of the *niaD* gene should be cloned and sequenced. It also important to identify transcription initation / termination sites.

Part of the genomic library clone may contain a novel fungal protease gene.

The remaining 7 kb EcoRI genomic fragment from the 15.8 genomic clone was found to be unrelated to the nitrate assimilation gene cluster, and it may contain a novel fungal protease gene. Partial sequence analysis of the ends of a 7 kb EcoRI genomic fragment indicated that this fragment can not be placed on a restriction map adjacent to any of the other three genomic fragments. This finding means that the adjacent fragment contains neither the remaining genomic sequences corresponding to the *niaD* gene nor the

remainder of the *crnA* gene sequence. The 7 kb EcoRI genomic DNA fragment is therefore unrelated to the nitrate assimilation gene cluster and not physically linked within the genome. It appears that during the preparation of the library, this 7 kb EcoRI genomic fragment may have become joined to the genomic fragment (8.8 kb) containing the nitrate assimilation gene cluster.

Some of the genomic sequence obtained from pYA211-2 showed extensive homology with alkaline protease protein sequences from *Erwinia chrysanthemi* and *Pseudomonas aeruginosa*. The sequence matches observed are very interesting because the proteases of *E. chrysanthemi* and *P. aeruginosa* are thought to play a key role in infection of host cells (Delepelaire and Wandersman, 1989, Dahler *et al.*, 1990, Kawasaki *et al.*, 1990, Miyatake *et al.*, 1995). The *A. fumigatus* putative amino acid sequence contains three motifs similar to the calcium binding domain GGXGDXUX (where X is any amino acid and U is a bulky hydrophobic amino acid) common to alkaline proteases (Baumann *et al.*, 1993). Thus it was important to determine if this 7 kb EcoRI fragment is actually a fragment native to the *A. fumigatus* genome, rather than one of the two *E. coli* strains (DH5α and KW251) used for propagation of the genomic clones.

Southern analysis of genomic DNA from two *A. fumigatus* strains and from two *E. coli* strains was carried out using two probes derived from the genomic insert of pYA1-2 . The probes did not detect any signal from the DNA of any of the organisms tested. The finding might suggest that the genomic fragment is strain-specific as the genomic library, from which the 2.2 kb EcoRI/EcoRV was originally derived, was made from the *A. fumigatus* isolate CHUV 192-88 (Jaton-Ogay *et al.*, 1992).

PCR analysis of the genomic DNA of the two *A. fumigatus* strains and the two *E.*

coli strains was also carried out and the primers used amplified multiple products including a band around the size expected, from all DNA templates used; which might be indicative of non-specific amplification. One would expect a single band if the amplification had been specific. Since the primers used were designed from a potential protease gene sequence and it is known that there are gene families of related proteases (Monod *et al.*, 1993), then using a given set of protease primers may give the expected specific product as well as products of other closely related genes. It is unlikely in this case that a specific product was amplified due to the fact that no signals were detected on a Southern blot using the same DNA. Therefore, both Southern and PCR analysis, confirm that the 7 kb EcoRI fragment is neither part of the genome of the two *A. fumigatus* strains nor of the two *E. coli* strains tested in this study. Hence, it is logical to conclude that the 7 kb EcoRI genomic fragment derived from the genomic clone λYA4-3 is either unique to the *A. fumigatus* isolate CHUV 192-88 from which the library was made, or it is a contaminant.

DNA typing methods have detected genomic polymorphism among different *A. fumigatus* isolates (Aufauvre-Brown *et al.*, 1992, Loudon *et al.*, 1993, Girardin *et al.*, 1994a, and Girardin *et al.*, 1994b). A 0.95 kb DNA fragment (a potential molecular marker randomly amplified) has been correlated with the virulence of some *A. fumigatus* isolates (Mondon *et al.*, 1995). Mondon and his group carried out PCR analysis on seven strains of *A. fumigatus* which were isolated from different patients with non-invasive (3 strains) and invasive (4 strains) aspergillosis and four environmental strains, using primers designed from the 0.95 kb molecular marker. The PCR results showed that five strains (2 strains isolated from patients with invasive, one strain isolated from a patient with non-invasive aspergillosis, and 2 enviromental strains) were positive isolates for the 0.95 kb molecular

marker, and the other six strains were negative (Mondon *et al.*, 1996). When the eleven strains were tested for virulence in an experimental murine model of invasive pulmonary aspergillosis, it was found that the 0.95 kb-positive strains caused significantly higher mortality rates in mice than the 0.95 kb-negative strains. The authors think that their findings support the hypothesis that certain isolates of *A. fumigatus* are more virulent than others and that their virulence may be associated with the 0.95 kb molecular marker. It should be noted that this 0.95 kb molecular marker does not show any homology with any sequence in the databanks. In light of the literature mentioned above, it would be very interesting to probe the genomic DNA of the *A. fumigatus* isolate CHUV 192-88 and other *A. fumigatus* isolates with DNA fragments derived from the 7 kb EcoRI genomic fragment. If future work proves that the 7 kb EcoRI genomic fragment in fact belongs to the *A. fumigatus* isolate CHUV 192-88 and/or other *A. fumigatus* isolates, it would suggest that the 1416 bp genomic sequence derived from the 7 kb EcoRI in this study might represent a novel fungal protease gene fragment. It would also present an even more interesting candidate as a putative virulence marker than the 0.95 kb genomic fragment proposed by Mondon *et al.*, (1995 and 1996). This is because the 1416 bp genomic fragment contains an open reading frame with high homology to secreted proteinase b from *E. chrysanthemi* and alkaline metalloproteinase from *P. aeruginosa*. Both proteases from *E. chrysanthemi* and *P. aeruginosa* have been suggested to contribute to the virulence of their respective organisms. The pathological aspects of the *P. aeruginosa* alkaline protease have been extensively studied and it has been found that this protease exhibits the potential anti-coagulant activity; hydrolysis of natural substrates such as fibrin and fibrinogen, and in addition it may inactivate various physiological activators such as immunoglobulins A and G and protease

inhibitors (Miyatake *et al.*, 1995). *P. aeruginosa* alkaline protease has also been investigated in the medical field as a target for anti-infection agents against the bacterium (Miyatake *et al.* 1995, Homma and Tanimoto, 1987). Therefore, it is worthwhile to determine if the 7 kb EcoRI genomic fragment belongs to the *A. fumigatus* isolate CHUV192-88, and to investigate whether it contains an active novel fungal protease gene.

DISCUSSION

Section II

Characterization of the *A. fumigatus* nitrite reductase gene (*niiA*). Analysis of the complete genomic sequence of the *niiA* gene obtained

The structural gene for *A. fumigatus* nitrite reductase (*niiA*) was cloned and sequenced and checked for homology with the corresponding *A. nidulans niiA* gene sequence (Johnstone *et al.*, 1990). The regions corresponding to the *niiA-niaD* intergenic region (promoter and regulatory regions), coding region, and the 3' untranslated region of the *A. fumigatus niiA* gene showed high homology in short functional regions mainly of the 5' end. The coding region is interrupted by eight small introns 42 to 74 bp long. All of the introns identified start with GT and end with CAG or TAG consensus sequences, respectively. In addition, the *A. fumigatus* introns also contain conserved internal sequences, such as TAAG, CTGAC, TAAC, and CAAT which are signature sequences characteristic of fungal introns (Ballance, 1986; Gurr *et al.*, 1987).

Introns number and position

The high amino acid sequence homology between *A. fumigatus* and *A. nidulans* NIIR sequences was useful in determining the position of seven corresponding introns among the eight introns identified within the nucleotide sequence of the *A. fumigatus niiA* gene. In addition *A. fumigatus* apparently has one extra intron (intron 4). Intron 4 of the *A. fumigatus niiA* gene was detected because it contains stop codons which disrupt the open reading frame. Since both strands of the *A. fumigatus niiA* gene were sequenced, it is likely that this sequence represents a true intron as also suggested by the homology of

the adjacent AA sequences. The ultimate proof that intron 4 exists will require obtaining and sequencing of the corresponding region from the *niiA* cDNA clone. Chang *et al.* (1996) recently reported the sequencing of some of the *A. parasiticus niiA* gene and found that the sequence obtained also contains an extra intron (intron 4) compared to its homologue from *A. nidulans* (Chang *et al.*, 1996). The presence of an extra intron in both *A. fumigatus* and *A. parasiticus* is an interesting finding because both species are classified as pathogenic fungi. Do all *niiA* genes from pathogenic *Aspergillus* species contain an additional intron, and if they do, what kind of evolutionary implications might it have? The answer to these questions has to await the cloning and sequencing of more *niiA* genes from other pathogenic *Aspergillus* species.

Conservation of intron position has also been observed in other fungal genes. Similarly, the *A. nidulans* and *N. crassa* glutamate dehydrogenase genes show a striking conservation of the positions of their two introns, although there is no conservation of intron sequence (Gurr *et al.*, 1987, Kinnaird and Fincham, 1983). In addition, both intron position and sequence conservation have been found in the *glaA* gene from *A. awamori* and *A. niger* (Numberg *et al.*, 1984; Boel *et al.*, 1984). However, lack of intron conservation has also been found , e.g. for the orotidine decarboxylase gene, *N. crassa* and yeast lack the only intron identified in the orotidine decarboxylase gene from *A. nidulans* (Campbell and Kinghorn, 1990). The occurrence and positions of introns within homologous genes from different organisms have been used to infer evolutionary gene relationships. The existence of introns in filamentous fungal genes but not in their budding yeast homologs, raises the question whether genes of multicellular eukaryotes gained introns, or whether yeast and prokaryotic genes have lost theirs at some point during evolution. The answer to this

question is not yet clear. However, current thinking favors the idea that intron loss occurred in unicellular organisms rather than intron gain in higher organisms. The hypothesis is based on the argument that growth and population turn-over of unicellular organisms are faster than that of multicellular organisms and therefore loss of non-coding, unused DNA, would be more extreme in unicellular organisms; hence, introns in filamentous fungal genes may be retained by this group of organisms which grow more slowly than yeast, and are of a multicellular nature (Gurr *et al.*, 1987). However, some increase of intron number and size, during evolution of large genomes of vertebrates appears obvious.

The open reading frame of the *niiA* gene

The *A. fumigatus niiA* gene encodes a putative protein sequence of 1110 amino acids when all of the eight introns are taken out. It also could encode a putative protein sequence of 776 amino acids if the last intron is retained as part of the coding sequence (intron 8 contains a stop codon). The longer open reading frame is terminated by the TAG stop codon while the shorter open reading frame terminates with the TAA stop codon. It has been reported by Johnstone *et. al* that intron 7 of the *A. nidulans niiA* gene contains a stop codon which may give a short transcript that could be translated into a polypeptide of 771 amino acids (if intron 7 is not spliced out). A longer transcript which translates to 1104 amino acids is obtained when intron 7 is spliced out (Johnstone *et al.*, 1990). Reverse transcriptase PCR experiments carried out by Johnstone *et al.* showed that two transcripts of the *A. nidulans niiA* gene were made. Their results indicated that the shorter transcripts accounts for 90% of the total *niiA* mRNA made in the cell under the conditions used for this analysis. However, no evidence has been reported for two

simultaneous transcripts, or for at least the shorter one on a Northern blot to confirm that the *A. nidulans niiA* gene is actually alternatively spliced.

The *A. fumigatus niiA* gene has the same gene structural organization found in the *A. nidulans niiA* gene. Evidence from partial sequence of the *A. fumigatus niiA* cDNA clone obtained in this study suggests that in this case the long open reading frame was translated. The cDNA fragment included the coding sequence upstream and downstream of intron 8 which meant that intron 8 was spliced out. In addition, Northern analysis of total RNA using the *niiA* gene fragment as the probe detected a single 3.3 kb band. The 3.3 kb band is likely to correspond to the longer transcript but it could correspond to a pre-mRNA transcript. Northern analysis of *A. fumigatus* or *A. nidulans* polyA$^+$ cytoplasmic mRNA will be required to decisively determine whether or not the *niiA* gene is alternatively spliced.

Protein sequence analysis

Pairwise comparison of the putative protein sequence from the *A. fumigatus niiA* to that of *A. nidulans* showed a 80% amino acid identity over 1104 amino acids (Figure 5b). The *A. fumigatus* NIIA sequence also shows a 71% amino acid identity with the partial *A. parasiticus* protein sequence (368 amino acids) reported by Chang *et al.*, 1996. The *A. fumigatus* protein sequence also shows a high level of amino acid identity with *N. crassa* (59%) and *E. coli* (44.9%). Comparison of the *A. fumigatus* and *A. nidulans niiA* protein sequence has also confirmed important functional domains. Amino acid sequences identified as FAD and NAD(P)H domains in *A. nidulans* were also found in the same position at residues 44 to 79 and 146 to 176 in *A. fumigatus*, respectively. A

comparison analysis of the *A. nidulans* NIIA sequence to other flavoproteins such as *Pseudomonas fluorescens* p-hydroxybenzoate (Weijer *et al.*, 1982), pig D-amino oxidase (Ronchi *et al.*,1982), *E. coli* respiratory lactate dehydrogenase (Campbell *et al.*,1984), showed no similarities among these enzymes. The *A. nidulans* NIIA sequence also showed no homology with FAD-containing human or bovine NAD cytochrome b5 reductase (Kinghorn and Campbell, 1989). Based on these comparisons the FAD-containing domain could not be identified with certainty in the *A. nidulans* NIIA enzyme (Kinghorn and Campbell, 1989). The assignment of the FAD- and NADPH- binding domains to the N-terminus in fungal NIIA is based on the fact that the N-terminus regions assigned contain the βαβ dinucleotide binding fold GXGXXG, a common feature within FAD- and NADH binding domains (Weirenga *et al.*, 1985).

The four cysteine residues identified in the *A. nidulans niiA* protein at positions 720, 726, 760, and 764, as metal-binding sites for iron-sulfur and siroheme were found to be conserved in the *A. fumigatus niiA* putative protein sequence at positions 725, 731, 765, 769. Sequence alignment also showed that the four cysteine residues identified as metal-binding sites were conserved in the nitrite reductase protein sequences from *N. crassa* and *E. coli* (Figure 6). The number and identity of residues between these conserved cysteine residues are similar, i.e. N-terminal, cysxxxxxcys Xn cys xxx cys, C-terminal, among fungal and *E. coli* nitrite reductase protein sequences. The five amino acids (**CVGTTWC**) separating the very N-terminus cysteines are identical in *A. fumigatus*, *A. nidulans*, *N. crassa*, and *E. coli*. The three residues (**CV/TREC**) separating the last two cysteine residues are also conserved. In *A. fumigatus* and *A. nidulans* the residues are VRE and in *N. crassa* and *E. coli* the residues were TRE. Fungal and *E. coli* NIR show an

extensive number of shared motifs which imply similarity of structure and function. The sequence homology observed from the multiple alignment analysis further confirms the notion the genes encoding these proteins once evolved from one ancestral gene (Kinghorn and Campbell, 1989).

In contrast to the high sequence homology observed for fungi and bacteria, fungal and plant nitrite reductase proteins show no or very little sequence homology between plant and fungal nitrate reductase proteins (Kinghorn and Campbell, 1989). For example, plant nitrite reductase enzymes have been found to be much shorter than the fungal enzymes. Such differences in size are attributed to the fact that fungal nitrite reductase enzymes contain a FAD domain, while plant enzymes lack this domain. These findings suggest that the nitrite reductase genes of filamentous fungi and plants may have evolved from different ancestral genes (Kinghorn and Campbell, 1989).

Codon usage

The codon usage for the nitrite reductase gene in *A. fumigatus*, *A. nidulans* (Johnstone *et al.*, 1990), *N. crassa* (Exley *et al.*, 1993), and *E. coli* (Peakman *et al.*, 1990) was analyzed. Codon counts are useful in showing varying patterns of codon preference. For example, the codon AAG-Lys is used twice as much as the codon AAA-Lys in *A. fumigatus*, *A. nidulans*, and *N. crassa,* whereas the codon AAA-Lys is used 4.3 times as much as the codon AAG-Lys in bacteria. It is clear that in the *E. coli* nitrite reductase gene the codon AAA-Lys was preferred over the codon AAG-Lys which suggests that the tRNA for the codon AAA-Lys is more abundant than the tRNA for the AAG-Lys. Another example of significant difference in codon usage is that the codon ACG-Thr is

used 21 times in *A. fumigatus*, 12 times in *A. nidulans*, 12 in *N. crassa*, and 1 time in *E. coli*. The fungal tRNA for the codon ACG-Thr is more frequently used than its counterpart in *E. coli*.

Analysis of the 5' upstream region and the 3' untranslated region

The 5' upstream sequence of the *A. fumigatus niiA* contains consensus sequences which are typical of regulatory regions. Two putative TATA and two putative CAAT boxes were identified. Four GATA motifs were also identified in all *niaD-niiA* intergenic regions investigated so far from *Aspergillus* species. Gel shift and DNA footprint experiments in *A. nidulans* , *A. parasiticus*, and *N. crassa* have shown that GATA motifs bind the positively-acting regulatory product of the *areA* gene (Punt *et al.*, 1995, Chang *et al.* 1996, Chiang and Marzluf, 1994). Comparison of the *niiA-niaD* intergenic regulatory region of *A. fumigatus* to that of *A. nidulans* (Johnstone *et al.*, 1990) revealed that they share 42% sequence identity, which is similar to those obtained among other *Aspergillus* species. The *A. fumigatus* promoter region is 43% and 37% identical to the regulatory regions from *A. parasiticus* and *N. crassa* (Chang *et al.*, 1996, Exley *et al.*, 1993). It should be noted that the *A. oryzae* and *A. parasiticus niiA-niaD* intergenic regions show 95% identity which is unusually high identity for a regulatory region.

Sequences of the *A. fumigatus niiA-niaD* intergenic region which are found to share significant homology with *A. nidulans* promoter sequences besides the GATA motifs included the consensus sequences TCCGCGGA, a complete palindrome, and CCCGCGGGG, an incomplete palindrome. These consensus sequences have been experimentally confirmed as binding sites for the regulatory proteins *NIRA* in *A.*

nidulans, *A. parasiticus*, and in *N. crassa* using mobility shift assays (Punt *et al.*, 1995, Fu and Marzluf, 1990, Chang *et al.*, 1996). The distance between the GATA motif and the palindrome CTCCGCGGA is found to be 40-80 nucleotides which may suggest possible protein-protein interactions between the products of the *areA* and *nirA* genes in the activation of *niiA* gene expression (Chang *et al.*, 1996). The presence of the GATA motifs and the CTCCGCGGA palindrome in the *niiA-niaD* intergenic regions suggest that different species share similar regulatory mechanisms of nitrate assimilation. No work has been done to identify transcription/termination starts within the *niiA-niaD* intergenic region of any of the *Aspergillus* species, and hence they need to be determined.

The *niiA* 3' untranslated region obtained in this study did not contain a polyadenylation signal. Hence, more sequence is required in order to determine whether or not the *niiA* gene contains a polyA addition site. However, absence of the polyadenylation signal is not unusual in fungal genes (Gurr *et al.*, 1987). The results discussed above strongly demonstrate that the genomic sequence obtained in this study corresponds to the functional *A. fumigatus* nitrite reductase (*niiA*) gene.

Northern and chromosomal mapping analysis

Northern analysis of total RNA from cells grown in minimal media containing either nitrate or ammonium indicate that in wild-type strains of *A. fumigatus* the expression of the nitrate assimilation gene cluster is induced by nitrate and repressed by ammonium as is the case reported in *A. nidulans* (Cove, 1979). The *A. fumigatus* chromosomes migrated as four bands on pulsed field electrophoresis, similar to those ,eight chromosomes, found in *A. niger* (Verdoes *et al.*, 1994, 1989, Debets *et al.*, 1990a).

A. nidulans also has eight chromosomes (Kafer, 1958) which when migrate as six bands on pulsed field electrophoresis (Brody and Carbon, 1989; Ehninger *et al.*, 1990). The nitrate assimilation gene cluster was mapped to the largest chromosomal band and the size was estimated to be about 5.3 Mb using *S. pombe* as a size marker. Since in *A. nidulans* the nitrate assimilation gene cluster was mapped to chromosome VIII (the largest chromosome; Cove, 1979), the chromosome detected by the probe in this study may correspond to chromosome VIII of *A. fumigatus* (if in fact the *A. fumigatus* genome is made up of eight chromosomes like its close relatives *A. niger* and *A. nidulans*). Therefore, some of four bands of *A. fumigatus* genome may represent a double or triple chromosomes migrating together as one, as found in *A. nidulans* and *A. niger*. The smallest band of the *A. fumigatus* genome may represent two chromosomes, the second smallest band may represent a single chromosomes, the second largest band may represent two chromosomes, and the largest band may represent three chromosomes.

DISCUSSION

Section III

Gene disruption of the *niiA* gene and complementation by *niiA* clones of *A. fumigatus* and *A. nidulans*.

Recovery of two *niiA*⁻ disruptants by transformation with part of the cloned *niiA* gene

The *A. fumigatus niiA* endogenous gene was disrupted by an integrative hygromycin-plasmid containing part of the *niiA* gene . Two *A. fumigatus* transformants (T3 and T6) unable to utilize nitrate as the sole nitrogen source were obtained (about 10% of the tested hyg^r-transformants). Southern analysis of the DNA from the *niiA*⁻ mutant T6 and the wild-type indicated that the disrupting plasmid (pYA10) was specifically integrated within the *A. fumigatus niiA* gene. Growth tests in liquid minimal media were very useful in identifying transformants defective in nitrate or nitrite utilization, being rapid and clear-cut: 24 hrs of growth in liquid media was enough to distinguish between a mutant (no growth at all) and wild-type strains (healthy growth). Transformants T3 and T6 showed some growth in liquid media after 72 hrs suggesting that a reversal of the integration process may have occurred in some cells or some of the dying cells are able to feed back ground growth. Rare cells which have the plasmid excised will have a selective advantage, but tests using transfer to nonselective medium indicated that such disruptants are mitotically stable. The *niiA*⁻ mutant T6 was found to be mitotically stable

in the absence of the selectable marker used, the antibiotic hygromycin B.

Southern analysis of T6 DNA

The molecular analysis results obtained in this study were consistent with the growth tests mentioned above, and showed that the *niiA* gene of the *A. fumigatus* (ATCC 13073) was disrupted and that the hygromycin plasmid containing a *niiA* gene fragment was inserted into the *niiA* sequence at a single site. The transformation work reported in this study provides all the elements for the development of an efficient homologous transformation system for *A. fumigatus*. In addition, heterologous transformation system should be possible using dominant resistance genes, such benomyl resistance (Orbach *et al.*,1986), or hygromycin resistance (Tang *et al.*, 1992, Punt *et al.*, 1987) have also been achieved. Other filamentous fungal transformation systems using the acetamidase gene (*amdS*; Debets *et al.*,1990b) or the nitrate reductase gene (*niaD*; Malardier *et al.*, 1989) have also been successfully applied to several species of filamentous fungi of agricultural and industrial, importance (Daboussi *et al.*, 1989), and are no doubts possible in *A. fumigatus*.

Since the *A. fumigatus* has seen little genetic characterization to date, developing a homologous as well as heterologous transformation systems for this organism will be very useful in future studies of other genes of interest. In fact, the disrupting plasmid pYA10 can be used in co-transformation providing a double selection system; namely selection for hygromycin B resistance and for nitrate utilization by *niiA*$^{-}$ complemented transformants.

Alternatively, the *A. fumigatus niaD* gene fragment cloned in this study (pYA61) could be used to disrupt the *niaD* gene. Development of a homologous transformation system for *A. fumigatus* based on the nitrate reductase gene (*niaD*) will offer certain advantages over the use of a dominant selectable marker such as hygromycin resistance or the use of *niiA* described here. For example nitrate reductase gene (*niaD*) mutants are easily generated provided good conditions for selection of chlorate resistant mutants can be found as reported for several filamentous fungal species (Unkles *et al.*, 1989, Daboussi *et al.*, 1989, Debets *et al.*, 1990c).

To assess the possibilities for isolation of chlorate resistant mutants (including *niaD*$^-$) the sensitivity of *A. fumigatus* wildtype to chlorate toxicity was investigated using three different chlorate concentrations and three different nitrogen sources. The results obtained showed that sensitivity *A. fumigatus* increased with increasing chlorate concentration (as judged by growth inhibition) when ammonium or urea were used as nitrogen sources, but the growth of *A. fumigatus* was even more inhibited by chlorate when L-arginine was used as a nitrogen source. Similar findings have been reported for chlorate toxicity in wild-type *A. nidulans* by Cove (1976). It was concluded that chlorate toxicity effects (100 mM chlorate) on wild-type *A. fumigatus* are seen best if (50 mM) L-arginine is used as the nitrogen source, so that arginine is the recommended nitrogen source for selection of *niaD* mutants and other chlorate resistant mutants. In fact, a number of *A. fumigatus* chlorate resistant colonies have been generated during the chlorate toxicity assays but these have not yet been characterized. Such colonies are likely to include mutants of several genes but can be classified based on their ability to grow on various N-sources as described for *A. niger* chlorate resistant

mutants by Debets *et al.*(1990c).

Complementation of the *niiA*$^-$ mutant (T6)

Complementation experiments using transformation by the *niiA*$^+$ gene from *A. fumigatus* and *A. nidulans* confirmed that the transformant T6 is *niiA*$^-$ as a result of gene disruption in the *niiA* locus. The results from the control experiments suggest that some *niiA*$^-$ mutant T6 protoplasts may spontaneously revert during mitosis at low frequency, presumably by mitotic recombination which leads to excision of the integrated plasmid when plated on *niiA*$^+$ selective medium containing nitrate as the sole nitrogen source. However, the sample tested in this study is too small to assess the frequency of spontaneous plasmid loss with any certainty. Because spontaneous plasmid loss of the disruption plasmid can occur, *niiA*$^+$ transformants postulated to be rescued by *niiA*$^+$ of *A. fumigatus* and *A. nidulans* (pYA2-3 or p*niiA*) need to be checked by Southern blot analysis to distinguish between those rescued by cloned *niiA*$^+$ DNA and spontaneous wildtype revertants.

CONCLUSIONS

Findings of this study include:

1). **Isolation of a clone of the niaD-niiA-crnA gene cluster of A. fumigatus:** a) a genomic clone containing a large (15.8 kb) EcoRI insert isolated from an *A. fumigatus* genomic library, using a cDNA probe corresponding to the *A. fumigatus* nitrite reductase gene (*niiA*); b) the larger part of this insert (an 8.8 kb EcoRI fragment) contained most of the nitrate assimilation gene cluster. The cluster was shown to contain the complete gene sequence of nitrite reductase (*niiA*), and partial sequences of nitrate reductase gene (*niaD*) , and nitrate transporter gene (*crnA*), and c) The remainder of the genomic clone (7 kb EcoRI insert) was found to be the *A. fumigatus* nitrate assimilation gene cluster, but sequencing results suggest that it may contain a novel fungal protease.

2). **Sequence Characterization**: Comparison of the deduced NIAD amino acid sequence of the far N-terminus region (of the clone) shared some homology with its homologs from fungal species while deduced amino acids of the middle region showed extensive sequence homology (79% identity over 197 amino acids) to both fungal and plant nitrate reductase protein sequences.

The partial DNA sequence determined from the *crn*A gene fragment exhibit a high degree of sequence homology with the corresponding region of the *A. nidulans* *crnA* gene sequence. The CRNA contained motifs motifs D/NRXGRR/K and IXXRXXGXXXC found conserved in a group of transporters known as the major facilitator superfamily (MFS) which include antibiotic efflux pumps and mammalian passive glucose transporters.

The complete genomic sequence of the *niiA* gene sequence contained an extra

intron with respect to that of *A. nidulans, which is also,* present in *A. parasiticus*. The NIIA putative protein sequence of 1110 amino acids shares 80% identity with the *A. nidulans* NIIA protein sequence.

3). **Gene Disruption**: a mitotically stable *niiA*⁻ mutant was obtained by disruption of the *niiA* locus and homologous integration of a gene fragment as well as the selective hygromycin resistance gene.

4). **Chromosome Mapping**: the *A. fumigatus* nitrate assimilation gene cluster was mapped to the largest migrating chromosomal band and the size was estimated to be 5.3 Mb (using *S. pombe* chromosomal DNA as a size marker). The expression of the *A. fumigatus* gene cluster nitrate assimilation gene cluster was shown to be induced by nitrate and repressed by ammonium (as had been found for several other species of filamentous fungi). Results indicate that the genes for nitrate assimilation in *A. fumigatus* are physically linked and are transcribed in the same direction as in *A. nidulans, A. niger,* and *A. parasitcus.*

5). **Chlorate Toxicity**: sensitivity of *A. fumigatus* to chlorate toxicity was investigated and was found to vary depending on the nitrogen source used. Growth of wild-type *A. fumigatus* was inhibited when 50 mM nitrogen source was used in presence of 100 mM potassium chlorate, and some putative mutants were obtained (which may include several types, including *niaD*).

Contributions of this study

Contributions to our knowledge of the molecular characterization of *A. fumigatus* in general, and to the nitrate assimilation gene cluster in particular, have been achieved in

this study. Lack of genetic markers in *A. fumigatus* makes use of transformation as a start of molecular analysis difficult. However, this study has made available all the important elements needed to develop an effective, homologous transformation system for this medically important fungal pathogen. Such a transformation system can make use of *niaD* mutants, which can be easily created by disrupting the *A. fumigatus niaD* gene (using an integrative recombination plasmid containing a segment of the *niaD* gene fragment cloned in this study). Insights about homologous transformation in *A. fumigatus* have been obtained with the demonstration that stable and specific single site integration can be achieved at reasonable frequencies using the *niiA* gene. Development of this and other homologous transformation systems will expedite the study of other *A. fumigatus* genes of interest, especially those encoding potential virulence factors.

RECOMMENDATIONS FOR FUTURE WORK

1. Cloning of the remainder of both the *niaD* and *crnA* genes.

2. Use of available niaD fragment to disrupt the *niaD* gene, i.e. to generate *niaD*$^-$ mutants, which can be selected and stably maintained on chlorate.

3. Perform gel mobility shift experiments on the *niiA-niaD* intergenic regulatory region using reporter gene constructs to determine if any additional regulatory elements can be identified.

4. Conduct polyA$^+$ mRNA analysis to determine splicing pattern of the *niiA* gene and its regulation.

5. Determine if the DNA unrelated to the *niaD* cluster found in the same library clone (pYA1-2) contains a novel functional protease gene.

APPENDIX

Section I

Preliminary work completed in attempting the isolation of the cytochrome P-450 lanosterol 14-α demethylase gene from *Aspergillus fumigatus*.

INTRODUCTION

The cytochrome P-450s are a group of ubiquitous, heme-containing enzymes which catalyze the oxidation of many diverse endogenous as well as xenobiotic substrates. The classification of these enzymes as cytochrome P450 is based on their absorbance spectrum. When they are complexed with carbon monoxide (Fe(II)-CO complex) (Guengerich, 1991), these enzymes show a characteristic absorption maximum near 450nm. The mono-oxygenases require both molecular oxygen and reducing equivalents from NADPH cytochrome P450 reductase to carry out their catalytic functions(Guengerich, 1991). Most eukaryotic cytochrome P450 enzymes are localized in endoplasmic reticulum (ER) membrane although mitochondrial cytochrome P450 enzymes have also been identified and are primarily involved in steroid biotransformation in the adrenal gland (Porter and Coon, 1991).

Cytochrome P-450 enzymes constitute a gene superfamily which is widely distributed among higher and lower eukaryotes and to a limited extent to prokaryotes (Porter and Coon, 1991). In the mammalian P-450 system, multiple P-450s are encoded with a gene family which may contain more than 100 genes (Nebert and Gonzolez, 1987). Some of these enzymes exhibit wide substrate specificities while other P-450s exhibit strict selectivity for their intrinsic substrates (Guengerich, 1991). The mammalian

P-450 system has been the most extensively studied and has been shown to be involved in the biosynthesis of such compounds as cholesterol, steroid hormones, and prostaglandins as well as in the biotransformation of lipophilic xenobiotics such as drugs, pesticides, and other chemical carcinogens (Kalb *et al.*, 1986). In contrast to the higher eukaryotic P450 system, cytochrome P450 enzymes in lower eukaryotes such as fungi are not well understood but biochemical evidence suggests that a wide range of P450 enzymes exists (Yoshida and Aoyama, 1991).

Only a few cytochrome P450 proteins have been isolated from fungi . The most extensively studied and characterized fungal enzyme is the lanosterol 14-α demethylase (14DM) from the yeast *Saccharomyces cerevisiae* (Kalb *et al.*, 1987, Aoyama and Youshida, 1978, Turi and Loper, 1992). This cytochrome P-450 catalyzes the demethylation of lanosterol which is the first step in the biosynthesis of cholesterol or ergosterol in mammalian and fungal cells, respectively (Guengerich, 1991, Porter and Coon, 1991, Yoshida and Aoyama, 1991, Aoyama *et al.*, 1984). The oxidative removal of the methyl group has been verified by reconstitution studies with purified 14DM in both systems (Yoshida and Aoyama, 1991, Trzaskos *et al.*, 1986). The structural gene for the 14DM has been cloned and sequenced from *S. cerevisiae* (Kalb *et al.*, 1987), *Candida tropicalis* (Chen *et al.*, 1988), and *Candida albicans* (Kirsch *et al.*, 1988). Sequences from these three yeast species share a significant homology (> 60% identity) at the amino acid level (Kirsch *et al.*, 1988)

The 14DM protein is of particular interest to both pharmaceutical and agrochemical industries because 14DM has been shown to be the specific target of azole antifungal

agents such as imidazole (Van den Bossche *et al.*,1980, Van den Bossche *et al.*, 1988). A number of azole antifungal agents are now being used both in clinical medicine to treat mycosis and in agriculture for crop protection from phytopathogenic fungi (Porter and Coon, 1991). The fungistatic action of azole is thought to be the result of a complex multi-mechanistic process arising from changes in membrane sterol composition brought about by the inhibition of P450 14DM (Guengerich, 1991, Porter and Coon, 1991). Studies investigating the interaction of 14DM from *S. cerevisiae* with azoles (imidazole and itraconozole) have indicated that azoles inhibit the function of 14DM by binding to P-450 14DM (Yoshida and Aoyama, 1977, Yoshida and Aoyama, 1984, Van den Bossche *et al.*, 1980, Van den Bossche, 1988, Aoyama *et al.*, 1983). The azole-treated fungi accumulate 14α sterols which then disrupt membrane structure and function in addition to membrane integrity (Porter and Coon, 1991). Azoles may also affect the level of sparking ergosterol which is thought to have a regulatory function in mitotic division (Guengerich, 1991). For example, fluconazole-treated *Candida albicans* has been shown to develop large multinucleated cells.

The potency of azoles against P-450 14DM has been evaluated by measuring the incorporation of ^{14}C-acetic or ^{14}C-mevalonic acids into the 14α-methylated sterols of azole treated cells and lysates (Guengerich, 1991, Porter and Coon, 1991). Both structure and lipophilicity are important in determining the potency of azoles against P-450 14DM. The P-450 14DM has a discrete active site which is capable of binding ligands that mimic the lanosterol substrates (Guengerich, 1991, Porter and Coon, 1991). Experiments by Yoshida and Aoyama (1987) have shown that ketoconazole is able to induce marked spectral changes (Type II) of the P-450 14DM purified from *S. cerevisiae* microsomes.

Ketoconazole has also been shown to inhibit the 14α demethylation of lanosterol in a reconstituted system consisting of 14DM and NADPH-cytochrome P-450 reductase (Yoshida and Aoyama, 1978).

Studies on the 14DM of fungi other than *S. cerevisiae* have shown that the azoles have a similar binding and inhibitory effects. Using microsomal fractions of *C. albicans*, a 50% decrease in the absorption spectrum at 448 nm (absorbance maximum for yeast cytochrome P-450-carbon monoxide complex) was reported at 3.1 x 10^{-8} M-ketoconazole (Van den Bossche *et al.*, 1980). In exponentially growing *C. albicans*, a 50% inhibition of ergosterol synthesis was achieved at 4 x 10^{-9} M-ketoconazole after 1 h of contact (Van den Bossche *et al.*, 1980). Similarly, inhibition of ergosterol synthesis in exponentially growing *Aspergillus fumigatus* was observed at 5.8 x 10^{-8} M-ketoconazole (Van den Bossche *et al.*, 1988). A ketoconazole concentration of 6 x 10^{-8} M, added to microsomal fractions of *A. fumigatus*, resulted in a 50% decrease in absorbance at 451 nm (absorbance maximum of fungal cytochrome P450-carbon monoxide complex; Marichal, 1989). These concentrations are approximately 10 times lower than those required to inhibit mammalian cytochrome P-450 enzymes (Van den Bossche *et al.*, 1990)

The increased occurrence of life-threatening, invasive fungal infections especially among individuals undergoing chemotherapy or with AIDS (Marichal, 1989) has stimulated research on the development of new antifungal agents. The filamentous fungus, *A. fumigatus* is one of the major causes of life threatening invasive fungal infections in immunocompromised patients (Holden *et al.*, 1994). To date, no work has been reported on the molecular cloning and characterization of the structural gene

corresponding to the cytochrome P-450 lanosterol 14α demethylase from this medically-important filamentous fungus. The initial purpose of this work was to clone and characterize the lanosterol 14α demethylase from *A. fumigatus*. The cloning of the 14DM gene would have provided the first cytochrome P-450 gene sequence from *A. fumigatus* to be characterized at the molecular level. The characterization of the *A. fumigatus* 14DM gene and its product would have been useful in examining structure-function relationships among 14DM genes in higher and lower eukaryotes. It might have contributed to our understanding of control of fungal disease by providing structural information to aid in the design of more effective azole antifungal agents.

The work presented in this section did not result in the isolation of the 14DM gene. But, it led to the recovery of a 1.5 kb EcoRI cDNA fragment corresponding to the *A. fumigatus* nitrite reductase gene (*niiA*). 250-300 nucleotides from each end of the cDNA clone were used to search DNA and protein data banks. The cDNA sequence showed extensive homology with *A. nidulans* and *Neurospora crassa* nitrite reductase DNA sequence, 73% identity in 237 bp overlap and 61.7% identity in 248 bp overlap respectively. The deduced amino acid sequence showed 80% identity with a 75 amino acid overlap with the *A. nidulans* nitrite reductase protein sequence.

MATERIALS AND METHODS

Fungal strain

The *Aspergillus fumigatus* (13073) and *Saccharomyces cerevisiae* (D273 10B) were obtained from ATCC. For isolation of DNA, the strains were grown on MYPD medium (as described above; p. 23).

Genomic DNA isolation

Isolation of DNA from *A. fumigatus* mycelia was carried out using standard phenol-chloroform methods (Sambrook *et al.*, 1989). For medium scale DNA extraction, mycelia were collected by filtration and were immersed in liquid nitrogen. The frozen mycelia were ground to a powder using a mortar and pestle. DNA extraction buffer (1 ml/100 mg) (50 mM Tris-HCL pH 7.5, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) was added to the frozen powder and the mixture gently shaken as it thawed. The mixture was then incubated at 65° C for 1 hr. An equal volume of phenol-chloroform was then added, and the mixture shaken and centrifuged at 14000 rpm for 20 min. The aqueous phase was extracted three more times with phenol-chloroform and twice with chloroform-isoamyl alcohol. The DNA was precipitated in an equal volume of isopropanol by centrifugation at 14000 rpm at 4° C. The pellet was resuspended in 500 ml sterile ddH2O, and 10 µl of RNAase (10mg/ml) was added to the DNA which was then incubated at 37° C for 1 hr. DNA was subsequently precipitated by adding 2.5

volume of 95% ethanol and 0.1 volumes of 3 M sodium acetate pH 5.2, and centrifuged at 4° C for 20 min. The pellet was washed with 70% ethanol and air dried. The dried pellet was resuspended in 10 mM Tris-HCL pH 7.5, 1 mM EDTA (TE). DNA from *Saccharomyces cerevisiae* was isolated using the same procedures.

Oligonucleotides

Two 18-mer oligonucleotides separated by 550 bp of sequence were designed from *S. cerevisiae* lanosterol 14α demethylase (14DM) gene sequence (Kalb *et al.*, 1987). The position of the two Oligos with respect to 14DM gene coding sequence was 959-917 for oligo 1 and 1487-1509 for oligo 2. The primers were synthesized on an oligonucleotide synthesizer (Applied Biosystems) in the Institute of Molecular Biology and Biochemistry, Simon Fraser University. Primers 1 and 2 were used to amplify a 0.5 kb PCR fragment which contained the conserved heme-binding region (HR-2) of the *S. cerevisiae* 14DM gene.

PCR

PCR reactions were carried out as described in p. 28.

Preparation of DNA fragments for oligolabeling

See page 25

Southern blotting

(see p. 25/26)

Hybridization.

The *S. cerevisiae* ^{32}P-labeled probe was hybridized at low stringency to *A. fumigatus* genomic DNA overnight at 42° C in a solution containing 5xSSC, 5% Denhardt's, and 0.5% SDS (Sambrook, 1989). The filter was washed with successive solutions of 3xSSC, 2xSSC, 1xSSC, and 0.5xSSC containing 0.1% SDS at 42° C. Hybridization of homologous probes to DNA was carried out at high stringency as described by Sambrook *et al.* 1989 (see p. 26).

Subcloning

(see p. 26)

Screening an *Aspergillus fumigatus* (CHUV 192-88) λEMBL3 genomic library.

The *A. fumigatus* (CHUV 192-88) λEMBL3 genomic library was screened with the 0.5 kb PCR fragment containing the conserved heme-binding region (HR-2) of the *S. cerevisiae* 14DM gene under low stringency conditions (as described above). Approximately twenty thousand phage were screened using standard methods as described in Sambrook *et al.* (1989). Three rounds of screening were carried out.

Screening an *A. fumigatus* λZAP cDNA library.

An *A. fumigatus* λZAP library kindly provided by Dr. M. Monod was screened with the 0.7kb EcoRI/SalI *A. fumigatus* genomic fragment derived from the genomic library clone λYA4. Three rounds of screening were carried out under high stringency conditions (as described in p. 26). The plasmid from one apparently hybridizing λZAP phage isolate was excised according to the standard protocol (Stratagene), producing a plasmid (pBluescript®) which contained a 1.5 kb EcoRI cDNA insert.

RESULTS

a) Southern analysis of *A. fumigatus* genomic DNA using a PCR fragment from the *S. cerevisiae* 14DM gene

It was important to establish that the *A. fumigatus* genome contains the gene of interest and that it can be detected by using a heterologous probe. A 0.5 kb PCR fragment containing the conserved heme-binding region (HR-2) (Figure 26A) of. *S. cerevisiae* 14DM gene (Kalb *et al.*, 1987) was used as a probe in the analysis of genomic DNA isolated from *A. fumigatus* at low stringency conditions (see Materials and Methods). The ^{32}P-labeled PCR fragment (probe) hybridized to a 1.8-2 kb MboI genomic fragment (Figure 27); suggested that the putative *A. fumigatus* lanosterol 14α - demethylase gene was present as a single copy.

b) Screening an *A. fumigatus* EMBL3 genomic library with the yeast 14DM probe

The 0.5 kb yeast 14DM probe was subsequently used to screen an *A. fumigatus* (CHUV 192-88) λEMBL 3 genomic library to determine if a genomic fragment containing the *A. fumigatus* 14DM gene could be recovered. Only 1/20,000 phage screened resulted in the recovery of a genomic clone containing a 15-20 kb EcoRI genomic fragment (λYA4).

Figure 26. Schematic diagram showing the region of *S. cerevisiae* lanosterol 14-α demethylase (14DM) gene used as a probe. Arrows indicate the position of the two oligonucleotides used to amplify a PCR product of approximately 550 bp. The HR2 is the conserved heme-binding region in 14DM genes. The 0.5 kb PCR product was labeled with ^{32}P and used for both Southern analysis of genomic DNA and screening of the genomic library of *A. fumigatus*.

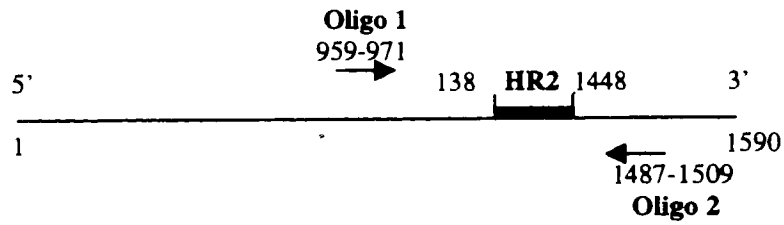
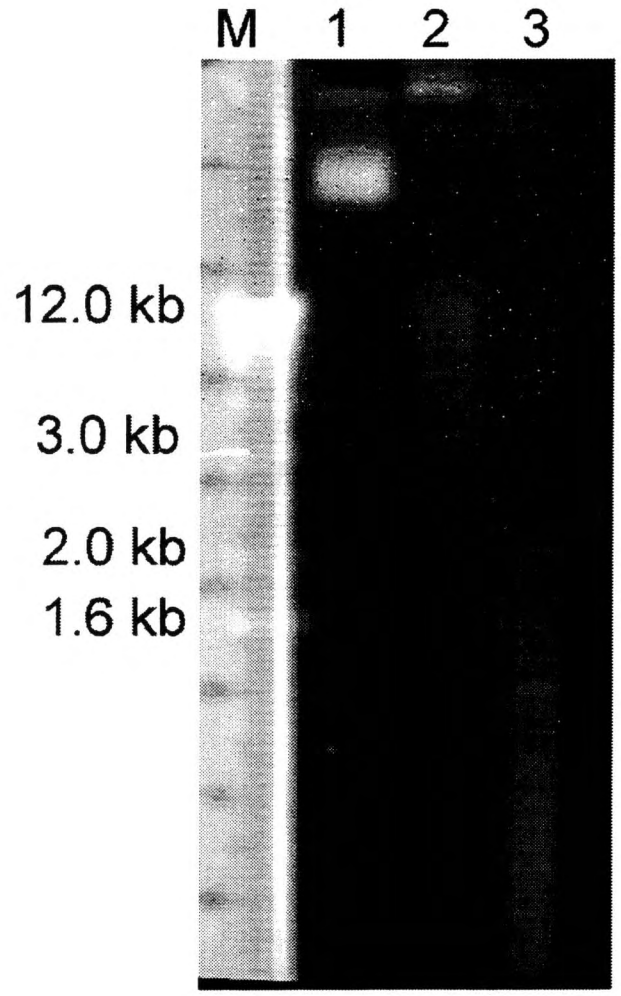

Oligo 1
959-971
5'
138
HR2
1448
3'
1
1590
1487-1509
Oligo 2

Figure 27. Southern analysis of *A. fumigatus* wildtype genomic DNA with the yeast 14DM probe:

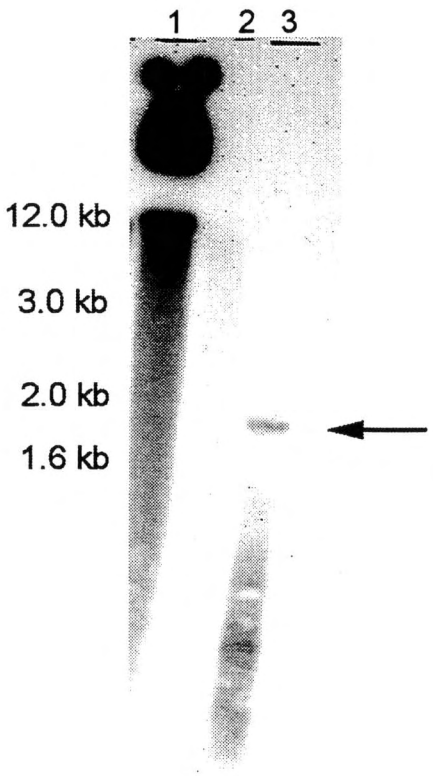
(a) Ethidium bromide stained gel containing both partial and complete MboI digests of *A. fumigatus* genomic DNA (lanes 2 and 3 respectively). Lane 1 contains a positive control (the plasmid pVK1 containing the entire yeast 14DM gene sequence) M_1 = 1 kb marker.

(b) A Southern analysis of *A. fumigatus* genomic DNA with the 14DM probe. The probe detected a 1.8 to 2 kb (lane 3) MboI genomic fragment from *A. fumigatus* (ATCC 13073) but no signal was detected in lane 2.

a



b



c) Probing a genomic clone from the phage DNA library with the yeast probe

An EcoRI digest of λYA4 DNA was hybridized to the 14DM probe. A 7 kb EcoRI genomic fragment was detected (Figure 28). This *A. fumigatus* 7 kb EcoRI fragment was then subcloned into the vector pBluescript® to produce pYA01.

d) Restriction and Southern analysis of the *A. fumigatus* 7 kb EcoRI genomic fragment (pYA01)

In order to narrow down the size of the genomic fragment of interest, the 7 kb EcoRI genomic fragment was subjected to Southern analysis after further digestion with restriction enzymes. A blot containing the 7 kb EcoRI DNA fragment digested with Sau3A, PstI, SalI, and XhoI/HindIII was hybridized to the 0.5 kb yeast probe. As shown in Figure 29, the probe detected two Sau3A genomic fragments of 0.3 and 0.4 kb (lane 1), one 1.7 kb PstI fragment (lane 2), one 0.7 kb PstI/SalI (lane 3), and a 5-6 kb XhoI/HindIII genomic fragment (lane 4), and uncut 7 kb EcoRI genomic fragment is shown in lane 5. Figure 30 shows a restriction map of the 7 kb EcoRI genomic fragment in pYA01. Based on the results obtained from the Southern analysis of the 7 kb EcoRI genomic fragment, it was apparent that the region detected by the yeast probe is contained within the 1.7 kb PstI genomic fragment (Figure 30).

e) Subcloning of the *A. fumigatus* 1.7 kb PstI/EcoRI genomic fragment derived from pYA01

The *A. fumigatus* 1.7 kb PstI/EcoRI genomic fragment detected by the yeast probe (Figure 30) was subcloned into the vector pBluescript® to produce pYA04 (Figure 31a). The pYA04 was digested with EcoRI/SalI and a 0.7 kb EcoRI/SalI genomic fragment was isolated and subcloned into pBluescript® to produce pYA05 (Figure 31b). This 0.7 kb EcoRI/SalI genomic fragment is the same fragment that was detected by the yeast probe in lane 3 of figure 29b.

f) Partial sequence analysis of the *A. fumigatus* 1.7 kb PstI/EcoRI fragment

Both ends of the 1.7 kb PstI/EcoRI genomic fragment (in pYA04) were sequenced using the reverse and forward primers (Figure 31a), and the genomic sequences obtained from both ends (Figure 32a and b) were used to search the databanks. In addition, the forward primer was used to sequence one end of pYA05, which corresponds to the middle of pYA04. These genomic sequences from the ends and the middle of the *A. fumigatus* 1.7 kb PstI/EcoRI genomic fragment did not match sequences of known lanosterol 14α-demethylase gene sequences (or any other known proteins of the data banks) (Figure 32a, b, and c). Hence, it is unlikely that the 1.7 kb PstI/EcoRI genomic fragment cloned in this study contained any sequence corresponding to the *A. fumigatus* lanosterol 14α demethylase gene.

g) Screening an *A. fumigatus* λ ZAP cDNA library with the 0.7 kb EcoRI/SalI *A. fumigatus* genomic fragment

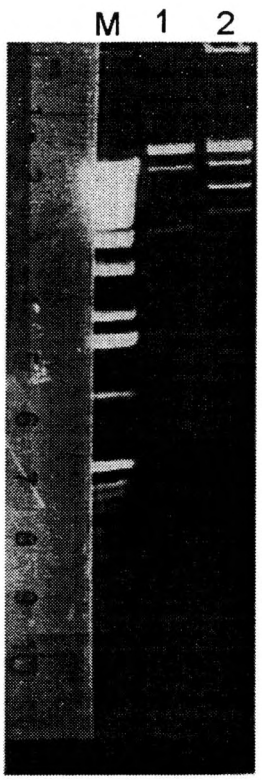
The 0.7 kb EcoRI/SalI genomic insert in pYA05 was used as a probe to screen an *A. fumigatus* (CHUV 192-88) λZAP cDNA library (gift from Dr. M. Monod) to determine if a cDNA (s) could be isolated. Twenty thousand phage were screened under high stringency conditions, resulting in the recovery of one cDNA clone (pYA06) which contained a 1.5 kb EcoRI insert. Various restriction digests of this 1.5 kb EcoRI cDNA fragment identified a single EcoRV site (Figure 34). Sequencing from both ends produced sequences (of Figures 33a and c) which were used to search the protein database using the BLAST Network Service. The sequences from the 1.5 kb cDNA clone showed extensive homology to the nitrite reductase gene (*niiA*) from *A. nidulans* (Johnstone *et al.*, 1990; Figures 34b and d). The deduced amino acid sequence of the *A. fumigatus* cDNA sequence matches amino acids of the $COOH^-$ half of the NIIA protein sequence of *A. nidulans* (Johnstone *et al.*, 1990). The extensive sequence homology observed indicated that the 1.5 kb cDNA clone recovered contains sequences corresponding to the *A. fumigatus* nitrite reductase gene (*niiA*). Figure 34 shows the position of the putative translated sequence obtained from the *A. fumigatus* cDNA clone with respect to the amino acid sequence of the *A. nidulans* nitrite reductase (NIIA) protein sequence.

Figure 28. Southern analysis of *A. fumigatus* genomic clone(λYA4) using the yeast probe:

(a) Photograph of an agarose gel showing an EcoRI digest of *A. fumigatus* (ATCC 13073) genomic clone (λYA4) containing a 15-20 kb EcoRI genomic insert (lane 2). Lane 1 contains a negative control and M = 1 kb marker.

(b) Southern analysis of an EcoRI digest of λYA4. The yeast 14DM probe detected a 7 kb EcoRI genomic fragment (lane 3) as indicated by the arrow.

a



b

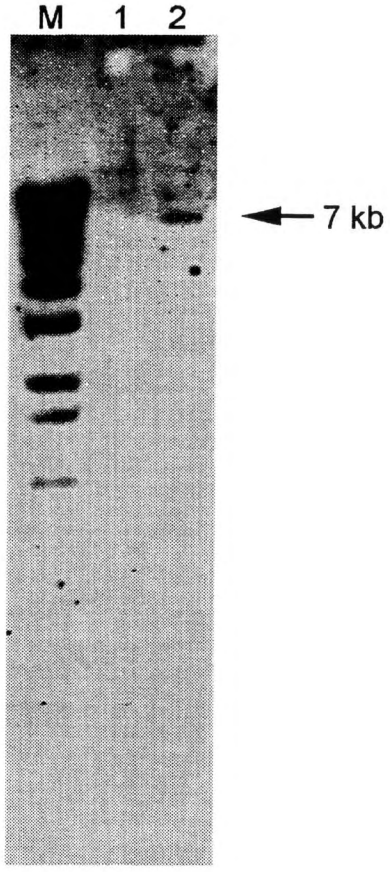
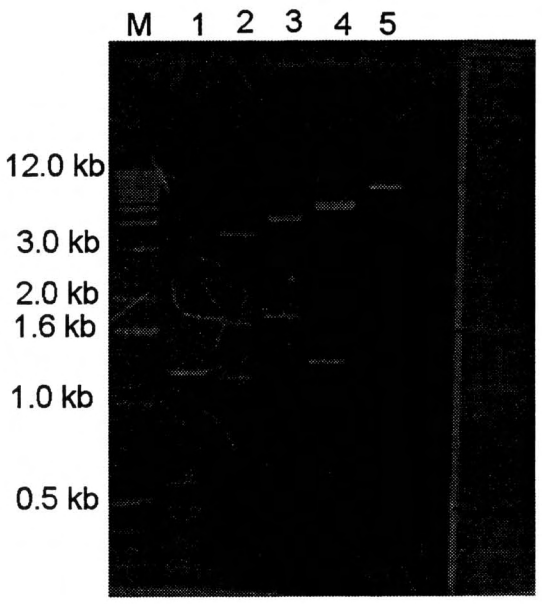


Figure 29. Southern analysis of *A. fumigatus* 7 kb EcoRI genomic fragment using the yeast probe:

(a) Ethidium bromide stained gel of the 7 kb EcoRI *A. fumigatus* genomic fragment (derived from λYA4) digested with a number of restriction enzymes. Lane 1 = Sau3A1 digest, lane 2 = PstI digest, lane 3 = PstI/SalI digest, lane 4 = XhoI/HindIII digest, and lane 5 = uncut 8 kb EcoRI fragment, M = 1kb marker.

(b) Southern analysis of the *A. fumigatus* 7 kb EcoRI genomic fragment (in pYA01). The yeast 14 DM probe detected the following signals: 0.6 and 0.7 kb Sau3A1 fragments (lane 1), a 1.7 kb PstI/EcoRI fragment (lane 2), a 0.7- 0.8 kb SalI fragment (lane 3), a 6-7 kb XhoI/HindIII fragment (lane 4), and uncut 7 kb EcoRI genomic fragment (lane 5).

a



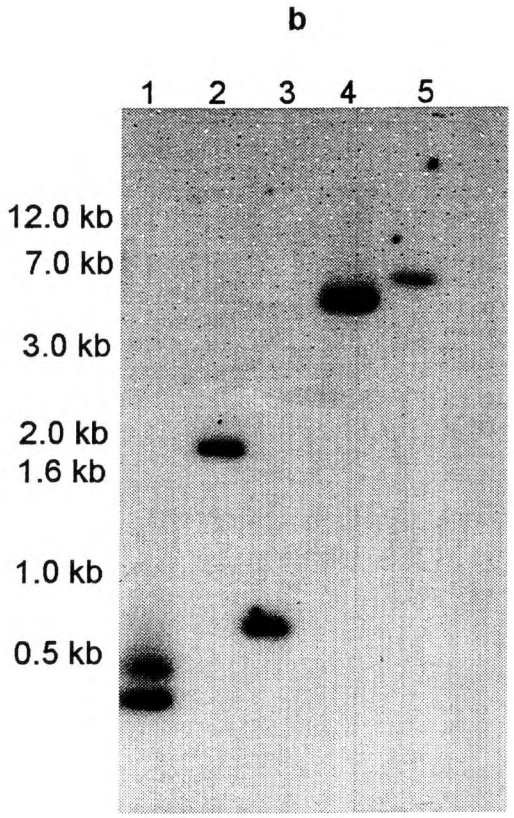

b
1
2
3
4
5
12.0 kb
7.0 kb
3.0 kb
2.0 kb
1.6 kb
1.0 kb
0.5 kb

Figure 30. A restriction map of the *A. fumigatus* 7 kb EcoRI genomic fragment in pYA01. The black bar shown below the map represents the 1.7 kb PstI/EcoRI fragment detected by the 14DM probe in figure 29b. E = EcoRI, P = PstI, and S = SalI. Numbers indicate fragment sizes in kb.

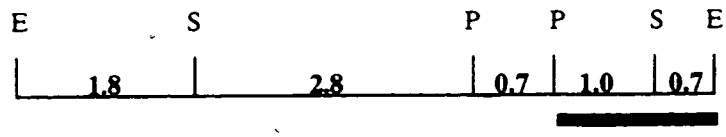

E
S
P
P
S
E
1.8
2.8
0.7
1.0
0.7

Figure 31. (a) Plasmid pYA04 produced by subcloning the 1.7 kb PstI/EcoRI genomic fragment derived from the 7 kb EcoRI fragment in pYA01. Arrows indicate the ends sequenced using the forward (F) and reverse (R) primers. (b) Plasmid pYA05 produced by subcloning of a 0.7 kb SalI/EcoRI genomic fragment derived from pYA04. F = forward and R = reverse.

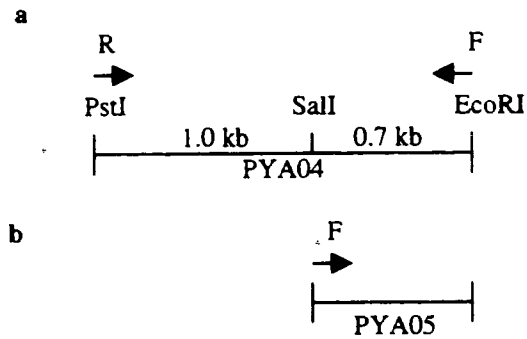

a
R
F
PstI
SalI
EcoRI
1.0 kb
0.7 kb
PYA04
b
F
PYA05

Figure 32. Nucleotide sequences obtained from the 1.7 kb PstI/EcoRI genomic fragment of *A. fumigatus*:

(a) Sequence of 399 bp obtained from pYA04 using the reverse primer.

(b) Nucleotide sequence of 420 bp obtained from pYA04 using the forward primer. (c) Nucleotide sequence of 479 bp obtained from pYA05 using the reverse primer. Nucleotide sequences a-c were used to search the protein database using Blastx (BLAST Network Service) but no significant matches were found.

a

```
TGCAGGTGCTTCAGAATAACGAACGCTTTTTACTATACAAGTCTTCGTTAATCAAACTGA 60
GCTTCATCAGATGACCGAGGGGCTTCAGAAGTATTATTGAGACTACCAACCTTCATAGGC 120
GCGAAACCACCGTACTGGTGTGCTGGGGGCATGCCCATANAGGGATACATCCGAAGCGGG 180
GGANGGGCANGGCGGTAAGGGCTGCCANCGGGGCCGGAGTTGTTCTGAGATCGACCCCAG 240
CTGAGACGCACACGGGAATTTNCCGATGGGGTATCCCCTGCATCTGGTTTGATGGCCATC 300
TCCCCCCGCGTTGGCGCCTGAACGAACTTGAAACGAAAACCGCCACCCTTTCCCAGAAGG 360
ATTCTTTGAACTTTAGTTTATCTCCCCCNAATCCCCTTG                       399
```

b

```
AATTCCCGGGTGAACGAGCCGTTTTCGGGATAAAAAGATTTCGTGCTTTAAACTTTAGTT  60
TCGAGGTATGTAATGCCGACGCGACTTTTCTGCCACTTCTGCGCCATCGCACTGATAGAA 120
ATCATTTTCGTGTTTTTCGCTAATACTAATTACTATGTTCCCAGATAGCATTAACGCGAT 180
TCCCATCTTTACGACTGTCGGAGTTTAGTCAACATGTCTTTCCAAAACTTTGACTCTTTC 240
CAAAACCAGCACCCGTCAGCCGACGCTGCTGCTGCTGCTGCCCCAGGCGCTCCGGCAACT 300
GCGGATACCACCATGACCGGGCAGACTGACCCTACCACTGGCTCTTTCCAAGGTCCTGCC 360
CCCCGGCGAGCCCTCAACTGCTCCCTGTTTGCTCCAGCAAGGCAATGAAAGGAAAAAACA 420
```

c

```
CGAAGCAATATCCGGCATTGCTCCTACTACCAACATTAGGATCGCCTTCAAAAGAGCAAG 60
AAGATTCCCATAAACTCACCCAGAAAACTTGTCGCGGATCATCTTGACATTAACCTGTTC 120
GCCCATCTGGAACCACAGATTGCGGATGAAGTTCTCATCGATCCAAGGCTCAAGCTCGCC 180
CATCCTGAATGATGGTCAGCAAAAAAAGAACGCGTATTTCCGAATATGTACATGAGCGCG 240
ATGATCGAGGCCAAGCGTACCAGAGAGTGGTCTTTCCCTCATTGCCCTGCTGAGCAACAG 300
GAGCAGCTGAAGGCTCGCCGGGGGCAAGACCCTTGGAAAGAACCCATGGGTAAGGTCAGT 360
TCTGCCCCGTCATGGTGGTATCCGCCATTTGCCCGAACGCCCTGGGGGCAACAACCACCA 420
CAACGTTTCGGTTGAAAGGTTTCTTGTTTTTT                              479
```

Figure 33. cDNA sequence analysis obtained from the *A. fumigatus* 1.5 kb EcoRI cDNA clone:

(a) cDNA sequence I (252 bp) obtained from the *A. fumigatus* (*Af*) 1.5 kb EcoRI cDNA clone using the forward primer, was used to search the protein database using the Blastx.

(b) Sequence I shares very extensive homology (80% identity and 90% similarity over 75 amino acids) with the *A. nidulans* (*An*) nitrite reductase NIIA protein sequence.

(c) cDNA sequence II (373 bp) obtained from the *A. fumigatus* 1.5 kb EcoRI cDNA clone using the reverse primer was also used to search the protein database.

(d) Sequence II shares very extensive homology (82% identity and 90% similarity over 52 amino acids) with the *A. nidulans* nitrite reductase NIIA protein sequence.

a

```
AAGAGGCTGAGAACATTTGTCGATGTCATGCAGGCCGTGGGAAGGAAGCCGGACTCTCTT 60
GGCTGCGAGATTTGCAAACCCTCCATTGCATCCATTCTTTCGAGTCTGTTTAACCAGCAC 120
ATCATGGACAAGGAGCTTCATGACCTTCAAGAGACAAATGACCGATTCCTTGCCAACATC 180
CAGAGAAACGGCACATTCTCTGTGGTTCCGCGAGTCCCTGGTGGGTGAGATC         232
```

b

```
Af     8   KRLRTFVDVMQAVGRKPDSLGCEICKPSIASILSSLFNQHIMDKELHDLQETNDRFLAXI
187
           ++LRTF DVM++ G+ PDSLGCEICKP+IASILSSLFN H+MDKE H+LQETNDRFLA I
An     579 RQLRTFDDVMKSAGKCPDSLGCEICKPAIASILSSLFNPHLMDKEYHELQETNDRFLANI
638

Af     188 QRNGTFSVVPRVPGG 232
           QRNGTFSVVPRVPGG
An     639 QRNGTFSVVPRVPGG 653
```

c

```
AACGGATCTGGTGCTTCCCCTTTCCTCACCTTCCATTTCTCCGTTCCCAAAACGGAATCT  60
AGTTCCTCAACGGGAGGCAGCTTCATATACACCCAGCCGTCGTCGCGTTCCTCAACCGGG 120
AAGGTCGCAATGTTCATGCTCTCGTCATTCGTGCAACGCCCTGCCTGCTCACCGTTGAGC 180
TCGAAGTTGTCGAAGTTGCGTTTGTGGTAAGGGCAGGAGACCCAATATTTACCCGCATCG 240
TCATCGCCAATCAAGCCATCAGATAGCACGAAGGCGCGTTTGTGCGGGCACATCTGTTGT 300
GTGGCGTAGTACTTGCCCTTGACCTTGAAGATGGCCAATTGAGTATCCCCGCGCTTGATG 360
TTGGCCGAGGAGA                                                 373
```

d

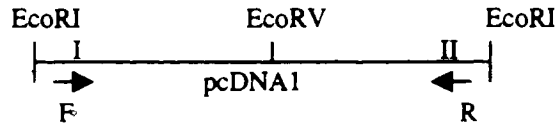
```
Af     157 CTNDESMNIATFPVEERDDGWVYMKLPPVEELDSVLGTEKWKVRKGEAPDPF 2
           C NDE+MNIATFPVEER+DGW+YMKLPPVEELDSVLGTEKWKV+KGEA DPF
An    1018 CQNDEAMNIATFPVEEREDGWIYMKLPPVEELDSVLGTEKWKVKKGEAVDPF 1069

Af     312 RKGKYATQQMCPHKRAFVLSDGLIGDDDAGKYWVSCPYHKRNFELNGEQAG 160
           .+KGKYATQQMCPHKR FVLSDGLIGDDD GKYWVSCPYHKRNFELNGEQAG
An     966 KGKYYATQQMCPHKRTFVLSDGLIGDDDNGKYWVSCPYHKRNFELNGEQAG 1016

Af     373 HPQISANIKRGDTQLAIFKVKGK 305
               SANIKRGDTQLAIFKVKGK
An     946 PAISSANIKRGDTQLAIFKVKGK 968
```

Figure 34. Partial restriction analysis of the *A. fumigatus* 1.5 kb EcoRI *niiA* cDNA clone:

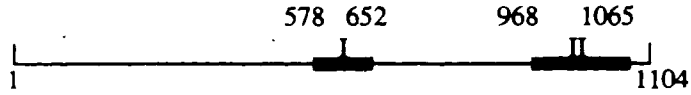
(a) Restriction map of *A. fumigatus niiA* cDNA clone; the ends sequenced are also shown.

(b) Position of the *A. fumigatus* deduced amino acid sequences (I and II) with respect to the *A. nidulans* nitrite reductase (NIIA) protein sequence of 1104 amino acids. Numbers correspond to amino acid positions of the *A. nidulans* NIIA sequence.

a



b



A.nidulans NIR sequence

DISCUSSION

Detection of a signal on a Southern blot

Southern analysis of *A. fumigatus* genomic DNA using the yeast 14DM gene fragment led to the detection of a 1.8 kb MboI genomic fragment which was expected to correspond to the *A. fumigatus* lanosterol 14α -demethylase gene. According to this result the *A. fumigatus* lanosterol 14α-demethylase gene is probably present in a single copy in the genome as is the case found in *S. cerevisiae* (Kalb *et al.*, 1987), *Candida tropicalis* (Chen *et al.*, 1987), and *Candida albicans* (Kirsch *et al.*, 1988). Therefore, the hybridization (at 42° C in a solution containing 5xSSC, 5% Denhardt's, and 0.5% SDS) and washing (at 42° C with salt concentrations of 3xSSC, 2xSSC, 1xSSC, and 0.5x SSC in a solution containing 0.1% SDS) conditions developed in this study were stringent enough to give a very clear signal on a Southern blot. These hybridization conditions can now be used as a starting point in future studies involving analysis of genomic DNA of filamentous fungi using yeast *S. cerevisiae* DNA as a probe or vice versa.

λYA04 does not contain any sequences corresponding to the *A. fumigatus* 14DM gene

The genomic sequences obtained from the ends of the 1.7 kb PstI/EcoRI of the genomic fragment in pYA04, and from one end of the 0.7 kb EcoRI/SalI in pYA05 (derived from pYA04) did not match any of the known 14DM gene sequences in the databanks and did not show any significant homologies with any other known protein sequences. The results therefore demonstrate that the 1.7 kb PstI/EcoRI genomic

fragment did not contain any sequences corresponding to the *A. fumigatus* 14DM gene.

To understand why this genomic clone was selected, the sequence results were further analyzed by comparing the genomic sequence obtained from the 0.7 kb PstI/SalI to the yeast probe sequence, and a region of 13 bp identity was observed over a stretch of 15 bp; the sequence stretch within the probe sequence was TTGATGGAGCAAAAG and the sequence stretch within the *A. fumigatus* genomic sequence was TTGCTGGAGCAAACAG. The question is, could this 15 bp sequence have been sufficient to account for the hybridization of the yeast 14DM probe to the 0.7 kb EcoRI/SalI in pYA05. The use of end labeled oligonucleotides as probes is not an uncommon practice, and hence it is possible that a ^{32}P-labeled fragment containing the 15 bp sequence (TTGATGGAGCAAAAG) could have been produced during the random-oligolabeling of the 0.5 kb template corresponding to the yeast 14DM gene. The ^{32}P-labeled 15 bp in turn could have formed a stable base-paired complex with a target genome. In addition, the low stringency conditions used to screen the genomic library with the yeast 14DM probe probably contributed to the stability of binding of the probe to genomic DNA.

Alternatively, the hybridization of the yeast 14DM probe to the *A. fumigatus* genomic clone could have been due to non-specific hybridization. Screening a genomic library can be troublesome especially if a heterologous probe is being used and the screening is carried out under non-stringent hybridization and washing conditions. One reason that non-specific binding can occur is that genomic filters often contain large amounts of phage DNA as well as some debris contributed by phage coat components and top agar. Therefore, the probe can non-specifically bind to phage DNA and remain

after washing the filter. This would eventually lead to a false signal showing on the autoradiograph which in turn, leads to the selection of a false positive clone.

Recently, the $P450_{14DM}$ gene was cloned and characterized from the filamentous fungal plant pathogen *Penicillum italicum* using the yeast *Candida tropicalis* 14DM gene as a probe (Van Nistelrooy *et al.*, 1996). It should be noted that using the *S. cerevisiae* 14DM gene as a heterologous probe did not detect any signals under low stringency conditions on a Southern blot containing *P. italicum* genomic DNA (Van Nistelrooy *et al.*, 1996).

The *P. italicum* $P450_{14DM}$ (*CYP51*) gene is the first 14DM gene to be cloned from filamentous fungi. Filamentous fungi $P450_{14DM}$ uses eburicol as a substrate instead of lanosterol which is the substrate for the yeast 14DM genes (Van den Bossche *et al.*,1988, Koller, 1992). Alignment of the *P. italicum CYP51* amino acid sequence with the 14DM amino acid sequences from *S. cerevisiae*, *C. tropicals*, and *C. albicans* showed 45.8%, 47%, and 47.2 % amino acid sequence identity (Van Nistelrooy *et al.*, 1996). Since the *P. italicum* P45014DM gene sequence is avaliable, it would be worthwhile to use it as a probe for Southern analysis and genomic library screening of *A. fumigatus* DNA. The *C. tropicals* may also be used as a heterologous probe for analysis of genomic DNA from *A. fumigatus* to determine if similar results can be obtained as found in the studies involving *P. italicum* DNA (Van Nistelrooy *et al.*, 1996).

Recovery of 1.5 kb EcoRI cDNA fragment corresponding to the *A. fumigatus* nitrite reductase gene

The *A. fumigatus* 0.7 kb EcoRI/SalI genomic fragment in pYA05 was used as a probe to screen an *A. fumigatus* λZAP cDNA library under high stringency hybridization and washing conditions. The screen resulted in the recovery of a 1.5 kb EcoRI cDNA fragment. The ends of the 1.5 kb cDNA clone were sequenced, and the sequences obtained were found to show extensive homology to the nitrite reductase (*niiA)* gene sequence from *A. nidulans* (Johnstone *et al.*, 1990). Thus, this cDNA clone corresponded to the *A. fumigatus* nitrite reductase gene (*niiA*). The cDNA clone obtained in this study did not contain the entire 3' translated end of the *niiA* gene.

It should be noted that there was a problem with the cDNA library; and the plaques used in the first round of screening did not look very healthy. The simplest explanation is that the hybridization of the *0. 7* kb *A. fumigatus* probe to the phage clone was non-specific and that the cDNA clone selected was a false positive clone. Nonetheless, the recovery of the cDNA clone corresponding to the *A. fumigatus* nitrite reductase presented an excellent opportunity to investigate the *A. fumigatus* nitrate assimilation gene cluster at the molecular level.

Section II

Summary of some methods used in this study

Protoplast preparations for pulsed field gel analysis and transformation

The method of Debets and Bos (1986) was used to isolate protoplasts from *A. fumigatus*. *A. fumigatus* (ATTC13073) conidia were collected and 5 x10^8 conidia were inoculated in 50 ml medium in a silanized 125 ml Erlenmeyer flask. The cultures were maintained overnight on a shaker at 37° C and agitated at 150 rpm. The next day the mycelia were collected on Miracloth® filters on a sterile Buchner funnel and were washed with 10 ml of NCC buffer (0.8 M NaCl and 50 mM $CaCl_2$). Two grams of slightly wet mycelia were transferred to a sterile 125 ml flask and 20 ml of Novo-buffer (5 mg/ml of Novozyme in NCC buffer[0.8 M NaCl and 50 mM $CaCl_2$) were added. The flask containing the mycelia and Novo-buffer was incubated at 30° C with gentle shaking at 100 rpm for approximately 2.5 hrs. To release the protoplasts, clumps of mycelia were broken by vigorous pipetting using a 10 ml sterile pipette after 1 and 2 hrs of incubation. Protoplast development was monitored microscopically. When the production of protoplasts was complete as evident by collapsed and broken hyphal fragments the suspension was filtered over a funnel containing a very thin layer of sterile glass wool. Subsequently, the protoplasts were pelleted at 2000 rpm for 10 minutes and resuspended in 5 ml of STC buffer (1.2 M Sorbitol, 10 mM Tris, 50 mM $CaCl_2$).). After two washes with this buffer, the protoplasts were spun down and were resuspended in 1 ml of STC buffer .

Protoplasting and Transformation procedures for gene disruption

Mycelial protoplasts of *A. fumigatus* wild type were obtained using the protocol of Debets and Bos (1986; see Appendix II). For genetic transformation the method of Osmani *et al.* (1989) was used. Protoplasts (200 µl) were mixed with 5 µg of plasmid DNA and 50 µl of 30% PEG (8000) in a microcentrifuge tube and were kept on ice for 20 minutes. Then 1 ml of 30% PEG solution was added to the microcentrifuge containing the protoplasts. The suspension was mixed by tipping and tapping, and was held at room temperature for approximately 20 minutes. Specified amounts (50, 100, and 500 µl) of the protoplast transformation mixture were added to 4-5 ml of selective overlays and were poured onto selective plates contained 250 µg /ml hygromycin B (in 1 M sucrose: minimal medium with $NH4^+$ as nitrogen source). The plates were incubated at 30° C for 7-14 days to permit hyg-resistant transformants to form conidiating colonies. As a negative control untransformed protoplasts were plated onto the same selective media. Serial dilutions of untransformed protoplasts were also plated onto the same medium without hygromycin to estimate numbers of viable protoplasts and transformation frequency

Fungal DNA extraction

DNA extraction was done using the method of May *et al.* (1985; updated 1992). Two loops of *A. fumigatus* conidia (from complete or supplemented minimal media plates) were inoculated in 100 ml of liquid minimal media (Cove, 1967) containing either nitrate (test MM) or ammonium (control) as the sole N-source. Cells were grown for 3 days with

shaking (200 rpm) at 28° C. Cells were grown overnight in 50 ml YAG inoculated with 10^6 conidia/ml and incubated at 37° C overnight with agitation at 200 rpm. The next day the mycelia were filtered on sterile Miracloth® using Buchner funnel, and 0.2 g of wet mycelia were frozen using liquid nitrogen and were ground to a fine powder using a mortar and pestle. One ml of a solution containing 10 mM Tris-HCl, 100 mM EDTA, 2% Sarkosyl, pH 8.0 was added to the ground mycelia in a microcentrifuge tubes. The tubes were mixed well and were incubated at 65° C for 30 minutes. Then the solution was split into 2 portions (2 fresh tubes) and was extracted with one volume of phenol (TE saturated phenol, pH 8.0). The aqueous layer was collected into fresh microcentrifuge tubes, and 5 µl of a solution containing RNAase A (10 mg/ml) and 50 mM Sodium acetate, pH 4.5 were added and the tubes were incubated at 37° C for 30 minutes. Then the aqueous layer was further extracted once with phenol, twice with phenol: chloroform (1:1), and twice with chloroform: isoamyl alcohol (1: 24). The aqueous layer was transferred to fresh tubes, and 0.5 ml of a solution containing 20 % PEG 8000 and 2.5 M NaCl was added and the tubes were incubated on ice for 2-3 hrs. The tubes were centrifuged at 14000 rpm for 10 minutes, and the pellet obtained was resuspended in 200 µl TE and 20 µl of 3 M Sodium acetate were added. Contents of tubes were combined and two volumes of ice cold 95% ethanol were added, and the tubes were centrifuged for 5 minutes at 14000 rpm.

The pellet recovered was washed once with 70% ethanol and the pellet was air dried. The dried pellet was resuspended in 100 µl sterile TE or H_2O. The DNA was stored at -20 ° C until required. The amount of DNA obtained was about 100 µg/ 0.2 g wet mycelia.

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