INACTIVATION OF *snfA1* AFFECTS CARBON CATABOLITE DEREPRESSION IN THE FILAMENTOUS FUNGUS *ASPERGILLUS NIDULANS*

By

LAVANYA AMIRNENI

Bachelor of Medicine; Bachelor of Surgery

Guntur Medical College

Guntur, India

2000

Submitted to Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July 2004

INACTIVATION OF *snfA1* AFFECTS CARBON CATABOLITE DEREPRESSION IN THE FILAMENTOUS FUNGUS *ASPERGILLUS NIDULANS*

Thesis approved:

Dr. Rolf A. Prade

Thesis Adviser

Dr. Jeffrey Hadwiger

Dr. Ulrich Melcher

Dr. Al Carlozzi

Dean of the Graduate College

DEDICATION

I dedicate this thesis to the loving memory of my beloved sister A. Hima Bindu. I love you and will never forget you.

PREFACE

Glucose, an easily metabolizable sugar, is the preferred energy source for most living organisms. In the absence of glucose, microbes rearrange their regulatory network in a way that enables them to utilize and feed on alternate energy sources. This mechanism is used by several fungi to invade and infect plants. The plant cell wall polysaccharides act as one of the plant defenses against fungal invasion. Certain fungi produce enzymes, which break these polysaccharides down and successfully infect the plants. An efficient method to protect the plants from fungal invasion would be to prevent these fungi from degrading the plant cell walls.

The aim of the research in Chapter 1 is to understand the genetic mechanisms involved in regulation of carbon source availability for the growth of the filamentous fungus *Aspergillus nidulans*. The specific objectives of this research are (i) creating a deletion strain of *snfA1*, a gene involved in alternate carbon source utilization, (ii) phenotypic characterization of *snfA1* deletion mutant, and (iii) determining the genes regulated by *snfA1* in order to understand its function.

The research in Chapter 2 is a group project that describes the gene ontology for filamentous fungi. This chapter reviews the gene ontologies designed to describe most known molecular functions, cellular components and biological processes. It has been published in a book series, *The Mycota VIII*.

ACKNOWLEDGEMENTS

I would like to sincerely thank my adviser Dr. Rolf A. Prade, for his unconditional support and valuable guidance in the successful completion of this work. His expertise in the field helped me to overcome several hurdles at all times. I also like to express my deepest appreciation for his constant encouragement and trust on my professional abilities during my graduate studies.

I would also like to thank my other committee members, Dr. Jeff Hadwiger and Dr.Ulrich Melcher for their time and consideration. I especially thank Dr. Patricia Ayoubi for sharing her expertise and experience in completing the microarray work. I extend my gratitude to Dr. Rosanna Penna Muralla who has done the initial cloning part of this work. A special thanks to the Department of Microbiology and Molecular Genetics for providing me financial support throughout my study. I would also like to express my appreciation to all my friends and colleagues in the department and Prade Lab, past and present, for being helpful and making my days at OSU memorable.

Last, but not the least, I would like to thank all my family: my loving husband Vamsee for his constant support and encouragement, and above all for his thorough faith on my capabilities that encouraged me to pursue this degree; my parents, in-laws and all other family members for their constant understanding, encouragement and financial support that let me to complete my graduate studies smoothly.

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

Chapter 1

Chapter 2

LIST OF FIGURES

Figure **Page**

Chapter 1

Chapter 2

NOMENCLATURE

μg	micrograms
μl	microliters
aa-dUTP	amino-allyl deoxyuracil triphosphate
ATP	Adenosine triphosphate
CCR	Carbon catabolite repression
cDNA	complementary DNA
creA / CreA	Carbon repressor protein A (gene/protein in *A.nidulans*)
CTD	C-terminal domain
CTP	Cytidine triphosphate
Cy	Cyanine
DAHP	3-Deoxy-D-arabino-heptulosonate-7-phosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
GO	Gene Ontology
GTP	Guanosine triphosphate
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
RNA	ribonucleic acid
SNF1 / Snf1	Sucrose non-fermenting 1 (gene/protein in yeast)
snfA1 / SnfA1	Sucrose non-fermenting A1 (gene/protein in *A.nidulans*)
TCA	Tri carboxylic acid
TTP	Thymidine triphosphate

CHAPTER 1

INACTIVATION OF *snfA1* AFFECTS CARBON CATABOLITE DEREPRESSION IN THE FILAMENTOUS FUNGUS *ASPERGILLUS NIDULANS*

LAVANYA AMIRNENI, ROSANNA PENNA-MURALLA, ROLF A. PRADE

INTRODUCTION

Microbes turn off the expression of a large number of genes in the presence of glucose. This is an energy-saving mechanism, as it primarily shuts off the genes involved in the metabolism of other carbon sources, which are not required in the presence of glucose. Genes involved in gluconeogenesis, respiration and peroxisomal functions are also repressed. This phenomenon, known as carbon catabolite repression, is an ubiquitously occurring regulatory principle and has been well studied in bacteria and lower eukaryotes like yeast and *Aspergillus nidulans* (Monod and Jacob, 1961; Bailey and Arst, 1975; Gancedo, 1992; Kelly, 1994; Ronne, 1995; Ruijter and Visser, 1997). In yeast, carbon catabolite repression is mediated by the zinc-finger protein Mig1p (Nehlin *et al.*, 1991; Lutfiyya and Johnston, 1996) which, in the presence of glucose, acts by recruiting the general repressor complex Tup1p/Ssn6p to the promoters of a variety of glucose-repressible genes (Tzamarias and Struhl, 1994; Treitel and Carlson, 1995). In the absence of glucose, Mig1p is phosphorylated and hence inactivated by Snf1-kinase (Johnston *et al.*, 1994; Lutfiyya *et al.*, 1998; Treitel *et al.*, 1998) thus relieving the transcriptional repression. The Snf1 kinase therefore plays a significant role in carbon catabolite derepression in yeast.

SNF1 was originally identified by mutational analysis as a gene essential for sucrose utilization where *snf1* mutants were isolated in a search for sucrose-nonfermenting mutants (Carlson *et al.*, 1981). Snf1p is a serine/threonine protein kinase (α subunit) complexed with other proteins – a γ activating subunit Snf4p and β subunits Sip1p, Sip2p or Gal83p depending on where Snf1p localizes (Celenza and Carlson, 1989; Celenza *et al.*, 1989; Yang *et al.*, 1992; Yang *et al.*, 1994; Vincent and Carlson, 1999).

In the filamentous fungus *A. nidulans*, *creA*, *creB* and *creC* have been identified to be involved in carbon catabolite repression. *creA* is the major regulatory gene

mediating this repression (Arst and MacDonald, 1975; Bailey and Arst, 1975; Hynes and Kelly, 1977). *creA* which is the homolog of yeast MIG1 gene, has been cloned and sequenced in *A. nidulans* (Dowzer and Kelly, 1989; Dowzer and Kelly, 1991) and its mutations have lead to derepression of many activities, which would normally be repressed in the presence of glucose (Arst and Cove, 1973; Bailey and Arst, 1975; Hynes and Kelly, 1977). *creA* homologs have been identified in other fungi like *A. niger* (Drysdale *et al.*, 1993), *Trichoderma reesei* (Strauss *et al.*, 1995; Ilmen *et al.*, 1996), *Trichoderma harzianum* (Ilmen *et al.*, 1996), *Metarhizium anisopliae* (Screen *et al.*, 1997), and *Cochliobolus carbonum* (Tonukari *et al.*, 2003). Studies so far have focused mainly on the *creA* regulatory element of carbon catabolite repression. But recently, some light has been shed on the elements that control the derepression mechanism, for example SNF1. Mutation of SNF1 homologue in *C. carbonum* caused significant reduction in cell wall-degrading enzyme activities and their transcripts under derepressive conditions and also caused reduced growth on certain carbon sources (Tonukari *et al.*, 2000). Similar studies in *Fusarium oxysporum* have shown diminished transcription of genes encoding cell wall-degrading enzymes (Ospina-Giraldo *et al.*, 2003). These studies indicate that SNF1 homologue is important in the derepression mechanism.

As part of our efforts to understand the mechanisms of carbon catabolite repression in *A. nidulans*, we characterized the role of *snfA1*, by creating a deletion mutant of *snfA1* and analyzing its phenotypic and functional aspects in comparison to the reference strain. Consistent with the data from previous studies, absence of *snfA1* in *A. nidulans* causes defects in glucose-derepression mechanism and reduced growth on various carbon sources. In order to see if *snfA1* interacts with *creA* (MIG1 homolog), which is thought to be responsible for the derepression, we have performed an allyl alcohol sensitivity test where the reference and Δ*snfA1* strains were grown on varying concentrations of the toxic chemical, allyl alcohol. The Δ*snfA1* strain appeared to be

hyper resistant to allyl alcohol compared to the reference strain suggesting an interaction between *snfA1* and *creA*. Further, the expression profiles of several genes involved in polysaccharide degradation were studied using northern analysis and consistent with our previous results, the northern data showed that the transcripts were differentially expressed in the two strains when grown on different carbon sources.

This study also involves DNA microarray technology to understand the effect of *snfA1* on gene expression. cDNA targets prepared from reference and *snfA1* deletions strains grown on carbon sources – glucose and pectin, were labeled with Cy3 or Cy5 dyes and hybridized to *A. nidulans* arrays containing 6,272 probes. Our results demonstrate the presence of regulatory interactions between *snfA1* and the genes involved in alternate carbon source utilization, indicating the role of *snfA1* in carbon catabolite repression. In addition, *snfA1* also appeared to be regulating the genes involved in stress, gluconeogenesis, glycolysis, fatty-acid metabolism and beta oxidation, sporulation, sterol biosynthesis, cell wall structure and cell cycle regulation. Yeast Snf1p has also been linked with other cellular responses like glycogen synthesis, lipid biosynthesis, stress response and heat shock (Sanz, 2003).

Taken together, our study points to a global response of *snfA1* in the filamentous fungus *A. nidulans*, redirecting cellular function towards glucose limitation.

MATERIALS AND METHODS

STRAINS, MEDIA AND GROWTH CONDITIONS

Aspergillus nidulans strain RMS011, *pabaA1 yA2, ΔargB::trpC ΔB, veA1 trpC801* (Stringer *et al.*, 1991) was used as a reference strain in transformation experiments, growth assays, allyl alcohol sensitivity tests and Southern and Northern blot experiments. *A. nidulans* strain A748, *biA1, niiA4, creA204*, obtained from the Fungal Genetics Stock Center (Kansas State University, Kansas), was used as a reference

(negative control) strain in growth assays and allyl alcohol sensitivity tests. The *snfA1* deletion mutant (*pabaA1 yA2, ΔargB::trpC ΔB, veA1 trpC801, snfA1::argB)* was constructed during this work. All fungal strains were grown at 37 ^{0}C in appropriately supplemented minimal media as described previously (Pontecorvo *et al.*, 1953; Kafer, 1977). Growth assays were done on minimal media with different carbon sources (0.1 % wt/vol). The diameter of the radial growth of the mutant strains was compared to that of the reference strain. *Escherichia coli* strain DH5α was used as a host for the propagation of the deletion plasmid.

TRANSFORMATION AND GENETIC MANIPULATION

snfA1 deletion strain was constructed by transformation of the reference strain RMS011 with the *snfA1* deletion plasmid pSnfD. Protoplasts were prepared from mycelium as described by Jung *et al.*, 2000. Transformation was done according to Yelton *et al.*, 1984 with minor modifications. Transformants were selected for growth on minimal media without arginine. A mycelial pooling approach was used to screen the transformants. The transformants were grown in 24 well plates and all mycelia from one plate were pooled into a group. The groups were screened for *argB*$^{+}$ transformants by using PCR with primers Arg (F) {forward primer} and Snf (R2) {reverse primer} that would amplify a 2.1 Kb fragment in a deletion strain (Table II) and (Fig. 1). Two groups, LHA and LHD showed the expected 2.1 Kb PCR fragment. Each transformant in the two groups was further screened using two sets of primers: (i) Arg (F) and Snf (R2) primers to screen for plasmid integration in the *snfA1* locus (ii) Snf 1299 for 5' {forward primer} and Snf (WT) Rev {reverse primer} primers (Table II) to screen for the deletion of *snfA1* gene from the genome. The second set of primers would amplify a 1.25 Kb fragment in a reference strain (Fig. 1). Possible *snfA1* deletion strains were further analyzed by genomic Southern blots to confirm the integration of the plasmid and deletion of the *snfA1* locus in the genome.

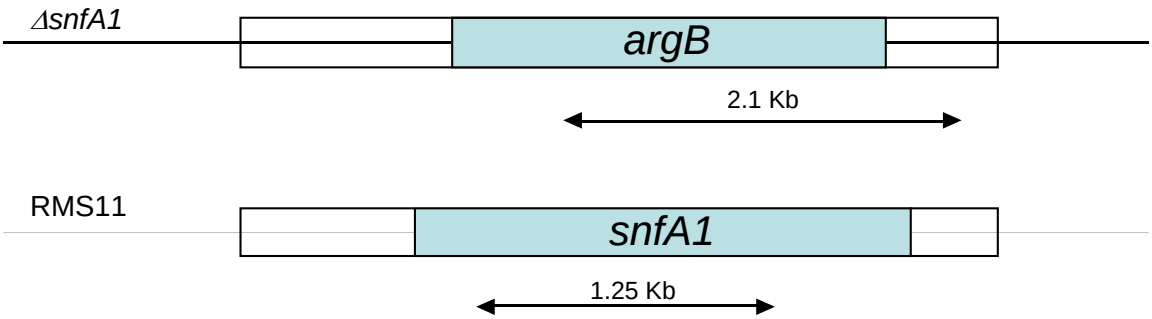

Figure 1. Mutant screening by PCR

PCR was used to screen for the possible *snfA1* deletion strain during the mycelial pooling approach. PCR was done using two sets of primers, the first set amplifying a 2.1 Kb fragment corresponding to a part of the *argB* gene and a part of the *snfA1* flanking sequence in a deletion strain and the second set amplifying a 1.25 Kb fragment corresponding to the middle region of *snfA1* ORF in a reference strain.

NUCLEIC ACID MANIPULATIONS

DNA and RNA manipulations followed standard procedures (Sambrook *et al.*, 1987). Plasmid DNA was extracted by the standard alkaline lysis method and purified by the PEG purification method. Genomic DNA, for PCR and Southern analysis, was isolated from overnight grown *Aspergillus nidulans* mycelium and the DNA extraction procedure was as described by others (Sambrook *et al.*, 1987; Aramayo and Timberlake, 1993). For Northern analysis, the reference and mutant strains were grown initially for 18 hours in minimal medium with 1 % glucose (wt/vol) as the sole carbon source and then washed and grown for an additional 6 hours in 0.25 % to 0.5 % (wt/vol) of various carbon sources. The experiment also included a starvation condition where the strains, initially grown on 1 % glucose medium for 18 hours were grown on minimal medium with no carbon source for another 6 hours. RNA extraction was done using Trizol reagent (Invitrogen), according to the manufacturer's recommendations.

CLONING OF *snfA1*

PCR primers (Table 1) were designed based on PipeOnline2 annotated *A. nidulans* EST databases (Ayoubi *et al.*, 2002). A 1.2 kb PCR product was amplified from *A. nidulans* genomic DNA, radioactively labelled with ^{32}P and used to probe a non-redundant minimal cosmid library. Cosmid AL31G07 identified by colony hybridization was partially digested with *Sau*3A and the 1.5-2.0 kb fragments were excised. The fragments were ligated to *Bam*H1 digested pBluescript and transformed into *E. coli*. Positive subclones were identified using the EST-derived probe. The full-length genomic DNA sequence of *snf1A* was assembled from the overlapping sequences of the insert subclones. Each stretch of sequence was derived from at least 3 individual sequencing reactions.

The deletion construct pSnfD was generated by first amplifying the whole *snfA1* gene and sequences flanking it by PCR (Table I). The resulting amplicon was

Table 1 Primers used for EST probe construction and cloning of *snfA1*

Name	Sequence
Snf-For300-1500	5' GCACCAACGAGCCCAATC 3'
Snf-Rev300-1500	5' CACGATGATGAAGAATGCGTC 3'
Snf-out-For-New	5' TGCCGTACCCGTGTCAGA 3'
Snf-out-Rev-New	5' GCGGAGGTGATGTGGAGG 3'
Snf-For-New2	5' CGAGGGGCCTGTGTCATC 3'
Snf-Rev-New	5' GGCCTTGCGGTTGGTTAG 3'

cloned into pGEM T-Easy vector (Promega) yielding pSnf. Next, the *snfA1* ORF was eliminated from pSnf by inverse-PCR with outward-directed primers Snf-out-For-New {forward primer} and Snf-out-Rev-New {reverse primer} (Table I). The inverse PCR product lacks the *snfA1* ORF while retaining the *snfA1* flanking regions. A 1.8 kb *argB* fragment derived from pJYargB was incorporated into a ligation mix used to recircularize pSnf. The resulting plasmid pSnfD contains the *argB* gene and *snfA1* flanking regions.

MICROARRAY PROCEDURES

MICROARRAY EXPERIMENTAL DESIGN

Four different microarray experiments were done during this study. The four types of cross-hybridizations done during the experiments are:

1) Reference strain grown in “glucose” vs “pectin”
2) *ΔsnfA1* strain grown in “glucose” vs “pectin”
3) Reference strain grown in “glucose” vs *ΔsnfA1* strain grown in “glucose”
4) Reference strain grown in “pectin” vs *ΔsnfA1* strain grown in “pectin”

For each of the two strains (reference and *ΔsnfA1*) and each carbon source (glucose and pectin) four technical replicates were used, out of which two were dye swaps. The technical replicates and cDNA labeling for each experiment were done as follows:

1) For experiments 1 & 2:
    A. Glucose vs pectin labeled as Cy3 vs Cy5 respectively
    B. Glucose vs pectin labeled as Cy5 vs Cy3 respectively
2) For experiments 3 & 4:
    A. Reference strain vs *ΔsnfA1* strain labeled as Cy3 vs Cy5 respectively
    B. Reference strain vs *ΔsnfA1* strain labeled as Cy5 vs Cy3 respectively

The labeling in A & B was done twice for each experiment thus generating four technical replicates.

The ratios in each experiment were calculated as follows:

1) For experiments 1 & 2 it was calculated as pectin vs glucose
2) For experiments 3 & 4 it was calculated as *ΔsnfA1* vs reference

RNA EXTRACTION

A. nidulans strains RMS11 and LHA6 were initially grown in minimal medium containing 1 % glucose for 18 hours. Fungal mycelium present in 125 ml of culture was collected, washed with sterile water and transferred to 250 ml minimal medium containing 0.25 % pectin and grown for an additional 6 hours. The same procedure of initial growth on glucose medium and then transfer to pectin medium was repeated on three consecutive days. Total RNA from lyophilized mycelia was extracted using Tri reagent (Sigma) according to the manufacturer's instructions. 3µl of RNA was analyzed on a denaturing formaldehyde/agarose gel. RNA was also quantitated using a spectrophotometer. RNA samples with sharp ribosomal RNA bands on agarose gels and an spectrophotometric A_{260}/A_{280} ratio between 1.8 – 2.0 were selected and pooled from the three biological replicates mentioned above. RNA was stored at –80 ^{0}C until use.

cDNA TARGET SYNTHESIS AND LABELLING

Fluorescently labeled cDNA target was synthesized using a two-step procedure which involved cDNA production from target RNA using a reverse transcriptase reaction that incorporated aminoallyl-modified deoxynucleotide (aadUTP), followed by chemical coupling of fluorescent dye (Cy3 or Cy5) to the introduced amino group on the newly synthesized cDNA. 25µg of total RNA was mixed with 3µg of oligo-dT primers in a total volume of 25µl DEPC (diethylpyrocarbonate) treated water, incubated at 65 ^{0}C for 5 minutes, room temperature for 2 minutes and on ice for 2 minutes. Subsequently, 25µl of a

premix containing 5µl 100 mM DTT (dithiothreitol), 10µl 5X Superscript II First Strand buffer, 1µl aadNTP mix (25 mM dA, C, and GTP, 8.75 mM dTTP, and 16.25 mM aadUTP {Sigma Cat.No.A0410}), 7µl DEPC treated water and 2µl Superscript II™ reverse transcriptase (Invitrogen) was added to each tube. The mixture was incubated at room temperature for 2 minutes, 37 ^{0}C for 5 minutes and 42 ^{0}C overnight. After overnight incubation, 10.5µl 0.5M NaOH / 50 mM EDTA was added to each tube and the tube incubated at 65 ^{0}C for 10 minutes to degrade the RNA. The reaction was neutralized by adding 15µl 1M Tris HCl pH 7.5. The cDNA was purified using Qiaquick PCR purification kit (Qiagen Cat.No. 28104) according to the manufacturer's instructions. The cDNA was dried at room temperature in a Speed Vac to 8µl and mixed with 2µl 0.1M Na_2CO_3 (pH 9.0) and 5µl of Cy3 or Cy5 dyes. The dyes were prepared in advance by dissolving one tube of powdered Cy3 or Cy5 dye (Amersham Pharmacia Biotech Cat.No.PA23001 & PA25001 respectively) in 55µl dimethyl sulfoxide and storing at –20 ^{0}C until use. After 1 hour at room temperature in the dark, the coupling reaction was quenched using 4.5µl 4M hydroxylamine, followed by incubation for an additional 15 minutes. The Cy3 and Cy5 labeled cDNA samples were combined and purified using the Qiaquick PCR purification kit.

HYBRIDIZATION OF DNA MICROARRAY

Labeled probes were adjusted to 19.5µl using a Speed Vac and mixed with hybridization components consisting of 5µg oligo dT, 12.5µl 20X SSC, 0.5µl 10 % SDS and 12.5µl formamide. The mixture was then briefly centrifuged at high speed to remove air bubbles, heated at 99 ^{0}C for 2 minutes and maintained at 42 ^{0}C until use.

A. nidulans microarrays were obtained from the School of Biological Sciences, University of Manchester, Manchester, UK and were fabricated as described by others (Sims *et al.*, 2004). The array consisted of 6,272 individual spots arrayed in 32 blocks of 14 X 14 spots on Corning CMT-GAPS II slides.

DNA microarray slides were washed once for 2 minutes at room temperature in 0.1 % SDS (sodium dodecylsulfate) and twice in sterile water to remove unbound material. The slides were then boiled for 3 minutes in sterile water to denature the printed DNA, rinsed in cold ethanol for a few seconds and dried using low speed centrifugation in a microscope slide-accommodating rotor (Telechem). The slides were prehybridized by incubating in a fresh mixture of 50 ml of 25 % formamide, 5X SSC, 0.1 % SDS and 1 % BSA for 2 hours at 42 ^{0}C. Finally, the slide was rinsed in sterile water and dried using low speed centrifugation.

The pre-warmed labeled sample was pipetted onto the arrayed surface of the slide and spread uniformly by inserting a 24 X 60 mM glass cover slip (Fisher Scientific, Cat. No. 12-548-5P). The cover slip was pre-cleaned for 1 minute in 0.1 % SDS, 1 minute in distilled water and 1 minute in 95 % ethanol at room temperature and dried. 15µl 3X SSC was added to each of the two reservoirs of a Corning hybridization chamber (Corning Inc., Corning, NY) and the slide was transferred to and enclosed within the chamber. The slide was incubated in a 42 ^{0}C waterbath for 16 hours.

The hybridization was completed by post-hybridization washes. Initially, the cover slips were allowed to slide off the slide after submerging in 2X SSC and 0.2 % SDS solution. Then the slides were washed separately for 15 minutes each in 2X SSC and 0.2 % SDS, 2X SSC, 0.2X SSC solutions at room temperature in the dark. This was followed by water rinsing for a few seconds and a 95 % ethanol wash for 2 minutes. The slides were finally dried by low speed centrifugation and stored in the dark until scanned.

IMAGE EXTRACTION AND DATA ANALYSIS

The arrays were scanned using Scan Array Express Scanner from Perkin-Elmer Biosystems. Scanned images were analyzed using the Gene Pix Pro 4.0 software package (Axon Instruments, Inc., Union City, CA, USA). The raw “gpr” files obtained from Gene Pix Pro were uploaded to the GenePix Pro AutoProcessor (GPAP)

(http://darwin.biochem.okstate.edu/gpap) (Weng and Ayoubi, 2004). GPAP uses the R-project statistical environment (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org) for processing and normalization of raw microarray data. This included: (1) removal of poor quality spots; (2) filtering of data points where signal was less than the background plus two standard deviations in both channels; (3) filtering of data points where signal was less than 500 (user defined baseline value) in both channels; (4) log transformation of the background subtracted Cy3/Cy5 median ratios; (5) normalization using local Loess pin-by-pin intensity dependent normalization (Yang et al., 2002) (6) averaging of normalized log2 ratios from replicate spots. Four technical replicates were used for microarray hybridization. The ratios of values obtained for each gene were averaged across the four replicates. Only those genes having an expression ratio of at least 2-fold increase or decrease in at least one of the four experiments done were counted as being differentially regulated.

RESULTS

DELETION OF *snfA1* GENE

The deletion construct pSnfD was used to transform *A. nidulans* strain RMS011, which is wild type with respect to the *snfA1* gene. The *argB*$^{+}$ transformants were initially screened by PCR, for plasmid integration in the *snfA1* locus using primers Arg (F) {forward primer} and Snf (R2) {reverse primer} and for the deletion of *snfA1* gene from the genome using primers Snf 1299 for 5' {forward primer} and Snf (WT) Rev {reverse primer} (Refer Table II for primer sequences). Transformants that produced a 2.1 Kb product with the first set of primers and no product with the second set of primers were further tested for gene deletion using Southern blotting and hybridization. Genomic DNA of the possible deletion strains was digested with the restriction enzymes *Hin*dIII, *Bam*HI and a combination of both. Southern hybridization of the digested genomic DNA

Table II Primers used for Polymerase chain reaction in this study

Name	Sequence
Snf 1299 for 5’	5’ CTATGCCCCCGCCCGCGC 3’
Snf (WT) Rev	5’ GCTTGTGCTGAATGGAGTGCCCTG 3’
Arg (F)	5’ CTTCGGTTCCAGTCATCAATGCTT 3’
Snf (R2)	5’ GAATCCCATCCGCAGAGTATCGCC 3’
1497 for	5’ ACGGCGTATATCGACTAGC 3’
Snf 3498 Rev	5’ TGATCGGATGCCATTCGG 3’

was done using two probes – a 3.1 Kb probe (middle region of *snfA1* genomic region) obtained from reference genomic DNA using the primers 1497 for {forward primer} and Snf 3498 Rev {reverse primer} and a 1.8 Kb probe (*arg* B fragment) obtained from the plasmid pDC1. *snfA1* was deleted by the transforming DNA in 2 transformants (LHA6 and LHD62) as seen in figure 2. When probed with *snfA1* probe, parental strain RMS011 showed >4 Kb fragment when digested with *Hin*dIII or *Bam*HI or double digested with both whereas *snfA1* deletion strains showed ~1 Kb fragment with *Hin*dIII digestion, ~2.7 Kb fragment with *Bam*HI digestion and ~1 Kb fragment with double digestion. When probed with *argB* probe, parental strain showed no fragments, as expected, and the mutant strains showed ~1 Kb and >4 Kb fragments with *Hin*dIII digestion, ~3 Kb fragment with *Bam*HI digestion and ~1 Kb & ~700 bp fragments with double digestion.

Since both the deletion strains showed exactly the same result in the Southern hybridization experiments, only one strain (LHA6) was chosen for further analysis and designated *ΔsnfA1*.

ANALYSIS OF GROWTH IN DIFFERENT CARBON SOURCES

Growth of the *snfA1* mutant was less than of the reference strain RMS11 on media containing different sugars as the sole carbon source. Figure 3 shows that the Δ*snfA1* grows better on glucose and quinic acid than on media containing cellulose, xylan, pectin or galacturonic acid as the sole carbon source. A748, the carbon derepressed mutant strain also showed similar type of growth as Δ*snfA1* on various carbon sources. Growth of the mutant strains on simple sugars such as fructose, cellobiose, arabinose, lactose or sucrose and on glycerol was not much different than that of the reference strain grown on these media. These results indicate that the mutants are partially defective in utilization of complex sugars.

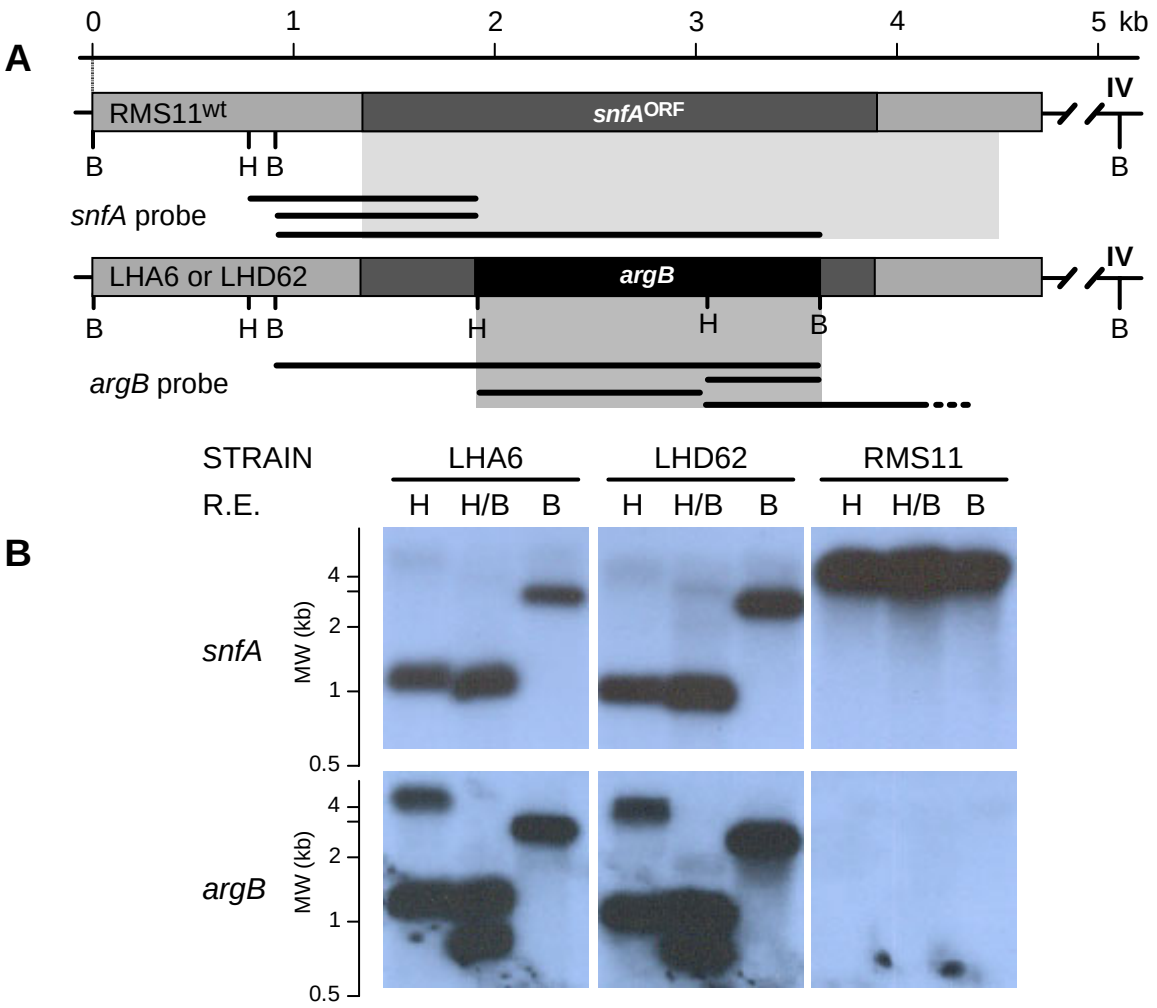

Figure 2. Southern analysis of the *snfA1* deletion strain

Deletion of *snfA1* gene from *A. nidulans* genome was confirmed using Southern blotting procedures. Southern transfers of *Hin*dIII, *Bam*HI or *Hin*dIII/*Bam*HI double digested genomic DNA of the reference strain RMS11 and two *snfA1* deletion strains LHA6 and LHD62 were probed with a 3.1 Kb insert corresponding to the middle region of *snfA1* genomic region and a 1.8 Kb *argB* insert obtained from the plasmid pDC1. Deletion strain LHA6 was chosen for further analysis.

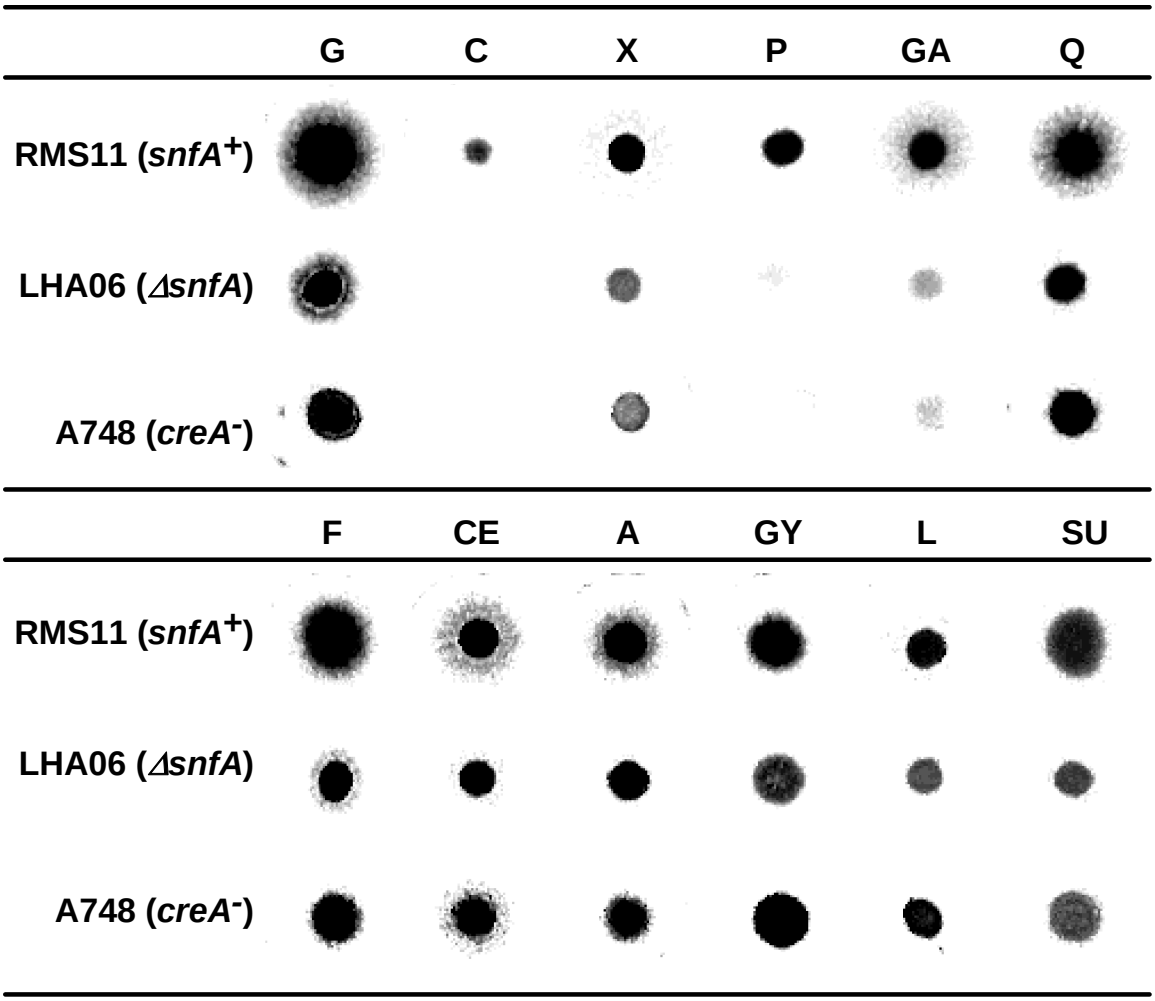

Figure 3. Phenotypic analysis of Δ*snfA1* strain as compared to the reference strain and *creA* mutant on different carbon sources

snfA1 reference strain RMS11, *snfA1* deletion strain LHA6 and carbon derepressed mutant A748 (*creA*$^-$) were grown in glucose (G) or alternate carbon sources like carboxymethylcellulose (C), xylan (X), pectin (P), galacturonic acid (GA), quinic acid (Q), fructose (F), cellobiose (CE), arabinose (A), glycerol (GY), lactose (L) and sucrose (SU). Growth of the mutant strains on various sugars was compared to that of the reference strain.

ALLYL ALCOHOL SENSITIVITY TEST

Figure 4 shows that the *snfA1* mutant strain is more resistant than the reference strain to allyl alcohol in 1% glucose medium, being resistant to allyl alcohol concentrations up to 150 mM as compared to the reference strain, which is resistant to a maximum of 50 mM. The A748 strain which serves as a negative control in this test, shows a total lack of growth on all concentrations of allyl alcohol, indicating derepression of the glucose-repressed *alcA*-encoded alcohol dehydrogenase I (ADHI) that is required to convert allyl alcohol to its toxic product, acrolein.

NORTHERN ANALYSIS OF *snfA1* DELETION MUTANT

Northern analysis of RNAs expressed by *snfA1* deletion and the reference strain was done. Both strains were initially grown on minimal medium with 1 % glucose for 18 hours; mycelia were washed subsequently and transferred to different media containing 0.5 % glucose, 0.5 % glucose + 0.25 % pectin, 0.25 % pectin, 0.5 % galacturonic acid, 0.25 % cellulose and 0.25 % xylan and also to minimal medium without any carbon source (starvation) and grown for an additional 6 hours. Mycelia from all the samples were harvested by filtration, lyophilized and RNA extracted from the lyophilized samples was used for northern analysis.

When probed with a 1.25 Kb *snfA1* fragment, the reference strain RMS11 showed a transcript of ~2.6 Kb size which appeared to be at slightly higher levels in glucose, xylan and starvation conditions compared to pectin, galacturonic acid and cellulose samples (Figure 5). The *snfA1* deletion strain showed complete absence of the transcript in all conditions as expected.

Since it was suggested that *sipA3*, the *A. nidulans* homolog of yeast SIP3, is involved in pectin utilization (Reddy S & Prade R, unpublished), we used *sipA3* as a probe in northern hybridization experiments to see if it is regulated by *snfA1*. Figure 5 shows that *sipA3* transcript levels were similar in all the conditions tested except in

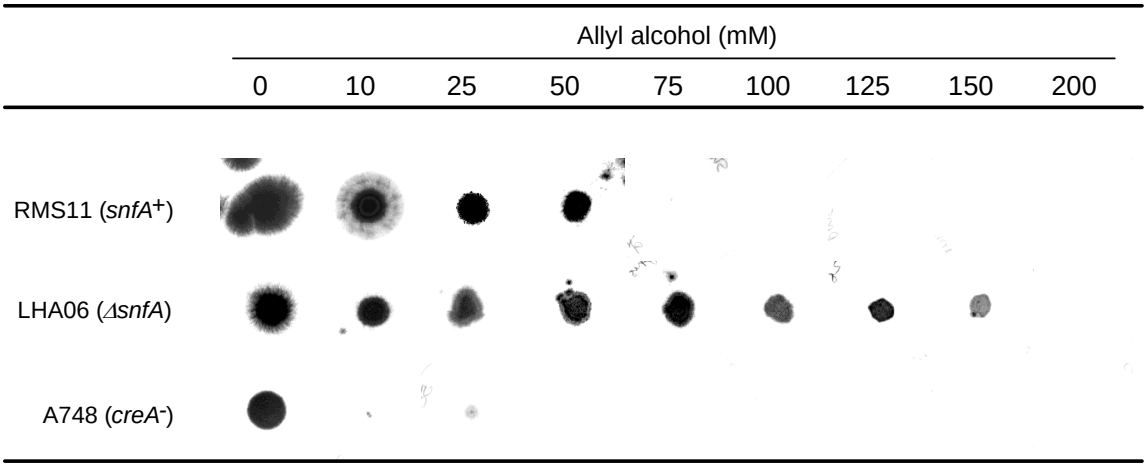

Figure 4. Allyl Alcohol Sensitivity Test

snfA1 reference strain RMS11, *snfA1* deletion strain LHA6 and carbon derepressed mutant A748 (*creA*$^-$) were grown in 1 % glucose combined with various concentrations of allyl alcohol, an ethanol analog. Growth of *ΔsnfA1* strain on various concentrations of allyl alcohol was compared to that of the reference strains RMS11 and A748.

cellulose where the transcript was more in the *snfA1* mutant than in the reference strain.

In *A. nidulans*, CreA plays a role in carbon catabolite repression and acts as a transcriptional repressor of the genes involved in utilization of less favored carbon sources. When *creA* was used as a probe, some differences in its transcript levels were observed between the mutant and reference strains, when grown in both glucose and non-glucose media (Figure 5), suggesting that *snfA1* might regulate *creA* under certain non-repressing conditions.

In the presence of glucose, the structural genes *alcA* and *aldA* encoding alcohol and aldehyde dehydrogenase respectively, which are involved in ethanol catabolism, are repressed by CreA (Flipphi *et al.*, 2002). Figure 5 shows that the *alcA* transcript was not expressed in glucose but expressed in non-repressing conditions in the reference but not the mutant strain, indicating once again that *creA* might be regulated by *snfA1* under some conditions. On the other hand, expression of the *aldA* transcript was not changed in non-repressing carbon sources between the mutant and reference strains, probably because of its indirect control by CreA.

Some pectin degrading enzymes from fungi (polygalacturonases and pectate lyases) are subject to carbon catabolite repression (Dean and Timberlake, 1989; Maldonado *et al.*, 1989; Tonukari *et al.*, 2000) and are regulated by CreA: for example, pectate lyase (Dean and Timberlake, 1989). Several genes encoding pectinolytic enzymes, like *pelA* and *galA*, are induced in the presence of galacturonic acid and pectin but not glucose (Dean and Timberlake, 1989). When *galA* (encoding galactanase) was used as a probe, expression was seen in pectin and galacturonic acid in the reference strain but only in pectin in the mutant strain (Figure 5). When probed with *pelA* and *pmeA* (encoding pectate lyase and pectin methyl esterase respectively), transcripts were not detected in pectin and galacturonic acid in both the strains.

When grown on media containing xylan as the sole carbon source, *A. nidulans* is known to produce at least three endo-(1,4)-β-xylanases (encoded by *xlnA, xlnB* and *xlnC*)

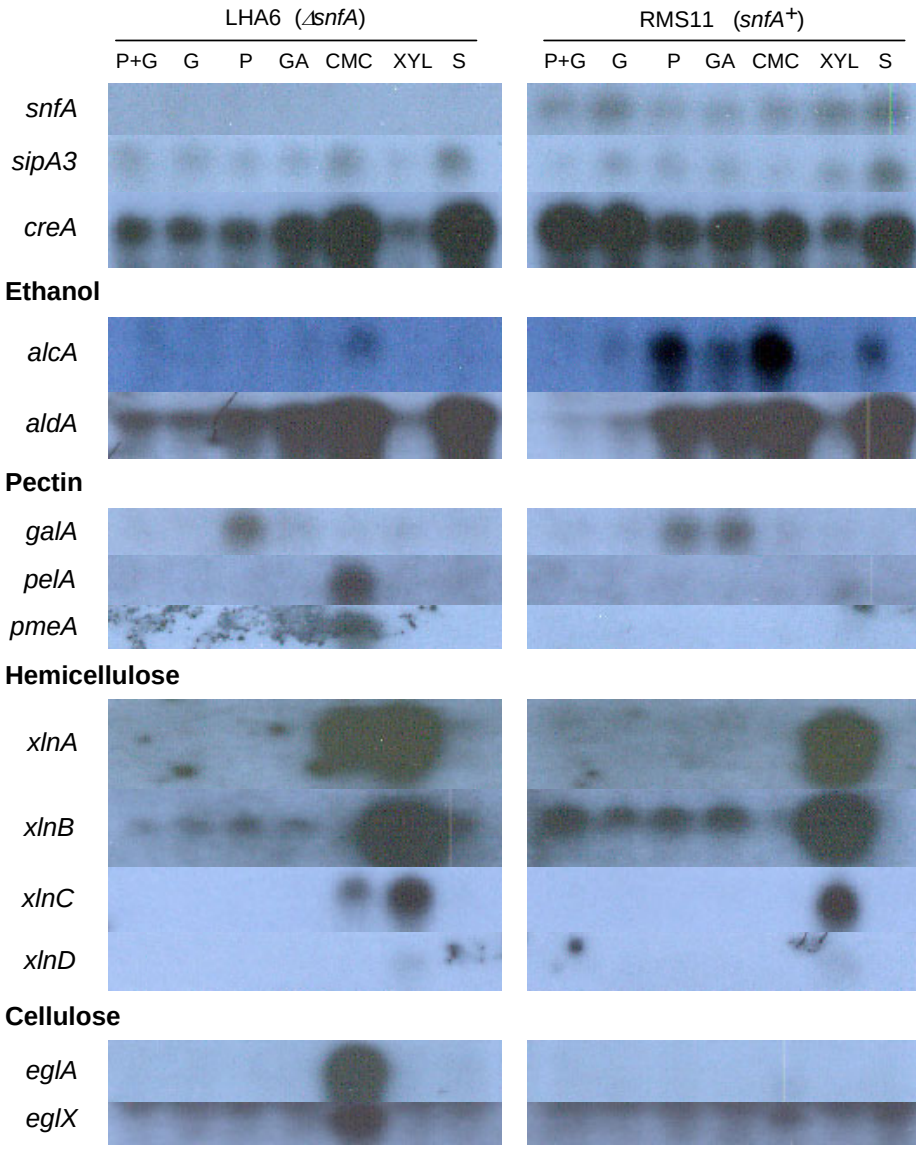

Figure 5. Northern analysis of the *snfA1* deletion strain

snfA1 wild type strain RMS11and *snfA1* deletion strain LHA6 were grown in glucose (G) or alternate carbon sources like pectin (P), galacturonic acid (GA), carboxymethylcellulose (C), xylan (X) and under starvation (without any carbon source) condition (S) and mycelia harvested after 6 hours. RNA was extracted and transferred and probed with *snfA1*, *sipA3* (encoding a transcriptional regulator), *creA* (encoding a carbon catabolite repressor), *alcA* and *aldA* (alcohol and aldehyde dehydrogenases involved in ethanol utilization), *galA, pelA* and *pmeA* (galactanase, pectate lyase and pectin methyl esterase involved in pectin utilization), *xlnA, B, C* and *D* (xylanases involved in xylan utilization) and *eglA* and *eglX* (endoglucanases involved in cellulose utilization).

(MacCabe *et al.*, 1996; MacCabe *et al.*, 1998; Orejas *et al.*, 1999) and one β-xylosidase (encoded by *xlnD*) (Perez-Gonzalez *et al.*, 1998; Orejas *et al.*, 2001). Expression of xylanases in *A. nidulans* is known to be under carbon catabolite repression (Pinaga et al, 1994) mediated by CreA (Perez-Gonzalez *et al.*, 1998; Orejas *et al.*, 1999; Orejas *et al.*, 2001). When probed with different xylanolytic genes, all transcripts were induced to the same extent by xylan in both the strains, but *xlnA* and *xlnC* transcripts were also induced by cellulose in the mutant strain (Figure 5).

Transcripts like *eglA* and *eglX*, which encode endoglucanases involved in cellulose utilization, are expressed in the mutant strain grown in cellulose but not in the reference strain (Figure 5). This indicates that the deletion of *snfA1* is causing overexpression of the cellulose utilizing genes in cellulose medium.

DIFFERENTIAL GENE EXPRESSION AMONG THE GLUCOSE AND PECTIN GROWN REFERENCE AND *snfA1* DELETION STRAINS

All the raw data files ("gpr" files) obtained by scanning the microarray slides were analyzed using the GPAP software (http://darwin.biochem.okstate.edu/gpap). Using the GPAP website we can upload up to 6 "gpr" files including the dye swaps. Data obtained from four individual replicates for each condition and each strain were used to compare the reproducibility of the data among the different replicates. Pin-by-pin intensity dependent local loess normalization was used. Once the data files were uploaded a result report was generated that contained a B-statistic value for each gene which determined if a gene was statistically significantly differentially expressed or not. 552 genes were selected based on a B-statistic $\geq$3 in at least one out of the four experiments that were done. After removing the redundancy, 480 genes were further analyzed out of which 281 were designated as unknowns, which included the genes with unknown functions, hypothetical proteins, predicted proteins and genes with unknown molecular function. The remaining 199 genes were further analyzed and depending on

their differential expression, they were classified into 3 categories: (1) *snfA1* regulated genes (93 genes – 47 %), (2) carbon source regulated genes (55 genes – 28 %) and (3) *snfA1* and carbon source regulated genes (51 genes – 25 %).

***snfA1* dependent regulation of gene expression:** 37 genes appeared to be up-regulated and 56 genes appeared to be down-regulated in a *snfA1* dependent manner either in glucose or pectin or both carbon sources (Tables III & IV). Most of the *snfA1* up-regulated genes are in the glucose grown condition compared to pectin. Some of the interesting genes in this group include *niA* encoding for nitrate reductase, DAHP synthase of the shikimate pathway, glucanase involved in cell wall organization and biogenesis, *pclA* encoding cell cyclin, transcriptional regulators *prnA* and *alkR* involved in proline and alkane metabolism respectively, glucose metabolism elements like *hxkG* encoding glucokinase, hexose transporter and phosphogluconate dehydrogenase, transaldolase involved in pentose phosphate pathway, and elements of translational control like RNA binding and ribosomal proteins. Some genes of significance that are *snfA1* up-regulated include dihydrolipoamide acetyltransferase involved in pyruvate metabolism, delta(24)-sterol c-methyltransferase involved in sterol biosynthesis, mannose-1-phosphate guanylyltransferase involved in cell wall mannoprotein biosynthesis, *stcL* involved in sterigmatocystin biosynthesis, *cpdC* encoding a CTD phosphatase that phosphorylates the C-terminal domain of RNA polymerase II, a serine threonine protein kinase, glutamate dehydrogenase involved in glutamate degradation and which is controlled by nitrogen catabolite repression, phosphoglycerate kinase involved in gluconeogenesis and glycolysis, β-1,3-glucosidase involved in cell wall organization and biogenesis and certain transcription factors.

Table III *snfA1* dependent up regulation of gene expression

Mechanism	**Up regulated in a *snfA1* dependant manner**	**Glucose (Δ*snfA1* vs reference)**			**Pectin (Δ*snfA1* vs reference)**		
		2 to 4	**4 to 6**	**6 to 8**	**2 to 4**	**4 to 6**	**6 to 8**
membrane - lipid synthesis	Delta(24)-sterol c-methyltransferase (p=E-154)						
membrane - undefined	HET-C protein (p=E-136)						
membrane - undefined	Membrane protein (p=1E-29)						
membrane - vesicle/vacuole	Synaptic vesicle protein (p=E-0)						
metabolism	Coproporphyrinogen oxidase (p=E-123)						
metabolism	Coproporphyrinogen oxidase (p=E-123)						
metabolism - aminoacid	Aspartate aminotransferase (p=E-122)						
metabolism - undefined	N,O-diacetylmuramidase (p=3E-71)						
metabolism -sugars	Mannose-1-phosphate guanylyltransferase (p=E-179)						
metabolism	Amylase - Taka A (p=E-167)						
metabolism	Phytase						
metabolism	Cinnamyl alcohol dehydrogenase (p=5E-29)						
regulation - transcription	Transcription factor - zinc finger (p=E-118)						
regulation - transcription	Transcription factor						
regulation - translation	Clathrin coat; (p=E-131)						
regulation - translation	Adenosine deaminase (p=7E-53)						
secondary metabolism	Sterigmatocystin biosynthesis *stcL*						
stress	Glutathione S transferase (p=4E-73)						
transport	Choline transport (p=3E-89)						
metabolism - nucleotide	Urate oxidase *uriC* (p=E-176)						
metabolism - undefined	CTD-phosphatase (cpdC)						
metabolism - vitamins	Inosine 5'-monophosphate nucleotidase (p=E-129)						
regulation - signalling	Protein kinase serine/threonine (*ran*)						
regulation - translation	Ribosomal 60S subunit (p=5E-21)						
regulation - translation	Translation factor - elongation ef3 (p=E-0)						
metabolism - aminoacid	Glutamate dehydrogenase						
metabolism	Endo polyphosphatase						
metabolism - central	Phospho glycerate kinase						
metabolism - nucleotide	Nucleoside diphosphatase (apyrase)						
metabolism	Fructofuranosidase beta						
metabolism	Glucan 1,3-beta-glucosidase						
regulation - signalling	SNF1A/AMP-activated protein kinase						
regulation - transcription	Transcription factor						
stress	Helicase						
stress	Heat shock protein						
stress	Heat shock protein						
transport	ATPase						

Table IV *snfA1* dependent down regulation of gene expression

Mechanism	Down regulated in a *snfA1* dependant manner	Glucose (*Δ snfA1* vs reference) >8	6 to 8	4 to 6	2 to 4
membrane - lipid synthesis	Fatty acid synthase, alpha subunit (p=E-0)				■
membrane - transport	Membrane trafficking events (Vps53)			■	
membrane - undefined	IgE-binging protein 29 kDa (p=3E-_8)				■
membrane - vesicle/vacuole	Vesicle protein Ydr100wp (p=8E-14)				■
membrane- extracellular	Caleosin				■
metabolism - alternate	Nitrate reductase NADPH *niA* (p=E-0)				■
metabolism - aminoacid	DAHP synthase (p=E-0)				■
metabolism	Peptide synthetase (p=E-0)				■
metabolism - central	Carbamoyl-phosphate synthase (*cpa*)				■
metabolism - central	Dehydrogenase - NADPH (old yellow enzyme) (p=2E-74)				■
metabolism - undefined	Oxidoreductase NADP-dependent (p=4E-85)				■
mitochondrial	Mitochondrial carrier *amcA* (p=E-178)				■
metabolism	Sulfonate/alpha-ketoglutarate Fe(II)-dependent (p=5E-32)				■
metabolism	Glucanase (p=8E-85)	■			
regulation - signalling	Cyclin *pclA* (p=6E-099)		■		
regulation - transcription	Transcription factor - (Zn(2)-Cys(6)*prnA*				■
regulation - transcription	Transcription factor - alkane regulation alkR	■			
regulation - transcription	Transcription factor - amino acid cross pathway control			■	
regulation - transcription	Transcription factor - zinc finger (p=E-0)				■
regulation - translation	Clathrin light chain (p=3E-23)		■		
stress	Glutathione S transferase (p=E-120)			■	
stress	Heat shock 70 kda protein				■
stress	Heat shock 70 kda protein (p=E-0)				■
stress	Heat shock 90kda protein (p=E-119)				■
stress	Heat shock protein (p=E-0)				■
stress	Heat shock protein 80 (p=E-0)			■	
stress	Heat shock protein 80 (p=E-0)			■	
stress	Heat shock protein *hs30* (3E-43)			■	
stress	Heat shock protein *hs30* (3E-63)		■		
stress	Heat shock protein *hsp70* and *hsp90* (p=E-158)				■
stress	Thioredoxin reductase (p=E-153)				■
transport	ATPase (p=E-0)			■	
transport	ATPase (p=E-0)			■	
transport	Glucokinase *hxkG* (p=E-0)			■	
transport	Sugar transporter - Hexose (p=E-0)				■
		Glucose & Pectin (*Δ snfA1* vs reference)			
stress	Heat shock protein (p=E-0)				■
stress	Heat shock protein (p=E-0)				■
		Pectin (*Δ snfA1* vs reference)			
membrane - transport	Ferric-chelate reductase				■
metabolism	Hydroxy methylglutaryl-CoA reductase (NADPH)				■
metabolism - aminoacid	Argininosuccinate lyase				■
metabolism - aminoacid	Glutamate dehydrogenase (NADP+)				■
metabolism - central	Acetyl-CoA C-acetyltransferase				■
metabolism - nucleotide	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase				■
metabolism - nucleotide	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase				■
metabolism - pentose shunt	Transaldolase				■
metabolism -sugars	Phospho gluconate dehydrogenase				■
mitochondrial	Cytochrome p450				■
mit/metabolism	Malic enzyme				■
regulation - transcription	Transcription factor				■
regulation - translation	Ribosomal protein				■
regulation - translation	RNA binding protein				■
regulation - translation	Threonine-tRNA ligase				■
regulation - translation	Translation factor - elongation				■
stress	Heat shock protein				■
transport	Sugar transporter - general alpha-glucoside:hydrogen symporter				■
metabolism	Dihydrolipoamide acetyltransferase (p=E-157)				■

Carbon source dependant regulation of gene expression: 15 genes were up-regulated and 40 genes down-regulated in a carbon source dependent manner (Tables V & VI). Among the up-regulated genes are present certain sugar transporters and alcohol dehydrogenase. The carbon source down-regulated genes include *erg3* coding for sterol delta 5,6-desaturase involved in ergosterol biosynthesis, pyruvate dehydrogenase alpha subunit involved in pyruvate metabolism, enoyl-CoA hydratase involved in fatty-acid beta oxidation, cellobiohydrolase essential for cellulose degradation, geranylgeranyl pyrophosphate synthetase involved in isoprenoid biosynthesis, GTPases and GTP binding proteins and transcriptional and translational factors.

***snfA1* and carbon source dependant regulation of gene expression:** This group included 51 genes that were up- or down-regulated in a *snfA1* and carbon source dependant manner in any of the four experiments done (Table VII). The most interesting gene among this group is the one coding for polygalacturonase, an enzyme involved in pectin degradation. As expected, its expression is regulated by the carbon source in a *snfA1* dependent manner. Others in this group are methylcitrate (2-) synthase and succinate dehydrogenase involved in TCA cycle, *sconC* and *metR* encoding transcription factors of the sulfur regulon, endopeptidase involved in cellular response to starvation and sporulation, polyketide synthase involved in secondary metabolism, *pbs2* encoding a serine/threonine protein kinase and certain heat shock proteins.

Unknown gene regulation: We have designated 281 genes, which were significantly differentially expressed, as unknowns. ~58 % (165 genes) of these unknowns were differentially expressed in a *snfA1* dependent manner, ~20 % (55 genes) were differentially expressed in a carbon source dependent manner and ~22 % (61 genes) were differentially expressed in a *snfA1* and carbon source dependent manner (Table VIII).

Table V Carbon source dependent up regulation of gene expression

Mechanism	Upregulated in carbon source dependant manner	Δ *snfA1* (Pectin vs glucose)				Reference (Pectin vs glucose)			
		2 to 4	4 to 6	6 to 8	>8	2 to 4	4 to 6	6 to 8	>8
metabolism	Arginase argI (p=E-176)								
metabolism - vitamins	Thiazole synthase (p=E-0)								
mitochodrial	Aconitase (p=E-0)								
membrane - undefined	Casein kinase I (p=E-168)								
membrane - undefined	Spore germination; *Krr1p* (p=E-137)								
metabolism	26S Proteasome (p=8E-107)								
mitochodrial	Alternative oxidase (p=E-0)								
mit/metabolism	Succinate dehydrogenase/fumarate reductase *sdhA*								
secondary metabolism	Trichothecene efflux pump (p=2E-07)								
transport	Proline permease								
transport	Sugar transporter								
metabolism - central	Fructose-1,6-bisphosphatase (p=E-0)								
metabolism	Arabinofuranosidase - alpha-l (p=E-0)								
metabolism	Alcohol dehydrogenase - NADPH (p=E-149)								
regulation - translation	RNA binding protein								

Table VI Carbon source dependent down regulation of gene expresssion

Mechanism	Down regulated in carbon source dependant manner	Reference (Pectin vs glucose)				Δ*snf A1* (Pectin vs glucose)			
		>8	6 to 8	4 to 6	2 to 4	>8	6 to 8	4 to 6	2 to 4
regulation - signalling	GTPase - beta subunit (p=4E-49)							■	
membrane	Glycosylphosphatidylinositol attachment (p=E-109)				■				
membrane - lipid synthesis	Long-chain-fatty-acid--CoA ligase (p=E-0)				■				
membrane - lipid synthesis	Sterol delta 5,6-desaturase *erg3* (p=9E-92)				■				
membrane - transport	Neomycin resistance protein (p=E-0)				■				
membrane - undefined	Hydroxy proline-rich glycoprotein				■				
membrane - undefined	Pseudohyphae (invasion) formation (p=2E-17)				■				
membrane - vacuole	Leucine aminopeptidase (p=E-103)				■				
membrane - vesicle/vacuole	ER translocation protein (p=E-0)	■							
membrane - vesicle/vacuole	Vesicular-fusion protein sec17 (p=9E-95)				■				
membrane - vesicle/vacuole	Vesicle protein inositol regulator (p=2E-27)				■				
metabolism	26S Proteasome subunit (p=E-170)			■					
metabolism	Protease (p=1E-96)				■				
metabolism	Peptidase c54 (p=5E-99)				■				
metabolism - central	Pyruvate dehydrogenase E1 - alpha subunit (p=E-0)				■				
metabolism - electron transport	Disulfide isomerase *pdi* (p=E-0)				■				
metabolism - undefined	Esterase (p=3E-27)				■				
metabolism - undefined	Esterase *ste1* (p=1E-58)				■				
mitochondrial	Alternative oxidase (p=E-0)				■				
mitochondrial	Mitochondrial splicing protein (p=1E-75)				■				
mitochondrial	Enoyl-CoA hydratase (p=3E-16)				■				
metabolism	Cellobiohydrolase (p=E-0)			■					
metabolism	Chitin synthase B *chsB* (p=E-0)				■				
metabolism	Alpha-beta hydrolase (p=2E-51)				■				
metabolism	Aldehyde dehydrogenase *aldH* (p=6E-71)				■				
regulation - signalling	GTP-binding protein *(ras)* (p=3E-76)				■				
regulation - translation	Ribosomal protein S2				■				
regulation - translation	RNA binding protein				■				
regulation - translation	RNA binding protein (p=E-0)				■				
secondary metabolism	Geranylgeranyl pyrophosphate synthetase (p=7E-67)				■				
stress	Chaperonin *groE1* (p=E-109)	■							
stress	Thioredoxin thio (p=1E-57)				■				
stress	Glutathione S-transferase (p=5E-21)				■				
transport	Sugar transporter (p=3E-20)			■					
transport	Purine-cytosine permease (p=5E-62)			■					
transport	Amino-acid permease (p=E-173)				■				
metabolism	Protease (p=E-0)	■						■	
regulation - signalling	GTPase - activator *(rab* -like) (p=E-108)	■						■	
regulation - transcription	Transcription factor - *tfiiD* (p=E-146)			■			■		
regulation - translation	Translation factor - elongation *ef1A* (p=E-0)	■				■			

Table VII *snfA1* and carbon source dependent regulation of gene expression

Mechanism	Upregulated in *snfA* and carbon source dependent manner	Glucose Δ*snfA1* vs reference				Pectin Δ *snfA1* vs		Reference (Pectin vs glucose)				Δ *snfA1* (Pectin vs glucose)			
		2 to 4	4 to 6	6 to 8	>8	2 to 4	4 to 6	2 to 4	4 to 6	6 to 8	>8	2 to 4	4 to 6	6 to 8	>8
regulation - translation	Ribosomal protein L32 (p=2E-43)														
stress	Heat shock protein *hs30* (3E-43)														
stress	Heat shock protein *clpA* (p=E-0)														
stress	Heat shock protein *hs30* (p=E-101)														
membrane - undefined	Cell wall biosynthesis kinase (p=E-125)														
mitochondrial	Alternative oxidase														
regulation - signalling	Protein kinase serine/threonine (*pbs2*)														
regulation - translation	Translation factor - initiation *eif-2b* (p=1E-64)														
stress	Ubiquitin-protein ligase E3 (p=E-178)														
transport	GABA transporter (p=6E-55)														
metabolism	Endo peptidase														
transport	Sugar transporter - general alpha-glucoside:hydrogen symporter														
stress	Ubiquitin-conjugating enzyme E2 (p=6E-21)														
stress	Heat shock protein *hs30* (3E-43)														
membrane - vesicle/vacuole	Synaptobrevin (p=8E-43)														
metabolism - aminoacid	Glutaryl-CoA dehydrogenase (p=E-0)														
metabolism - catabolism	Proteinase (p=E-0)														
metabolism - undefined	Flavodoxin														
metabolism -sugars	Glycosyl transferase (p=1E-20)														
regulation - signalling	GTP-binding protein (small subunit) (p=8E-99)														
regulation - transcription	Transcription factor - sulphur regulator *metR* (p=E-155)														
regulation - translation	Translation factor - elongation ef1g (p=3E-36)														
regulation - translation	Aspartyl-tRNA synthetase (p=E-140)														
secondary metabolism	Sterigmatocystin biosynthesis p450 monooxygenase *stcS* (p=E-0)														
stress	Heat shock protein *hs30* (3E-43)														
transport	Myo-inositol transport protein (p=E-126)														
metabolism	Dihydrolipoamide acetyltransferase (p=E-157)														
membrane - undefined	Antigenic determinant of rec-A protein (p=\2E-51)														
metabolism	Methylcitrate (2-) synthase *prpC*														
metabolism - lipid	Phosphatidylglycerophosphate synthase (p=E-111)														
metabolism	Succinate dehydrogenase/fumarate reductase (p=5E-83)														
regulation - transcription	Transcription factor - *sconC*														
secondary metabolism	Polyketide synthase (p=E-171)														
stress	Heat shock protein *hs30* (p=E-101)														
stress	Heat shock protein *hs30* (3E-43)														
transport	Quinate permease *qutD* (p=5E-58)														
mitochondrial	Glycylpeptide N-tetradecanoyltransferase														
stress	DNA (cytosinE-5-)-methyltransferase														
metabolism	Polygalacturonase														
metabolism	Polygalacturonase														
stress	Heat shock protein *hs30* (3E-43)														
reg - sig	GTPase - alpha-subunit														
met - lipid	Isopropylmalate (3-) dehydratase														
secondary metabolism	Polyketide synthase (p=E-0)														
membrane - lipid synthesis	Acyl-CoA sterol acyltransferase (p=E-171)														
membrane - transport	Vesicle traffic protein (p=E-149)														
mitochondrial	Ketoacyl (3-)-CoA thiolase (p=E-141)														
mit/metabolism	Monooxigenase (p=E-152)														
regulation - signalling	Calmodulin-dependent protein kinase *cmkA* (p=E-0)														
stress	Ubiquinol-cytochrome C reductase (p=E-12)														
stress	Ubiquitin fusion protein (p=E-0)														
		>8	6 to 8	4 to 6	2 to 4	4 to 6	2 to 4	>8	6 to 8	4 to 6	2 to 4	>8	6 to 8	4 to 6	2 to 4

Scale for up-regulation

Scale for down-regulation

Table VIII Unknown genes and their regulation

Regulation	Number of Genes	Total number of genes
snfA1 regulated in Glucose	92	
snfA1 regulated in Pectin	65	165 (~ 58 %)
snfA1 regulated in Glucose & Pectin	8	
Carbon source regulated in reference	48	
Carbon source regulated in Δ*snfA1*	3	55 (~ 20 %)
Carbon source regulated in reference & Δ*snfA1*	4	
snfA1 & Carbon source regulated in Glucose & Pectin	9	
snfA1 & Carbon source regulated in Glucose, Pectin, reference, Δ*snfA1*	41	61 (~ 22 %)
snfA1 & Carbon source regulated (miscellaneous)	11	

From these data we can conclude that *snfA1* is in general regulating cellular events like glucose derepression, lipid synthesis, sterol metabolism, iron homeostasis, secondary metabolism, stress response, cell cycle regulation and transcriptional regulation.

DISCUSSION

All organisms should have a mechanism that enables them to adapt to glucose-limiting conditions. Snf1 protein kinase and the AMP-activated protein kinase (AMPK) which are highly conserved from yeast to mammals play an important role in cell survival by activating or derepressing alternative genes in response to the stress of glucose deprivation. The significance of Snf1p has been demonstrated in yeast not only for derepression of glucose-repressed genes, but also for many other cellular processes like glycogen, sterol and fatty acid biosynthesis, fatty acid β-oxidation, peroxisome biogenesis, thermotolerance and sporulation (Sanz, 2003). Considering the diverse roles carried out by Snf1p in yeast, we set out to identify and characterize its homolog in *Aspergillus nidulans*, as this organism is considered to be an important model for genetic studies among the filamentous fungi (Martinelli, 1994).

The *snfA1* gene in *A. nidulans* has been cloned and characterized in this study. In order to better understand the full range of functions of *snfA1*, a deletion mutant has been generated and several comparative studies conducted between the reference and mutant strains. When grown on repressing (glucose) and nonrepressing (pectin, xylan, etc) carbon sources, the *snfA1* deletion strain shows less sporulation than the reference strain (data not shown). The number of genes involved in conidiation in *A. nidulans* is believed to be around a few hundred to one thousand genes (Timberlake and Clutterbuck, 1994). SNF1 in yeast has been shown to be involved in sporulation and *snf1* deletion mutants failed to form spores (Carlson *et al.*, 1981). Based on our results and previous studies, we

believe that *snfA1* is involved in sporulation either directly or indirectly by regulating some transcriptional activators of sporulation genes. Apart from decreased sporulation effects, absence of *snfA1* also caused decrease in the growth rates on complex carbon sources like pectin, xylan, cellulose and galacturonic acid and to a lesser extent on simple sugars like fructose, lactose, sucrose, etc., compared to the growth on glucose. This gives us a clue that *snfA1* might be essential for the metabolism of these alternate carbon sources during glucose deprivation. This could be either due to direct lack of derepression of glucose- repressed genes in a *snfA1* mutant or due to the impairment of some other growth factors as a consequence of *snfA1* deletion. To rule out the latter possibility, we have calculated the enzyme activities in the reference and Δ*snfA1* strains grown on glucose and pectin. As expected, pectinase activity was not detected in the mutant grown on pectin (Table IX), thus indicating that *snfA1* is involved in the derepression mechanism.

Another piece of evidence that supports our hypothesis of the involvement of *snfA1* in carbon catabolite derepression is the allyl alcohol sensitivity test. CreA, which is a transcriptional repressor, mediates the carbon catabolite repression system. In the presence of glucose, *alcA* encoding alcohol dehydrogenase I (ADHI) that converts allyl alcohol to a toxic product acrolein, is repressed by CreA. Hence the reference strain is able to grow on media containing certain concentrations of allyl alcohol after which probably CreA is being repressed by SnfA1 due to unknown mechanisms. Our results also showed that the Δ*snfA1* mutant is super resistant to allyl alcohol than the reference strain, which indicates that CreA is repressing the *alcA* gene at all allyl alcohol concentrations unlike in the reference strain. This clearly provides evidence that SnfA1 regulates CreA and hence involved in carbon catabolite derepression.

Our northern data also points to the fact that *snfA1* is involved in regulating the genes encoding certain polysaccharide degrading enzymes. This regulation can be either induction or repression. For example *alcA*, which is a glucose-repressed gene appears to

Table IX Pectinase activity determined in 1 ul of culture filtrate of vegetative mycelium grown in liquid medium for 48 hrs at 37 C.

STRAIN	PECTINASE (Prod of GAL Ac./min/ul)		FOLD INDUCTION
	GLUCOSE	PECTIN	
RMS11 - WT	0.12 +/- 0.03	3.01 +/- 0.21	25.08
LHA6 -*Δ snfA1*	0.35 +/- 0.51	0.08 +/- 0.03	0.23

Δ snfA1 does not produce detectable levels of pectin degrading activities

be induced by *snfA1* as the *alcA* transcript could not be seen in a Δ*snfA1* mutant. Transcripts like *pelA, xlnA and eglA* are expressed in the mutant strain though in the inappropriate carbon source, cellulose. This shows that *snfA1* might be repressing their expression when grown in other carbon sources. The expression of transcripts like *galA*, *aldA, xlnA, B,C* and *D* did not change in both the reference and mutant strains suggesting that either there is an alternate pathway that is regulating these genes when grown on alternate carbon sources or *snfA1* is not involved in their regulation. But we can rule out the latter explanation based on our results from growth assays and Northern blot experiments. Another interesting feature that is observed in the Northern data is the mis-scheduling of gene expression in the Δ*snfA1* mutant. For example, certain transcripts involved in pectin and xylan utilization are expressed in inappropriate carbon sources like cellulose in Δ*snfA1*. This indicates that *snfA1* coordinates gene expression with carbon source utilization.

The role of *snfA1* in regulating cellular processes during carbon source shift was further studied using microarray technology. Microarrays could help identify other genes that are being regulated by *snfA1* thus enabling us to understand the various functions of *snfA1* apart from carbon catabolite derepression. The most interesting result from our microarray experiment is the differential expression of polygalacturonase, an enzyme involved in pectin degradation. The microarray result shows that the enzyme is down-regulated in a Δ*snfA1* strain when grown on pectin (Table VII). This clearly shows that *snfA1* plays a role in the derepression of the enzyme in the absence of glucose. However, we also notice that the enzyme is up-regulated in both the strains when grown on pectin. This might suggest that there is an alternate pathway that is regulating the glucose derepression mechanism in the absence of *snfA1* or may be, in addition to *snfA1*.

snfA1 appears to play a role in glucose metabolism since a glucokinase encoding gene, *hxkG*, and a hexose transporter are being down-regulated in a *ΔsnfA1* strain. Glucokinase is essential for the first step in glycolysis to phosphorylate glucose to

glucose 6-phosphate. Previous studies have shown the dependency of fatty acid synthase expression on the production of glucose 6-phosphate rather than on glucose (Foufelle *et al.*, 1992; Prip-Buus *et al.*, 1995). Our microarray results also show that fatty acid synthase is down-regulated in a *ΔsnfA1* strain which could be an indirect effect due to the lack of glucose 6-phosphate production as a result of glucokinase down regulation.

There are several transcription factors that appear to be regulated in a *snfA1* dependent manner. Examples include the transcription factors *metR* and *sconC*, which encode the positive and negative regulators of sulfur metabolism respectively. *metR* is up-regulated in a reference strain when grown on pectin. This might be due to the glucose derepression effect. *metR* appears to be negatively regulating *sconC,* based on the previous work (Piotrowska *et al.*, 2000). We also see from our microarray data that *sconC* is up-regulated in *ΔsnfA1* grown on pectin compared to the reference strain, agreeing with the facts mentioned above. This still does not explain the involvement of *snfA1* in regulating the transcription factors of the sulfur regulon.

We also see from our microarray results that other transcriptional factors like *prnA* and *alkR,* encoding the transcriptional activators of proline utilization and alkane degradation pathways respectively, are also differentially expressed in a *snfA1* dependent manner. The proline utilization pathway is subject to both nitrogen and carbon catabolite repression in *A. nidulans* (Gomez *et al.*, 2002) and previous work has shown that the expression of *prn* genes is strongly repressed only by the simultaneous presence of glucose and ammonium (Gonzalez *et al.*, 1997). Our microarray results show that *prnA* expression is down-regulated in *ΔsnfA1* grown on glucose indicating that *snfA1* might be controlling its *creA* mediated glucose repression in a reference strain. Similarly, *alkR* has been shown to be transcribed at a low level (Ratajczak *et al.*, 1998) and we see from our microarray data that *alkR* is down-regulated in *ΔsnfA1* grown on glucose. These results indicate that *snfA1* might act as a general regulator of certain transcription factors either directly or indirectly by controlling some other regulatory mechanisms. Among these

transcription factors there are some that are zinc-finger containing (for example, *prnA*) which might be *creA* regulated. *snfA1* might regulate these factors via its interactions with *creA*. But none of the transcription factors have a phosphorylation domain, which indicates that they might not be direct targets for *snfA1* dependent phosphorylation.

Recently, genetic studies in yeast have demonstrated that under glucose starving conditions the heat shock transcription factor (HSF) induces the expression of a subset of its target heat shock proteins (for example Hsp30) in a Snf1 dependent manner (Hahn and Thiele, 2004). It was also shown that the induction levels of *HSP26* and *HSP30* were reduced in *Δsnf1* under glucose starving conditions. Our microarray data shows that several heat shock proteins like Hsp80, Hsp30, Hsp70 and Hsp90 are down-regulated in *ΔsnfA1* grown on glucose. It is surprising to note that heat shock proteins are down-regulated in the mutant even in the presence of glucose, considering the fact that Snf1 dependent activation of HSF target genes occurs only in response to glucose starvation in yeast. This can be explained based on our hypothesis that *snfA1* is essential for glucose utilization. The improper glucose metabolism in a *ΔsnfA1* strain could be a condition of stress for the fungus, which should induce the transcription of certain heat shock proteins. The absence of *snfA1* itself might be inhibiting this induction leading to the down regulation of heat shock proteins in a Δ*snfA1* strain. We also see that some heat shock proteins are differentially expressed in a *snfA1* independent manner when the strains are grown on glucose and pectin. So, there might be other mechanisms controlling the induction of these genes in response to changes in carbon source status. In *A. nidulans*, the expression of *gstA*, a gene encoding glutathione S transferase, has been shown to be transcriptionally regulated by the presence of toxic compounds and oxidative stress (Fraser *et al.*, 2002). Surprisingly, *gstA* expression has also been shown to increase when grown on galactose as a carbon source. These data suggest that glutathione S transferase is involved in stress response in the fungus. Consistent with this data, we found that there is significant differential expression of glutathione S transferase between

ΔsnfA1 and reference strain when grown on glucose, suggesting that *snfA1* might be involved in regulating its expression and hence plays a role in stress response.

In plants, SNF1-related protein kinases (SnRKs) have been shown to inactivate HMG-CoA reductase and nitrate reductase (Sugden *et al.*, 1999). These target enzymes catalyze regulatory events in isoprenoid biosynthesis and nitrogen assimilation respectively. Interestingly, we have seen such kind of *snfA1* dependent regulation of both the enzymes in our microarray data showing that *snfA1* plays a role in these metabolic pathways.

Another interesting feature that we found in our microarray data is the regulation of certain polysaccharide degrading enzymes in a *snfA1* dependent manner. Examples include beta fructofuranosidase, glucanase and beta1,3-glucosidase. Metabolic enzymes like chitin synthase B, cellobiohydrolase, aldehyde dehydrogenase (*aldH*) and NADPH alcohol dehydrogenase are differentially expressed in a carbon source dependent manner. It is not clear as to what mechanisms are governing the regulation of metabolic genes and this needs further experimentation.

Previous work in *A. nidulans* has shown that conidiophore development and spore formation requires a fine-tuning of cell cycle (Schier and Fischer, 2002). Loss of function of *pclA*, a cell cyclin gene involved in cell cycle regulation, caused a decrease in sporulation. Our microarray data shows that the absence of *snfA1* causes down regulation of *pclA* indicating that *snfA1* plays a role also in cell cycle regulation. This result also explains the fact that *ΔsnfA1* has decreased sporulation than reference strain, when grown on minimal media.

snfA1 also appears to play a role in controlling the expression of genes involved in sterol biosynthesis, pentose phosphate pathway, amino acid and pyruvate metabolism. The mechanisms governing these regulations need to be understood by further experimentation.

There are certain protein kinases that appear to be regulated in a *snfA1* dependent manner. For example, a serine threonine protein kinase (*ran* like) is being up-regulated in *ΔsnfA1* when grown on glucose and pectin. This indicates that *snfA1* also has a (negative) regulatory role in mechanisms not related to carbohydrate metabolism. Another serine threonine protein kinase (*pbs2* like), which is involved in osmotolerance is differentially regulated between *ΔsnfA1* and reference strain in a carbon source dependent manner. This kind of regulation may not be a direct consequence of *snfA1* deletion but could be an indirect effect.

Apart from the results discussed above, there are genes that are differentially expressed in a carbon source dependent manner. These include genes involved in TCA cycle, gluconeogenesis, amino acid catabolism, sterol metabolism, transcriptional and translational regulation and stress. It is beyond the scope of this work to explain the rationale behind these expression patterns. However it appears that there are several other as yet unidentified mechanisms controlling the gene expression in response to carbon source availability.

Apart from the genes with known molecular functions, there were 281 genes with unknown functions that were differentially expressed in a *snfA1* or carbon source or *snfA1* and carbon source dependent manner. Greater than 50 % of these genes appear to be regulated by *snfA1*. This could be a direct consequence of *snfA1* deletion from the genome or could be an indirect effect. This data indicates that *snfA1* plays as essential role in some unknown mechanisms also.

Based on all our results we have proposed a model for *snfA1* in *A.nidulans* that shows the various cellular activities regulated by *snfA1* both in carbon source dependent and independent manner (Fig. 7). According to this model, in the presence of low glucose SnfA1 protein inactivates CreA and derepresses the carbon catabolite repressed genes like those involved in alternate carbon source utilization, ethanol utilization, respiration and gluconeogenesis. Also, SnfA1 interacts with SipA3, a positively acting

transcription factor, and derepresses the genes involved in polysaccharide utilization like those encoding pectinases, xylanases and cellulases. The model also shows that SnfA1 plays a role in other cellular mechanisms irrespective of the carbon source availability.

In conclusion, we have successfully isolated and deleted *snfA1* gene in the filamentous fungus *A. nidulans*. *snfA1* plays an essential role in coordinating the cellular response with nutritional availability. But it is important to note that there could be other pathways contributing to the glucose response. Apart from its role in glucose derepression mechanism, *snfA1* also appears to act as a regulator of other cellular mechanisms like lipid synthesis, sterol metabolism, iron homeostasis, secondary metabolism, stress response, cell cycle regulation and transcriptional regulation. *snfA1* might exert its effect in these mechanisms either directly or indirectly, considering the differential expression of various transcription factors in a *snfA1* dependent manner.

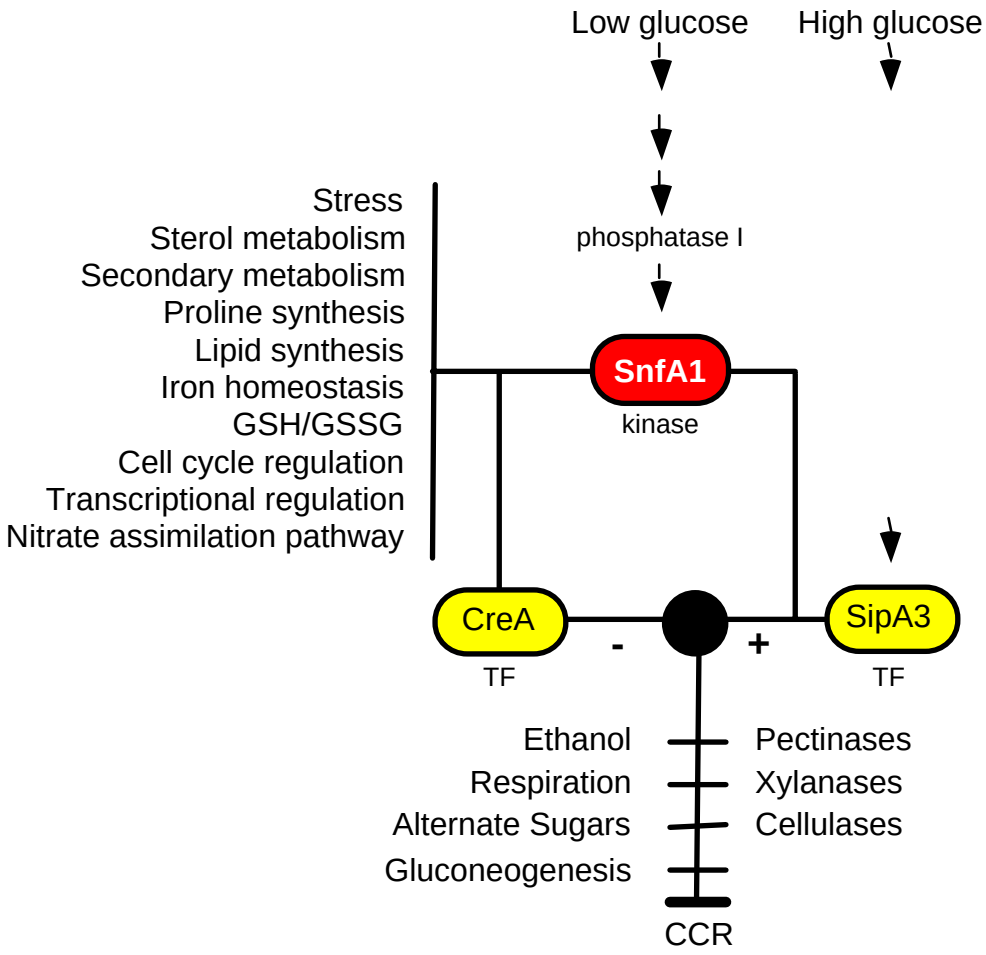

Fig 6. *snfA1* model in *Aspergillus nidulans*

Model proposed to show the role of *snfA1* in glucose limiting condition, during which SnfA1 protein interacts with CreA and SipA3 proteins and derepresses the CreA mediated carbon catabolite repressed genes involved in alternate sugar utilization, ethanol utilization, respiration and gluconeogenesis. The model also shows the involvement of SnfA1 in other cellular mechanisms.

REFERENCES

Aramayo, R. and Timberlake, W. E. (1993). The *Aspergillus nidulans yA* gene is directly regulated by the *abaA* gene. *EMBO J* **12**: 2039-2048.

Arst, H. N., Jr. and Cove, D. J. (1973). Nitrogen metabolite repression in Aspergillus nidulans. *Mol Gen Genet* **126**: 111-41.

Arst, H. N., Jr. and MacDonald, D. W. (1975). A gene cluster in Aspergillus nidulans with an internally located cis-acting regulatory region. *Nature* **254**: 26-31.

Ayoubi, P., Jin, X., Leite, S., Liu, X., Martajaja, J., Abduraham, A., Wan, Q., Yan, W., Misawa, E. and Prade, R. A. (2002). PipeOnline 2.0: automated EST processing and functional data sorting. *Nucleic Acids Res* **30**: 4761-9.

Bailey, C. and Arst, H. N., Jr. (1975). Carbon catabolite repression in Aspergillos nidulans. *Eur J Biochem* **51**: 573-7.

Carlson, M., Osmond, B. C. and Botstein, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98**: 25-40.

Celenza, J. L. and Carlson, M. (1989). Mutational analysis of the Saccharomyces cerevisiae SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. *Mol Cell Biol* **9**: 5034-44.

Celenza, J. L., Eng, F. J. and Carlson, M. (1989). Molecular analysis of the SNF4 gene of Saccharomyces cerevisiae: evidence for physical association of the SNF4 protein with the SNF1 protein kinase. *Mol Cell Biol* **9**: 5045-54.

Dean, R. A. and Timberlake, W. E. (1989). Regulation of the Aspergillus nidulans pectate lyase gene (pelA). *Plant Cell* **1**: 275-84.

Dowzer, C. E. and Kelly, J. M. (1989). Cloning of the creA gene from Aspergillus nidulans: a gene involved in carbon catabolite repression. *Curr Genet* **15**: 457-9.

Dowzer, C. E. and Kelly, J. M. (1991). Analysis of the creA gene, a regulator of carbon catabolite repression in Aspergillus nidulans. *Mol Cell Biol* **11**: 5701-9.

Drysdale, M. R., Kolze, S. E. and Kelly, J. M. (1993). The Aspergillus niger carbon catabolite repressor encoding gene, creA. *Gene* **130**: 241-5.

Flipphi, M., Kocialkowska, J. and Felenbok, B. (2002). Characteristics of physiological inducers of the ethanol utilization (alc) pathway in Aspergillus nidulans. *Biochem J* **364**:

25-31.

Foufelle, F., Gouhot, B., Pegorier, J. P., Perdereau, D., Girard, J. and Ferre, P. (1992). Glucose stimulation of lipogenic enzyme gene expression in cultured white adipose tissue. A role for glucose 6-phosphate. *J Biol Chem* **267**: 20543-6.

Fraser, J. A., Davis, M. A. and Hynes, M. J. (2002). A gene from Aspergillus nidulans with similarity to URE2 of Saccharomyces cerevisiae encodes a glutathione S-transferase which contributes to heavy metal and xenobiotic resistance. *Appl Environ Microbiol* **68**: 2802-8.

Gancedo, J. M. (1992). Carbon catabolite repression in yeast. *Eur J Biochem* **206**: 297-313.

Gomez, D., Cubero, B., Cecchetto, G. and Scazzocchio, C. (2002). PrnA, a Zn2Cys6 activator with a unique DNA recognition mode, requires inducer for in vivo binding. *Mol Microbiol* **44**: 585-97.

Gonzalez, R., Gavrias, V., Gomez, D., Scazzocchio, C. and Cubero, B. (1997). The integration of nitrogen and carbon catabolite repression in Aspergillus nidulans requires the GATA factor AreA and an additional positive-acting element, ADA. *Embo J* **16**: 2937-44.

Hahn, J. S. and Thiele, D. J. (2004). Activation of the Saccharomyces cerevisiae heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase. *J Biol Chem* **279**: 5169-76. Epub 2003 Nov 10.

Hynes, M. J. and Kelly, J. M. (1977). Pleiotropic mutants of Aspergillus nidulans altered in carbon metabolism. *Mol Gen Genet* **150**: 193-204.

Ilmen, M., Thrane, C. and Penttila, M. (1996). The glucose repressor gene cre1 of Trichoderma: isolation and expression of a full-length and a truncated mutant form. *Mol Gen Genet* **251**: 451-60.

Johnston, M., Flick, J. S. and Pexton, T. (1994). Multiple mechanisms provide rapid and stringent glucose repression of GAL gene expression in Saccharomyces cerevisiae. *Mol Cell Biol* **14**: 3834-41.

Jung, M. K., Ovechkina, Y., Prigozhina, N., Oakley, C. E. and Oakley, B. R. (2000). The use of beta-D-glucanase as a substitute for Novozym 234 in immunofluorescence and protoplasting. *Fungal Genet Newsletter* **47**: 65-66.

Kafer, E. (1977). Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. *Adv Genet* **19**: 33-131.

Kelly, J. M. (1994). Carbon catabolite repression. *Prog Ind Microbiol* **29**: 355-67.

Lutfiyya, L. L., Iyer, V. R., DeRisi, J., DeVit, M. J., Brown, P. O. and Johnston, M. (1998). Characterization of three related glucose repressors and genes they regulate in Saccharomyces cerevisiae. *Genetics* **150**: 1377-91.

Lutfiyya, L. L. and Johnston, M. (1996). Two zinc-finger-containing repressors are responsible for glucose repression of SUC2 expression. *Mol Cell Biol* **16**: 4790-7.

MacCabe, A. P., Fernandez-Espinar, M. T., de Graaff, L. H., Visser, J. and Ramon, D. (1996). Identification, isolation and sequence of the Aspergillus nidulans xlnC gene encoding the 34-kDa xylanase. *Gene* **175**: 29-33.

MacCabe, A. P., Orejas, M., Perez-Gonzalez, J. A. and Ramon, D. (1998). Opposite patterns of expression of two Aspergillus nidulans xylanase genes with respect to ambient pH. *J Bacteriol* **180**: 1331-3.

Maldonado, M. C., Strasser de Saad, A. M. and Callieri, D. (1989). Catabolite repression of the synthesis of inducible polygalacturonase and pectinesterase by Aspergillus sp. *Curr MIcrobiol* **18**: 303-306.

Martinelli, S. D. (1994). Aspergillus nidulans as an experimental organism. *Prog Ind Microbiol* **29**: 33-58.

Monod, J. and Jacob, F. (1961). On the regulation of gene activity. *Cold Spring Harb Symp Quant Biol* **26**: 193-211.

Nehlin, J. O., Carlberg, M. and Ronne, H. (1991). Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response. *Embo J* **10**: 3373-7.

Orejas, M., MacCabe, A. P., Perez Gonzalez, J. A., Kumar, S. and Ramon, D. (1999). Carbon catabolite repression of the Aspergillus nidulans xlnA gene. *Mol Microbiol* **31**: 177-84.

Orejas, M., MacCabe, A. P., Perez-Gonzalez, J. A., Kumar, S. and Ramon, D. (2001). The wide-domain carbon catabolite repressor CreA indirectly controls expression of the Aspergillus nidulans xlnB gene, encoding the acidic endo-beta-(1,4)-xylanase X(24). *J Bacteriol* **183**: 1517-23.

Ospina-Giraldo, M. D., Mullins, E. and Kang, S. (2003). Loss of function of the Fusarium oxysporum SNF1 gene reduces virulence on cabbage and Arabidopsis. *Curr Genet* **44**: 49-57. Epub 2003 Jul 5.

Perez-Gonzalez, J. A., van Peij, N. N., Bezoen, A., MacCabe, A. P., Ramon, D. and de Graaff, L. H. (1998). Molecular cloning and transcriptional regulation of the Aspergillus nidulans xlnD gene encoding a beta-xylosidase. *Appl Environ Microbiol* **64**: 1412-9.

Piotrowska, M., Natorff, R. and Paszewski, A. (2000). sconC, a gene involved in the regulation of sulphur metabolism in Aspergillus nidulans, belongs to the SKP1 gene

family. *Mol Gen Genet* **264**: 276-82.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., McDonald, K. D. and Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**: 141-238.

Prip-Buus, C., Perdereau, D., Foufelle, F., Maury, J., Ferre, P. and Girard, J. (1995). Induction of fatty-acid-synthase gene expression by glucose in primary culture of rat hepatocytes. Dependency upon glucokinase activity. *Eur J Biochem* **230**: 309-15.

Ratajczak, A., Geissdorfer, W. and Hillen, W. (1998). Expression of alkane hydroxylase from Acinetobacter sp. Strain ADP1 is induced by a broad range of n-alkanes and requires the transcriptional activator AlkR. *J Bacteriol* **180**: 5822-7.

Ronne, H. (1995). Glucose repression in fungi. *Trends Genet* **11**: 12-7.

Ruijter, G. J. G. and Visser, J. (1997). Carbon repression in *Aspergillii*. *FEMS Microbiology Letters* **151**: 103-114.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1987). In *Molecular cloning: A laboratory manual*. Cold Spring Harber,

Sanz, P. (2003). Snf1 protein kinase: a key player in the response to cellular stress in yeast. *Biochem Soc Trans* **31**: 178-81.

Schier, N. and Fischer, R. (2002). The Aspergillus nidulans cyclin PclA accumulates in the nucleus and interacts with the central cell cycle regulator NimX(Cdc2). *FEBS Lett* **523**: 143-6.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. (1997). Carbon regulation of the cuticle-degrading enzyme PR1 from Metarhizium anisopliae may involve a trans-acting DNA-binding protein CRR1, a functional equivalent of the Aspergillus nidulans CREA protein. *Curr Genet* **31**: 511-8.

Sims, A. H., Robson, G. D., Hoyle, D. C., Oliver, S. G., Turner, G., Prade, R. A., Russell, H. H., Dunn-Coleman, N. S. and Gent, M. E. (2004). Use of expressed sequence tag analysis and cDNA microarrays of the filamentous fungus Aspergillus nidulans. *Fungal Genet Biol* **41**: 199-212.

Strauss, J., Mach, R. L., Zeilinger, S., Hartler, G., Stoffler, G., Wolschek, M. and Kubicek, C. P. (1995). Cre1, the carbon catabolite repressor protein from Trichoderma reesei. *FEBS Lett* **376**: 103-7.

Stringer, M. A., Dean, R. A., Sewall, T. C. and Timberlake, W. E. (1991). Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes Dev* **5**: 1161-71.

Sugden, C., Donaghy, P. G., Halford, N. G. and Hardie, D. G. (1999). Two SNF1-related

protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. *Plant Physiol* **120**: 257-74.

Timberlake, W. E. and Clutterbuck, A. J. (1994). Genetic regulation of conidiation. *Prog Ind Microbiol* **29**: 383-427.

Tonukari, N. J., Scott-Craig, J. S. and Walton, J. D. (2000). The Cochliobolus carbonum SNF1 gene is required for cell wall-degrading enzyme expression and virulence on maize. *Plant Cell* **12**: 237-48.

Tonukari, N. J., Scott-Craig, J. S. and Walton, J. D. (2003). Isolation of the carbon catabolite repressor (CREA) gene from the plant-pathogenic fungus Cochliobolus carbonum. *DNA Seq* **14**: 103-7.

Treitel, M. A. and Carlson, M. (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc Natl Acad Sci U S A* **92**: 3132-6.

Treitel, M. A., Kuchin, S. and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae. *Mol Cell Biol* **18**: 6273-80.

Tzamarias, D. and Struhl, K. (1994). Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. *Nature* **369**: 758-61.

Vincent, O. and Carlson, M. (1999). Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. *Embo J* **18**: 6672-81.

Weng, H. and Ayoubi, P. (2004). GPAP (GenePix Pro Auto-Processor) for online preprocessing, normalization and statistical analysis of primary microarray data. *In preparation for submission to BMC Bioinformatics*

Yang, X., Hubbard, E. J. and Carlson, M. (1992). A protein kinase substrate identified by the two-hybrid system. *Science* **257**: 680-2.

Yang, X., Jiang, R. and Carlson, M. (1994). A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. *Embo J* **13**: 5878-86.

Yelton, M. M., Hamer, J. E. and Timberlake, W. E. (1984). Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc Natl Acad Sci U S A* **81**: 1470-4.

CHAPTER 2

GENE ONTOLOGY FOR FILAMENTOUS FUNGI

PATRICIA AYOUBI, LAVANYA AMIRNENI, LEO HALL, SHOBANA KRISHNAN, SAUL LEITE, SUNITA MACWANA AND ROLF PRADE

INTRODUCTION

The large amounts of biological information, linked to proteomes by means of the amino acid sequence, produce a need for algorithms that automate the use of functional descriptors. A requirement for functional computation is the availability of fully controlled descriptive terms (Karp, 2000). Examples of basic computational tasks requiring functional terms are queries that use descriptions of a gene product, a cellular component, or a biological process to determine functional equality, predict new functions, reconstruct metabolic pathways, define gene expression profiles or recreate protein interaction maps. Functional ontologies provide descriptive terms for a nearly complete array of known biological processes, cellular components and molecular functions, including enzymatic reactions involving small- and macro-molecular substrates, signal-transduction processes, transport events and mechanisms of regulation of gene expression (Karp, 2000; Lewis et al., 2000; Hodges et al., 2002; Raychaudhuri et al., 2002).

Here we describe and review gene ontology for uni- and multi-cellular fungal genomes designed to deliver comprehensive and controlled descriptors for most known molecular functions, cellular components and biological processes. The ontology is based on term definitions developed for *Saccharomyces cerevisiae* (Yeast GO), *Schizosaccharomyces pombe*, *Candida albicans*, bacteria and other fully sequenced eukaryotes. BLASTp (Altschul et al., 1990) associations of homology (E=1x10-20) and word-by-word matching algorithms were used to establish direct relationships between NCBI - GenBank accession numbers (fungal subset) with GO terms or previously GO associated peptide records. Input of novel terms, or modifications of existing GO terms

describing functions, components or processes can be done via an open-source, curated process at http://www.geneontology.org.

Fungal gene ontology

Ontology terms linked to current fungal NCBI (National Center for Biotechnology Information) non-redundant records (f-$NCBI_{nr}$), and corresponding amino-acid sequences enable automated functional term assignment to new ORFs that might be similar or homologous. Controlled descriptors of gene function do not address all the uncertainties related to the complexity of some gene products, and do not identify novel functions. However, when controlled descriptors are applied to the annotation of ORFs with known function, they become useful for functional computation algorithms.

DNA sequence data describing whole genomes have generated ambiguity surrounding annotation of function. The major problem lies within uncontrolled text describing biological function that researchers have linked voluntarily to amino-acid sequences. The Gene Ontology (GO) project seeks to provide a set of structured vocabularies for specific biological domains that can be used to describe gene products in any organism (The Gene Ontology Consortium, 2001). GO includes all biological information in three extensive ontologies that describe molecular function, cellular component and biological process (The Gene Ontology Consortium, 2001). Currently, the majority of GO descriptions are based on the information derived from model organisms such as *Saccharomyces cerevisiae* (SGD), *Drosophila melanogaster* (FlyBase), and Mouse (MGD/GXD).

Fungal ontology overview

Figure 1 shows the distribution of f-NCBI$_{nr}$ deposited amino acid sequences according to species and the multiplicity of the deposited peptides. The f-NCBI$_{nr}$ peptide database, contains over 1,437 unique fungal species, representing 449 genera and 139 strains of unknown species, with 33.6% of all fungal peptide records belonging to *S. cerevisiae* and 22.5% to *S. pombe* (May 2002).

Table I summarizes the classification of f-NCBI$_{nr}$ records according to GO terms. From 75,949 records deposited in GenBank (May 2002) that describe fungal DNA amino acid sequences, 65,982 can be classified to a biological function, cellular component and/or biological process as described by previously annotated GO terms including *S. cerevisiae* and *S. pombe* records (Table I). Conversely, the 75,949 f-NCBI$_{nr}$ records represent 29,987 truly non-redundant amino acid sequences of which 22,850 are classifiable with GO terms. Thus, 7,137 peptides cannot be annotated functionally with a direct match to an existing GO term. We have inspected these 7,137 unclassified peptides and eliminated for GO entry assignments all peptides belonging to *S. cerevisiae* and *S. pombe* (~3,000), records with less than 50 amino acids, apparent nonsense peptides (e.g., xxxxx or "xxpxaxaqxvxglxpvxxeqx") and peptides annotated with unknown, hypothetical, unnamed or putative functions (~1,440). The remaining 2,697 peptides were manually matched to existing GO terms (~25%), novel GO terms or redefinition of existing GO terms (~30).

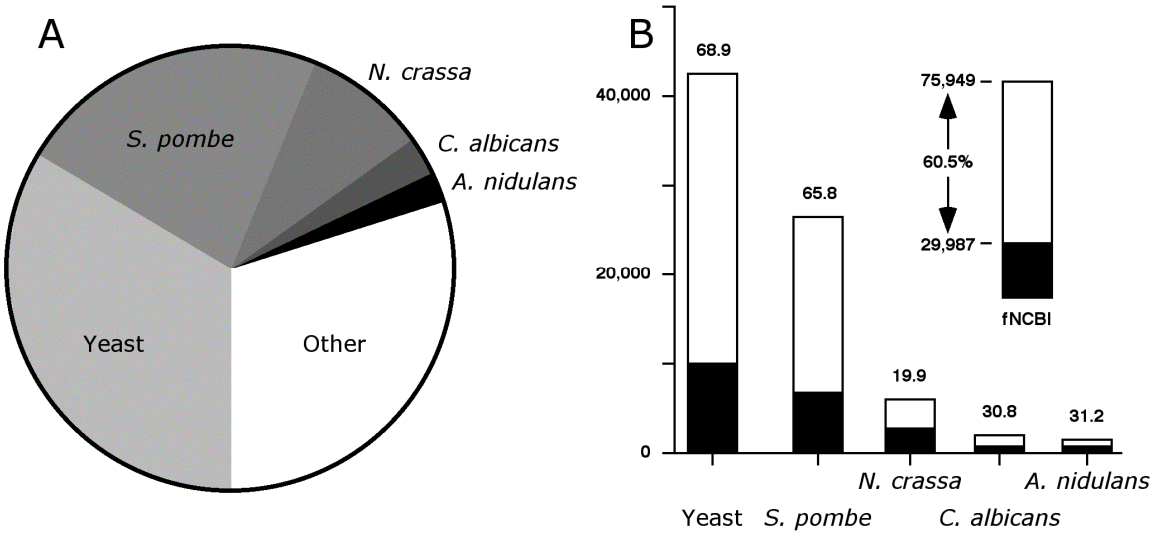

Figure 1 Distribution of fungal peptides in f-NCBI$_{nr}$. **A** Distribution of f-NCBI$_{nr}$ records according to major fungal species. **B** Redundancy of peptides present in f-NCBI$_{nr}$ records according to major fungal species. Closed bars indicate the number of unique peptide sequences present within the complete f-NCBI$_{nr}$ records of a fungal species (open bars).

	Records	%	
		Rec	Pep
All f-NCBInr records	75,949		
f-NCBInr classifiable records	65,982	86.9	
f-NCBInr unclassifiable records	9,967	13.1	
f-NCBInr unique peptides	29,987	39.5	
f-NCBInr classifiable peptides	22,850		76.2
f-NCBInr unclassifiable peptides	7,137		23.8

Table I Direct associations of GO terms with public f-NCBInr records.

Descriptive terms found in Gene Ontology

As described by the gene ontology consortium (The Gene Ontology Consortium, 2001) molecular functions include descriptive terms for enzymes and enzyme regulators, signal transducers, transcription, translation and its regulators, transporters, motors, protein stabilization, protein tagging, storage proteins, structural molecules, and other functions such as anti-toxin, anticoagulant, antifreeze, antioxidant; apoptosis regulator, cell adhesion, chaperone and chaperone regulators, surfactant and triplet codon-amino acid adaptor.

Cellular components include external protective structures such as cell (spore and hypha) wall (*sensu* Fungi), periplasmic space (*sensu* Fungi) and cellular fractions partitioned in insoluble, membrane and soluble fractions. The membrane system includes endoplasmic reticulum, Golgi, nucleus, vesicle, plastid, chloroplast, mitochondrion, lysosome, peroxysome, vacuole and flagellum (*sensu* Eukarya). The cellular fraction contains nucleus and nuclear components such as chromosomes, the cytoplasm and its components and unlocalized components.

Biological processes describe cell growth and maintenance, transport, metabolism, membrane, cytoplasm, nuclear and cell biogenesis, cell shape and size control, physiological processes (e.g., nutrition, thermoregulation, germination and pathogenesis), stress response, cell differentiation, communication, growth, cell-cycle, motility, chemo-mechanical coupling, membrane fusion, cell-cell fusion, sporulation (*sensu* Fungi), budding, fruiting body formation, mating, autophagy, epigenetic control of gene expression, aging and death.

New specific descriptors for filamentous fungi

Secondary metabolism

Secondary metabolites are non-essential compounds produced by filamentous fungi in a variety of chemical structures including β-lactam rings, cyclic peptides, unusual sugars, nucleosides, polyacetylenes, polyenes, and macrolide rings (Lampen, 1969; Kerridge, 1979). The main types of secondary biosynthetic pathways involve formation of polyketides, isoprenes, beta-lactam rings, mycotoxins e.g., aflatoxins, fumonisins and sterigmatocystin (Brown et al., 1999). Fumonisins and trichothecenes are chemically diverse; however they share with tricyclic sesquiterpenes, a 12,13 epoxy-trichothec-9-ene ring (Sweeney and Dobson, 1999). Penicillins and cephalosporins are beta-lactam containing antibiotics produced by numerous fungal genera (Diez et al., 1990; MacCabe et al., 1990). Isoprenoids are derived from mevalonate and examples are fungal plant hormones such as gibberellins, abscisic acid, cytokinines and trichothecenes (Homann et al., 1996). The structural genes encoding enzymes are part of a secondary metabolic pathway, often found in large gene clusters (Mathison et al., 1993; Martin and Gutierrez, 1995; Prieto et al., 1996; Keller and Hohn, 1997; Meyers et al., 1998; Sweeney and Dobson, 1999). Fungi are highly variable in whichever type of secondary metabolite they produce. Thus, even though we have added all currently known secondary metabolism functions, components and processes into gene ontology, a significant number of activities remain unknown and will need to be added in the future.

DEVELOPMENT

Filamentous fungi reproduce through sexual, asexual and parasexual developmental processes (Pontecorvo et al., 1953; Clutterbuck, 1974; Käfer, 1977; McGinnis et al., 1992). A large number of fungi develop exclusively through a meiotic based reproductive process (teleomorph) while others undergo asexual mitosis (anamorph) (Subramanian, 1965; de Hoog and Smith, 1984). Holomorphic fungi are able to reproduce both mitotically and meiotically (Parry and Parry, 1987; Raju and Perkins, 1994). Fungi may reproduce in a self-fertile (homothallic) or self-sterile (heterothallic) mode (Poggeler, 1999; Yun et al., 1999; Poggeler and Kuck, 2000; Yun et al., 2000; Poggeler and Kuck, 2001). Fungi can be bipolar and tetrapolar (incompatibility) which leads to out- and in-breeding (Bakkeren and Kronstad, 1993; Bakkeren and Kronstad, 1996; Hibbett and Donoghue, 2001). Asexual or vegetative reproduction involves various modes of spore production (sporulation) such as a sporangium, a pored cell, which extrudes a new spore, an annelid cell which constricts the formation of a new cell with a ring, simple segmentation of a cell or a complex multicellular spore producing structure the conidiophore. Only partial molecular information is currently available that describe these varied asexual reproductive processes and a limited understanding is only available for conidiation in *A. nidulans* (Clutterbuck, 1969; Timberlake and Clutterbuck, 1993; Adams et al., 1998) and N. crassa (Ebbole, 1998; Maheshwari, 1999; Yatzkan and Yarden, 1999). Independent of the mode by which spores are produced, they all germinate and produce a new vegetative tissue, termed the mycelium (Osherov and May, 2001). Thus, the ontology for developmental processes of sporulation, sexual or asexual is mostly incomplete and unlikely to be matched with other developmental systems

among plants and animals. Furthermore, fungi engage in parasexual developmental reproduction involving recombination in vegetative cells that form a diploid nucleus within a heterokaryotic haploid mycelium, multiply and haplodize into a recombined homokaryon (Pontecorvo et al., 1953; Clutterbuck, 1974).

pH regulation

Fungi frequently control the type of enzymes they secrete to the surrounding medium by sensing the extracellular pH. Gene regulation based on pH involves several genes such as *pacC, palA, B, C, F, H* and *I* (Denison, 2000). There are many examples of genes whose expression is dictated by ambient pH in fungi (Espeso and Arst, 2000). In every case examined in fungi where a regulatory system mediates gene expression by ambient pH a homolog of *A. nidulans pacC* has been identified (Caddick et al., 1986). In *S. cerevisiae* (Tilburn et al., 1995), *Y. lipolytica* (Lambert et al., 1997) and *C. albicans* (Ramon et al., 1999) homologs of *A. nidulans pal* genes have been identified as components of the signal transduction pathway regulating the activity of the *pacC* homolog in response to ambient pH. However, there are also differences among these pH regulatory pathways. There is no homolog in *S. cerevisiae* of *A. nidulans palC,* whereas there are *S. cerevisiae* homologs of *palA, B, F, H* and *I.* The homolog of *A. nidulans pacC, palB* and *palH* in *S. cerevisiae* have been located and recorded in gene ontology (Denison, 2000).

GO unnanotated f-NCBInr peptides	Recs.	%	
	1,276	###	
No homolog in f-NCBInr	256	20	
Homologous only to itself	402	32	
Same function non-homologous	618	48	100
Length, 11-50 residues	110		18
Mating type factors	26		
Uncertain fragments or proteins	84		
Length, 51-100 residues	60		10
Antifungal proteins	5		
Mating type factors	6		
Heterokaryon incompatibility	2		
Hydrophobins	4		
Uncertain fragments or proteins	43		
Length, 101-200 residues	231		37
Mating type factors	9		
Hydrophobins	3		
Heterokaryon incompatibility	69		
Unclear function - gene name only	150		
Length, >201residues	217		35
Mating type factors	19		
Heterokaryon incompatibility	12		
Unclear function - gene name only	186		

Table II Fungal peptides currently not annotated with GO terms.

SHARED FUNCTIONS NOT READILY IDENTIFIED BY AMINO ACID HOMOLOGY

Numerous molecular functions require species-specific annotation because the function is independent of the linear sequence of amino acids. Table II illustrates some of these classes: hydrophobins (Ebbole, 1997; Wessels, 1999; Scholtmeijer et al., 2001; Wosten, 2001), mating-type pheromones (Kothe, 1996; Nelson, 1996; Casselton and Olesnicky, 1998), and heterokaryon incompatibility factors (Glass et al., 2000; Saupe, 2000; Powell et al., 2001; Wu and Glass, 2001; Xiang et al., 2002). Fungal hydrophobins maintain conserved hydrophobic domains allowing a wide range of amino acid sequence options, and mating-type factors whose function in many cases are amino acid sequence-specific but non-conserved among species.

NEW DEFINITIONS AND UPDATING OF EXISTING DESCRIPTORS

Submission of new terms and revisions of GO annotated terms can be made directly on Gene Ontology or submitted to the accompanying Internet resource (http://fungal.geneontology.org). To add new descriptions or make changes to existing GO entries, go to the Gene Ontology curator request page at https://sourceforge.net/tracker/?atid=440764&group_id=36855&func=browse/.

Fungal Gene Annotator (FGA)

The Fungal Gene Annotator (FGA) is accessible via the Internet from http://aspergillus-genomics.org, and includes a tool allowing users to input a large collection of FASTA formatted ORFs or amino acid sequences and retrieve GO-based functional annotation(s) from sequences matching records in Fungal GO. Thus, ORFs of

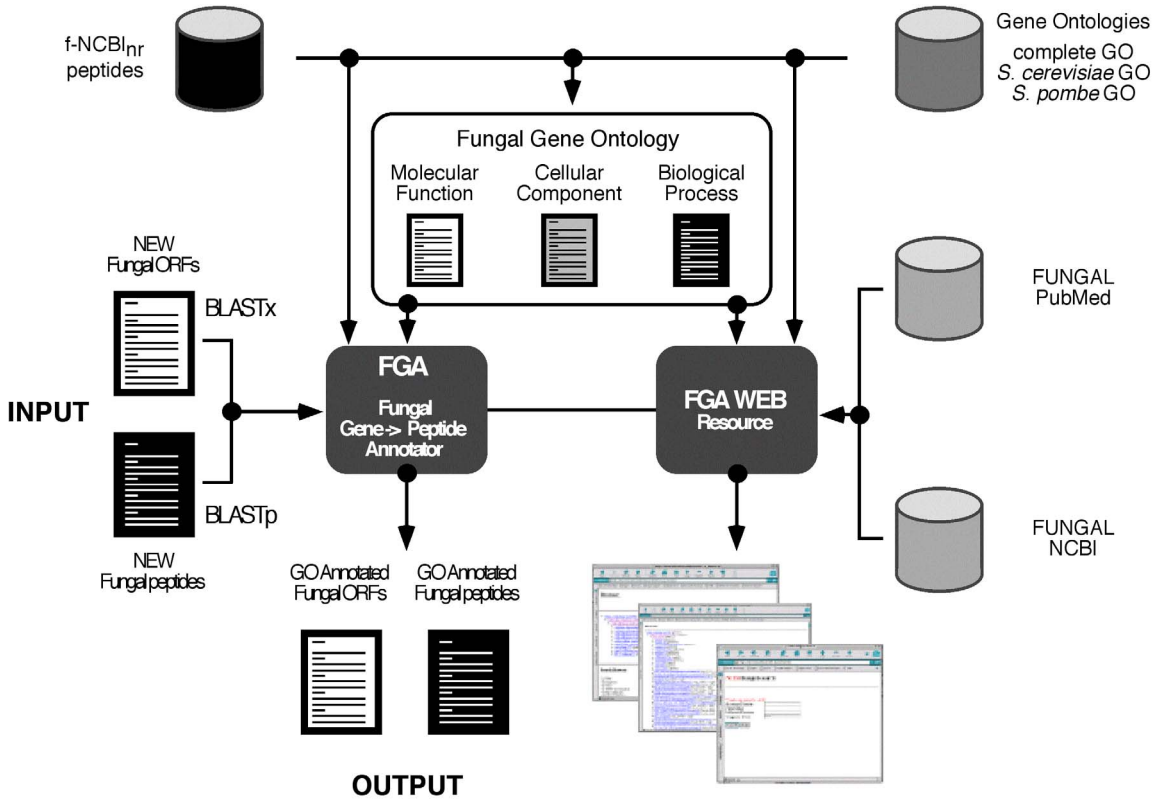

Figure 2 Schematic overview of the Fungal Gene Annotator. A MySQL database contains all the descriptive terms, curated and updated by the Gene Ontology project f-NCBI$_{nr}$ database contains all free-text annotated records deposited in NCBI, which report fungal DNA sequences including the fully sequenced genomes of *Candida albicans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Fungal Medline database contains all records reporting fungal genes deposited in NCBI.

an entire fungal genome can be rapidly annotated with functional terms, cellular components and/or biological processes. The resulting annotation can be downloaded and used for further computation and analysis. A summarized schematic overview of FGA is shown in Figure 2. Annotation of genomic DNA by FGA is achieved by a BLASTx or BLASTp (Altschul et al., 1990) search of the fungal GO database and the retrieval of the GO term(s) if preexisting matches have been established. With a valid GO term (or terms) and the species definition of the "input" ORF collection, a dynamic query retrieves reliable MEDLINE (PubMed) publications regarding each ORF. The advantage of this composite method is that any given ORF receives a controlled term (or set of terms) that describes a specific function, a cellular component or a biological process while retaining the useful information submitted in an uncontrolled form on the NCBI database. The accuracy of assigning a controlled term to a given ORF is determined by the stringency (E-value cutoff for the BLAST search) by which a given amino acid sequence similarity match is considered homologous. This decision is left to the user "annotator" who sets a threshold to validate homologous amino acid sequences.

A collection of unknown fungal cDNA-derived peptides from an *A. nidulans* EST's sequencing project (Macwana and Prade 2002, unpublished) were used to test the FGA. Table III shows that input of 1,429 non-redundant EST's into FGA generates 458 (32%) fungal-GO annotated peptides with an expectation value of 1E-20 and 645 (45%) with an E-value of 1E-5. Moreover, out of the 784 records not matching FGA peptides (expectation of 1E-5), 84% also failed to match peptides from the complete f-$NCBI_{nr}$ database (expectation value of 1E-5), suggesting that matches to the fungal GO term database reflects a majority of the annotation content deposited in the public domain. For

Database	number of ORFs			
	E	match	no match	%
FGA	1E-20	458	971	32
FGA	1E-10	567	862	40
FGA	1E-05	645	784	45
NCBInr	1E-05	758	671	53

FGA - Fungal Gene Annotator database
NCBInr - complete NCBI

E - expectation value as determined by BLASTx

Table III Automated annotation of 1,429 random *A.nidulans* generated ORFs with fungal GO terms.

each of the 645 FGA-annotated records, the corresponding GO terms were compared manually by visual inspection with descriptions from matching peptides from the complete $NCBI_{nr}$ database (BLASTx expectation value 1E-5) to reveal >82% of term/function correlation.

ACKNOWLEDGEMENTS

We wish to thank the National Science Foundation from which our work has received funding.

REFERENCES

Adams, T. H., Wieser, J. K. and Yu, J. H. (1998). Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol Rev 62: 35-54.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. J Mol Biol 215: 403-10.

Bakkeren, G. and Kronstad, J. W. (1993). Conservation of the b mating-type gene complex among bipolar and tetrapolar smut fungi. Plant Cell 5: 123-36.

Bakkeren, G. and Kronstad, J. W. (1996). The pheromone cell signaling components of the *Ustilago* a mating-type loci determine intercompatibility between species. Genetics 143: 1601-13.

Brown, M. P., Brown-Jenco, C. S. and Payne, G. A. (1999). Genetic and molecular analysis of aflatoxin biosynthesis. Fungal Genet Biol 26: 81-98.

Caddick, M. X., Brownlee, A. G. and Arst, H. N., Jr. (1986). Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol Gen Genet 203: 346-53.

Casselton, L. A. and Olesnicky, N. S. (1998). Molecular genetics of mating recognition in basidiomycete fungi. Microbiol Mol Biol Rev 62: 55-70.

Clutterbuck, A. J. (1969). A mutational analysis of conidial development in *Aspergillus nidulans.* Genetics 63: 317-327.

Clutterbuck, A. J. (1974). In *Aspergillus nidulans*. Plenum Publishing Corp., New York.

de Hoog, G. S. and Smith, M. T. (1984). Morphological taxonomy of little-differentiated Hyphomycetes. Antonie Van Leeuwenhoek 50: 807-13.

Denison, S. H. (2000). pH regulation of gene expression in fungi. Fungal Genet Biol 29: 61-71.

Diez, B., Gutierrez, S., Barredo, J. L., van Solingen, P., van der Voort, L. H. and Martin, J. F. (1990). The cluster of penicillin biosynthetic genes. Identification and characterization of the *pcbAB* gene encoding the alpha-aminoadipyl-cysteinyl-valine synthetase and linkage to the *pcbC* and *penDE* genes. J Biol Chem 265: 16358-65.

Ebbole, D. J. (1997). Hydrophobins and fungal infection of plants and animals. Trends Microbiol 5: 405-8.

Ebbole, D. J. (1998). Carbon catabolite repression of gene expression and conidiation in *Neurospora crassa*. Fungal Genet Biol 25: 15-21.

Espeso, E. A. and Arst, H. N., Jr. (2000). On the mechanism by which alkaline pH prevents expression of an acid-expressed gene. Mol Cell Biol 20: 3355-63.

Glass, N. L., Jacobson, D. J. and Shiu, P. K. (2000). The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. Annu Rev Genet 34: 165-186.

Hibbett, D. S. and Donoghue, M. J. (2001). Analysis of character correlations among wood decay mechanisms, mating systems, and substrate ranges in homobasidiomycetes. Syst Biol 50: 215-42.

Hodges, P. E., Carrico, P. M., Hogan, J. D., O'Neill, K. E., Owen, J. J., Mangan, M., Davis, B. P., Brooks, J. E. and Garrels, J. I. (2002). Annotating the human proteome: the Human Proteome Survey Database (HumanPSD) and an in-depth target database for G protein-coupled receptors (GPCR-PD) from Incyte Genomics. Nucleic Acids Res 30: 137-41.

Homann, V., Mende, K., Arntz, C., Ilardi, V., Macino, G., Morelli, G., Bose, G. and Tudzynski, B. (1996). The isoprenoid pathway: cloning and characterization of fungal FPPS genes. Curr Genet 30: 232-9.

Käfer, E. (1977). Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genetics 19: A1-A28.

Karp, P. D. (2000). An ontology for biological function based on molecular interactions. Bioinformatics 16: 269-85.

Keller, N. P. and Hohn, T. M. (1997). Metabolic Pathway Gene Clusters in Filamentous Fungi. Fungal Genet Biol 21: 17-29.

Kerridge, D. (1979). The polyene macrolide antibiotics. Postgrad Med J 55: 653-6.

Kothe, E. (1996). Tetrapolar fungal mating types: sexes by the thousands. FEMS Microbiol Rev 18: 65-87.

Lambert, M., Blanchin-Roland, S., Le Louedec, F., Lepingle, A. and Gaillardin, C. (1997). Genetic analysis of regulatory mutants affecting synthesis of extracellular proteinases in the yeast *Yarrowia lipolytica*: identification of a RIM101/pacC homolog. Mol Cell Biol 17: 3966-76.

Lampen, J. O. (1969). Amphotericin B and other polyenic antifungal antibiotics. Am J Clin Pathol 52: 138-46.

Lewis, S., Ashburner, M. and Reese, M. G. (2000). Annotating eukaryote genomes. Curr Opin Struct Biol 10: 349-54.

MacCabe, A. P., Riach, M. B., Unkles, S. E. and Kinghorn, J. R. (1990). The *Aspergillus nidulans npeA* locus consists of three contiguous genes required for penicillin biosynthesis. Embo J 9: 279-87.

Maheshwari, R. (1999). Microconidia of *Neurospora crassa*. Fungal Genet Biol 26: 1-18.

Martin, J. F. and Gutierrez, S. (1995). Genes for beta-lactam antibiotic biosynthesis. Antonie Van Leeuwenhoek 67: 181-200.

Mathison, L., Soliday, C., Stepan, T., Aldrich, T. and Rambosek, J. (1993). Cloning, characterization, and use in strain improvement of the *Cephalosporium acremonium* gene *cefG* encoding acetyl transferase. Curr Genet 23: 33-41.

McGinnis, M. R., Sigler, L., Bowman, B. H., Masuda, M. and Wang, C. J. (1992). Impact of conidiogenesis, teleomorph connections, pleomorphism and molecular genetics on evolving hyphomycete systematics. J Med Vet Mycol 30 Suppl 1: 261-9.

Meyers, D. M., Obrian, G., Du, W. L., Bhatnagar, D. and Payne, G. A. (1998). Characterization of *aflJ*, a gene required for conversion of pathway intermediates to aflatoxin. Appl Environ Microbiol 64: 3713-7.

Nelson, M. A. (1996). Mating systems in ascomycetes: a romp in the sac. Trends Genet 12: 69-74.

Osherov, N. and May, G. S. (2001). The molecular mechanisms of conidial germination. FEMS Microbiol Lett 199: 153-60.

Parry, J. M. and Parry, E. M. (1987). Comparisons of tests for aneuploidy. Mutat Res 181: 267-87.

Poggeler, S. (1999). Phylogenetic relationships between mating-type sequences from homothallic and heterothallic ascomycetes. Curr Genet 36: 222-31.

Poggeler, S. and Kuck, U. (2000). Comparative analysis of the mating-type loci from *Neurospora crassa* and *Sordaria macrospora*: identification of novel transcribed ORFs. Mol Gen Genet 263: 292-301.

Poggeler, S. and Kuck, U. (2001). Identification of transcriptionally expressed pheromone receptor genes in filamentous ascomycetes. Gene 280: 9-17.

Pontecorvo, G., Roper, J. A., Hemmons, L. M. and MacDonald, K. D. (1953). The genetics of *Aspergillus nidulans*. Adv Genetics 5: 141-238.

Powell, A. J., Jacobson, D. J. and Natvig, D. O. (2001). Allelic diversity at the het-c locus in *Neurospora tetrasperma* confirms outcrossing in nature and reveals an evolutionary dilemma for pseudohomothallic ascomycetes. J Mol Evol 52: 94-102.

Prieto, R., Yousibova, G. L. and Woloshuk, C. P. (1996). Identification of aflatoxin biosynthesis genes by genetic complementation in an *Aspergillus flavus* mutant lacking the aflatoxin gene cluster. Appl Environ Microbiol 62: 3567-71.

Raju, N. B. and Perkins, D. D. (1994). Diverse programs of ascus development in pseudohomothallic species of *Neurospora*, *Gelasinospora* and *Podospora*. Dev Genet 15: 104-18.

Ramon, A. M., Porta, A. and Fonzi, W. A. (1999). Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *PRR2*. J Bacteriol 181: 7524-30.

Raychaudhuri, S., Chang, J. T., Sutphin, P. D. and Altman, R. B. (2002). Associating genes with gene ontology codes using a maximum entropy analysis of biomedical literature. Genome Res 12: 203-14.

Saupe, S. J. (2000). Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. Microbiol Mol Biol Rev 64: 489-502.

Scholtmeijer, K., Wessels, J. G. and Wosten, H. A. (2001). Fungal hydrophobins in medical and technical applications. Appl Microbiol Biotechnol 56: 1-8.

Subramanian, C. V. (1965). Spore types in the classification of the Hyphomycetes. Mycopathol Mycol Appl 26: 373-84.

Sweeney, M. J. and Dobson, A. D. (1999). Molecular biology of mycotoxin biosynthesis. FEMS Microbiol Lett 175: 149-63.

The Gene Ontology Consortium (2001). Creating the gene ontology resource: design and implementation. Genome Res 11: 1425-33.

Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Penalva, M. A. and Arst, H. N., Jr. (1995). The *Aspergillus* PacC zinc finger transcription factor

mediates regulation of both acid- and alkaline-expressed genes by ambient pH. Embo J 14: 779-90.

Timberlake, W. E. and Clutterbuck, A. J. (1993). In Genetic regulation of conidiation. Chapman and Hall, London.

Wessels, J. G. (1999). Fungi in their own right. Fungal Genet Biol 27: 134-45.

Wosten, H. A. (2001). Hydrophobins: multipurpose proteins. Annu Rev Microbiol 55: 625-46.

Wu, J. and Glass, N. L. (2001). Identification of specificity determinants and generation of alleles with novel specificity at the het-c heterokaryon incompatibility locus of *Neurospora crassa*. Mol Cell Biol 21: 1045-57.

Xiang, Q., Rasmussen, C. and Glass, N. L. (2002). The ham-2 locus, encoding a putative transmembrane protein, is required for hyphal fusion in *Neurospora crassa*. Genetics 160: 169-80.

Yatzkan, E. and Yarden, O. (1999). The B regulatory subunit of protein phosphatase 2A is required for completion of macroconidiation and other developmental processes in *Neurospora crassa*. Mol Microbiol 31: 197-209.

Yun, S. H., Arie, T., Kaneko, I., Yoder, O. C. and Turgeon, B. G. (2000). Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. Fungal Genet Biol 31: 7-20.

Yun, S. H., Berbee, M. L., Yoder, O. C. and Turgeon, B. G. (1999). Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. Proc Natl Acad Sci U S A 96: 5592-7.

VITA

Lavanya Amirneni

Candidate for the Degree of

MASTER OF SCIENCE

Thesis: INACTIVATION OF *SNFA* AFFECTS CARBON CATABOLITE DEREPRESSION IN THE FILAMENTOUS FUNGUS *ASPERGILLUS NIDULANS*

Major Field: Microbiology and Cell and Molecular Biology

Biographical:

Personal Data: Born in Hyderabad, India, on December 1, 1974. Married to Vamsee K.Amirneni March 2000. Have twin babies, Spandana Amirneni & Siddharth Amirneni

Education: Received a Bachelor of Medicine; Bachelor of Surgery (M,B:B,S) degree from Guntur Medical College, Guntur, India, 2000. Completed the requirements for the Master of Science degree with a major in Microbiology, Cell and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in July, 2004.

Experience: Teaching Assistant, Oklahoma State University, Department of Microbiology and Molecular Genetics, August 2001 through August 2004.

Name: Lavanya Amirneni Date of Degree: July 2004

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: INACTIVATION OF *snfA1* AFFECTS CARBON CATABOLITE DEREPRESSION IN THE FILAMENTOUS FUNGUS *ASPERGILLUS NIDULANS*

Pages in Study: 65 Candidate for the Degree of Master of Science

Major Field: Microbiology, Cell & Molecular Biology

Glucose, the simplest and most abundant sugar in nature, is the preferred carbon source for many organisms. In the presence of glucose, genes that are essential to metabolize alternative carbon sources are repressed, a phenomenon known as carbon catabolite repression. In yeast, the Snf1p has been shown to play a central role in carbon catabolite repression and causes derepression of the genes involved in alternate carbon source utilization, by phosphorylating Mig1p. During this work, a SNF1 homolog, *snfA1*, has been cloned and characterized in the filamentous fungus *Aspergillus nidulans*. A *snfA1* deletion strain has been constructed and its phenotypic characteristics studied. The mutant grew poorly on complex carbon sources like pectin, xylan, cellulose and galacturonic acid, suggesting the role of *snfA1* in polysaccharide degradation. Allyl alcohol sensitivity test showed that *ΔsnfA1* strain was super resistant compared to the reference strain indicating that *snfA1* interacts with *creA* (global transcriptional repressor) during glucose derepression mechanism. *ΔsnfA1* strain also had differential expression of several genes encoding cell wall degrading enzymes, in comparison to the reference strain as evidenced by Northern blot analysis. Absence of pectinase enzyme activity in culture filtrates of *ΔsnfA1* strain grown on pectin, further supports our hypothesis that *snfA1* is involved in the regulation of the genes encoding cell-wall degrading enzymes. Further, in order to discover the other genes being regulated by *snfA1* apart from glucose-repressed genes, microarray technology has been used to compare the expression profiles of Δ*snfA1* and wild type strains grown on glucose and pectin. Our data shows that *snfA1* also regulates genes involved in other cellular processes like lipid synthesis, sterol metabolism, polysaccharide metabolism, stress and transcriptional and translational regulation. Taken together, these data suggest that *snfA1* of *Aspergillus nidulans* is involved in carbon catabolite derepression and its deletion prevents the fungus from properly metabolizing the alternate carbon sources in the absence of glucose.

Adviser's Approval: Dr. Rolf A. Prade