SUMMARY

Awareness of *Candida albicans*, the human fungal pathogen, has risen during recent years. Although infections by *C. albicans* can be relatively mild and superficial, systemic mycoses often occur in immunocompromised patients, or even as a consequence of long-term therapy with broad-spectrum antibiotics or of chemotherapy (reviewed by Odds, 1988). Effective antifungal agents which are free of side-effects are urgently needed. There are several predisposing factors for *C. albicans* to alter from a state of a relatively quiescent communalism, to an aggressive pathogenic lifecycle. *C. albicans* acts as an alert opportunist in the presence of these factors. Natural factors like infectious, idiopathic, congenital, and other debilitating diseases, or a digression from the natural physiological status inclusive of a hormonal variation can cause an impaired state of immune function which is a prerequisite for candidiasis. Dietary factors, like excess or deficiency of certain nutrients may alter the endogenous microbial flora; mechanical factors, like trauma or occlusive injury can alter the microenvironment; medical factors like drugs used to depress the immune activity after surgery, and medication, which alters the host defenses against specific infections are all causes for this predisposition towards candidiasis (Odds 1988). The unique feature possessed by the pathogenic strains of *Candida* in being able to utilize the aminosugar N-acetylglucosamine (GlcNAc) certainly demanded attention. We suspected a correlation between this feature and virulence of the organism, and we were successful in adding this trait to the already long list of virulent properties of *C. albicans*.

The metabolism of N-acetyl-D-glucosamine (GlcNAc) by *C. albicans* has attracted interest, since the strains which are associated with the disease candidiasis were able to grow on N-acetylglucosamine (GlcNAc) as the sole carbon source (Singh and Datta, 1979b). GlcNAc is also capable of inducing cellular morphogenesis of *C. a/bicans*. This fungus frequently causes infections in the gastrointestinal, respiratory, and genital tracts. The mucous membranes at the site of infection are rich in aminosugars. The organism has an efficient catabolic system for the uptake and subsequent catabolization of N-acetylglucosamine into fructose-6-phosphate, which is then fed into glycolysis. Investigations into the GlcNAc catabolic pathway had begun by the study on the induction and regulation of N-acetylglucosamine kinase in *C. albicans* (Bhattacharya *et al.*, 1974), N-acetylglucosamine-6-phosphate deacetylase (Rai and Datta, 1982). GlcNAc is transported by a membrane bound permease and is sequentially metabolised by GlcNAc kinase, GlcNAc-6-phosphate deacetylase and GlcNAc-6-phosphate deaminase. GlcNAc is also an efficient inducer of cellular morphogenesis. However, there is evidence that
the germ tube formation, induced by GlcNAc, and N-acetylglucosamine metabolism may be mutually exclusive events. The purification of the GlcNAc-6-phosphate deaminase, the terminal enzyme of the GlcNAc catabolic pathway, and cloning of its cDNA, \textit{NAG1} (Natarajan and Datta, 1993), has thrown more light on GlcNAc catabolism in \textit{Candida albicans}.

**DISRUPTION OF N-ACETYLGLUCOSAMINE CATABOLIC PATHWAY IN \textit{CANDIDA ALBICANS} AND CHARACTERIZATION OF THE MUTANTS**

**Disruption of \textit{NAG1} in \textit{C. albicans}**

The clone pED4 was used for creating the deletion insertion mutants of \textit{nag1} gene. pED4 is the 3.915 Kb \textit{Sal I} fragment from \textit{C. albicans} SC5314, cloned into pBSKS (Kumar et al., 2000), housing the glucosamine-6-phosphate deaminase (\textit{NAG1}) gene. Sequence analysis revealed that it contains the 747 bp \textit{NAG1} ORF, with a 1.725 Kb upstream region, and a 1.442 Kb downstream region. The \textit{NAG1} ORF is without introns, and encodes a protein of 248 amino acids. pED4 was used for the construction of disruption cassette. Plasmid pCUB6 housing the \textit{hisG-URA3-hisG} sequence was obtained from Dr. W. Fonzi (Fonzi and Irwin, 1993). The strategy adopted for disruption of \textit{NAG1} was based on the gene replacement theory, in which, a cloned DNA fragment introduced by transformation is substituted by homologous recombination for a related region in the recipient genome (Orr-Weaver et al., 1981). The replacement event can even be monitored, by including an appropriate selection marker on the incoming molecule (Scherer and Davis, 1979). The \textit{hisG-URA3-hisG} construct was developed to make repeated use of a convenient selectable marker, in which the \textit{URA3} gene of \textit{C. albicans} is flanked by direct repeats of the bacterial \textit{Salmonella typhimurium} sequence \textit{hisG} (Alani et al., 1987). Such a construct if introduced into a cloned gene, and integrated into the yeast genome, would undergo frequent \textit{cis} recombination between the \textit{hisG} repeats to eliminate the \textit{URA3} gene, and leave behind a single copy of the \textit{hisG} sequence at the site of integration. This excision would lead to a Ura minus phenotype, and derivatives that have undergone such events could be directly selected with 5-Fluoro-orotic acid (5-FOA) (Boeke et al., 1984). The 5-FOA negative selection procedure is very efficient and selective, and under appropriate conditions one in several hundred 5-FOA resistant cells would be Ura positive. Both alleles of \textit{NAG1} were disrupted and checked by Southern.
Inability of the Null Mutant to Utilize N-acetylglucosamine and Glucosamine for Growth

Since \textit{NAG1} is involved in metabolism of the aminosugars N-acetylglucosamine and glucosamine, the mutants obtained should not be able to utilize these aminosugars. To confirm this, the mutants were first checked for utilization of the aminosugar N-acetylglucosamine. SC5314 (wild-type), N-2 (heterozygous mutant) and N-2-1-6 (null mutant), were checked for growth on YNB plates supplemented with 2% GlcNAc. It was observed that the null mutant N-2-1-6 could not utilize GlcNAc. The \textit{NAG1/nag1} heterozygous mutant N-2, showed growth on GlcNAc, with a slightly reduced rate. The wild-type strain SC5314, did not display any difference in rate of utilization of glucose (YNB with 2% glucose), and GlcNAc. N-2-1-6 also showed growth on SD (YNB-2% glucose) plate at a rate similar to the wild-type.

The mutants N-2 and N-2-1-6 were also checked for growth on YNB-2% glucosamine, since this aminosugar on phosphorylation is the substrate for deaminase \textit{NAG1}. The homozygous mutant N-2-1-6 was impaired in utilization GleN, and the single allele mutant N-2 displayed a slow growth. Overall, a slower rate of utilization of GleN by \textit{C. albicans} was observed, as compared to the rate of utilization of GlcNAc by this organism. The reason for slow growth on GleN plates could be because of the low affinity of the GlcNAc transporter for GleN.

Identification of Genomic Cluster Containing GlcNAc Catabolic Pathway Genes

\textit{Deacetylase (DAC1)}, \textit{Deaminase (NAG1)} and \textit{Hexokinase (HXK1)}

To ensure that any physiological change of a mutant is because of deletion of a particular gene, the respective gene has to be introduced back into the null mutant and check for restoration of altered function(s). So, a single copy of \textit{NAG1} was introduced at the \textit{NAG1} locus in the null mutant N-2-1-6-1, to create a revertant strain. To select for the transformants the \textit{hisG-URA3-hisG} cassette was inserted into pED4. The strain with one copy of \textit{NAG1} was expected to behave like the heterozygous mutant N-2, and grow on GlcNAc, but the strain failed to restore growth on YNB-GlcNAc plates. In fact, all the Ura positive transformants failed to grow on GlcNAc, even after screening a number of transformants from multiple transformation. The complete failure of \textit{NAG1} to restore function came as a total surprise. Southern was performed to ensure integration at the right position. When Southern showed integration at the right position, there was only one possibility: that some gene adjacent to \textit{NAG1} was disturbed while constructing the cassette for reintroduction of \textit{NAG1}, and, this gene was indispensable for
GlcNAc catabolism. Homology search with the region 3' to the NAG1 open reading frame, showed homology to hexokinase of Kluyveromyces, mouse and other species in the region spanning +1287 to +2130 with respect to NAG1 translation start site, in the reverse direction. Clustal W analysis of the hexokinase revealed its homology with other hexokinases. The regions responsible for sugar binding were identified, but the ATP-binding domain and the kinase-domain could not be identified. This suggests that the gene may be partially represented in the clone.

The region spanning -1402 to -243, with respect to the NAG1 translation start site +1, in a reverse orientation frame, showed strong homology to GlcNAc-6-phosphate deacetylase gene from Caenorhabditis elegans, Haemophilus influenzae, and many other species, on homology search. Clustal W analysis of the translated sequence with other GlcNAc-6-phosphate deacetylase sequences showed a good similarity. Possible start site of the DAC1 coding region has been located at -243 with respect to the NAG1 ATG. The promoter regulating NAG1 gene is a bidirectional one, also responsible for regulation of DAC1. While creating NAG1 mutant, DAC1 was also functionally impaired. Though clusters of functionally related genes are less prevalent in eukaryotes, it has often been reported that, genes for dispensable metabolic pathways in fungi are organized in clusters (reviewed by Keller and Hohn, 1997).

**Functional Defect was Restored with the Sal I Fragment of pED4**

As pED4 contained only the partial hexokinase, it was not possible to create a revertant by placing the 3.915 Kb Sal I fragment on a plasmid. So, a different approach was taken in which the Ura positive homozygous mutant N-2-1-6 was transformed with the 3.91 Kb Sal I fragment of pED4, and the transformants were selected for growth on YNB-GlcNAc plates. Integration in the right orientation was checked by Southern, as it was important for restoration of function, though the fact that it could grow on GlcNAc and restore the functional defect, was also confirmatory. The revertant strain was named P-4. Growth of P-4 on GlcNAc and GlcN confirmed that the growth defect of the null mutant on GlcNAc and GlcN, was solely because of disruption of the GlcNAc catabolic pathway genes.

**Null Mutant Impaired in Germ Tube Formation on Induction with GlcNAc**

GlcNAc is known to induce cellular morphogenesis in *C. albicans*. The yeast cells start producing germ tubes on induction with GlcNAc at 37°C (Shepherd *et al.*, 1980). Since the null mutant of the GlcNAc catabolic pathway was impaired in utilization of the aminosugar, we
wanted to check if the mutants still retained the ability to undergo dimorphic transition with GlcNAc as the inducer. The results showed that there was a complete block in production of germ tubes in the homozygous mutant N-2-1-6, while the heterozygous mutant N-2 and the revertant strain P-4 formed germ tubes as efficiently as the wild-type strain SC5314. Since it is not necessary for GlcNAc to be transported inside the cells to induce germ tube formation (Shepherd and Sullivan, 1983), it is probable that disruption of this pathway might have disturbed the receptor(s) on the cell surface responsible for reception and transmission of signal. So, it can be concluded that, a functional catabolic pathway is essential for GlcNAc-induced germ tube formation in *C. albicans*.

**EFFECT OF DISRUPTION OF N-ACETYLGLUCOSAMINE CATABOLIC PATHWAY ON VIRULENCE, GROWTH, ADHERENCE, CELL WALL STRUCTURE, ENZYME PRODUCTION, AND MORPHOGENESIS OF *CANDIDA ALBICANS* **

The Null Mutant of GlcNAc Catabolic Pathway is Avirulent

The lethal dose of $10^7$ could kill all the mice in just three days in case of wild-type *Candida albicans*. The heterozygous mutant and the revertant strain also showed comparable virulence, in which all mice got eradicated in a period of three to four days. But, the homozygous null mutant showed a prolonged period of survival with such high dose of inoculum. Statistical analysis was performed to confirm the significance of the results obtained. For sublethal dose of $10^6$ cells, mice infected with the null mutant N-2-1-6, continued to show healthy animals. The first death was recorded on day twenty-eight, showing a drop in the survival to 87.5%. No further death was noted until day sixty-seven when it dropped to 75%. Observation was kept until day seventy-five, and no additional death was recorded. The doses of the null mutant cells of GlcNAc catabolic pathway used in the experiments, and the length of period in which mice infected were followed up, both strongly indicate complete avirulence of the null mutant. The GlcNAc catabolic pathway therefore is important for the virulence of *C. albicans*.

Splenomegaly was observed in all animals infected with the wild-type and revertant strains. In case of infection with null mutant, some mice showed splenomegaly, but not as severe as in other cases. The fungal burden in the wild-type and revertant strains was very high after 24 hours, in all the three vital organs tested, evidenced from the high cfu recovered. The homozygous mutant N-2-1-6, in contrast, showed very low fungal burden in kidneys on the first day. Colonization of the null mutant progressed gradually in the kidney, at least in the initial
few days. From liver, the mutant was observed to clear fast, showing that on the third day, the organism could be recovered from only 25% of the samples. Also in case of spleen, the mutant showed a steady decrease in fungal load with time. The load in case of homozygous mutant was also significantly low, compared to the wild-type and revertant strains. The organisms could still be recovered from kidneys of one-third of the sample of mice after a period of 75 days, but showed total clearance from liver and spleen. The histology sections stained with periodic acid-Schiff, revealed huge focal collections of \textit{Candida} hyphal and pseudohyphal cells, with severe host inflammatory reactions on the first day (24 hours), in the wild-type and revertant strains. In contrast, the null mutant showed a very small area of infection, though the ability to form hyphae remained unaltered. The hematoxylin-eosin stained sections, after three days of infection (72 hours), showed massive infiltrates of neutrophils in wild-type and revertant strains. Infiltration of neutrophils could not be detected in case of null mutant. Whether the paucity of inflammatory cells was due to the low number of organisms present in the tissue, or a diminished host response elicited by this homozygous mutant, could not be ascertained.

The inability of \textit{C. albicans} with a mutated GlcNAc catabolic pathway to colonize the vital organs could be because of a number of reasons. It could be because of retarded growth of the GlcNAc null mutant as compared to the wild-type. Another reason could be because it is unable to form germ tubes. Germ tubes are known to adhere and invade tissues better than the yeast form. In systemic infection, vascular endothelium is reported to play a critical role, since blood-borne \textit{Candida} species likely adhere to and penetrate through the endothelial cell lining of the blood vessels, to gain access to the tissue parenchyma. It was speculated that the initial low count of fungus recovered from the kidneys infected with the homozygous mutant could be because of lowered ability of the null mutant to adhere to endothelial cells \textit{in vivo}, resulting in rapid clearance from the blood.

\textbf{No Effect on Growth of the Null Mutant}

Generation times (time required for one doubling) were calculated, by growing the wild-type and mutant derivatives in YEPD and serum, to check if they were significantly different from one another, as that would also account for avirulence of the null mutant. Growth was studied in serum to provide the physiological conditions encountered by the strains \textit{in vivo}. There was almost no difference in generation time among the wild-type and mutants in any of the cases.
Reduced Adherence to HBEC *in vitro*

The very low fungal count in kidneys infected with the null mutant was speculated to be because of reduced ability of the mutant to adhere *in vivo*. We examined the ability of wild-type *C. albicans* SC5314, the null mutant N-2-1-6, and the revertant strain P-4, to adhere to human buccal epithelial cells (HBEC) *in vitro*, using a visual assay of stained cell preparation. The percentage of adherence of SC5314, N-2-1-6, and P-4 was 70.55% ± 3.55%, 58.00% ± 1.00%, and 66.00% ± 2.00% respectively (the results are expressed as the mean of two independent experiments ± the standard error). In addition to this observation, the number of *C. albicans* that adhered per BEC was also noted which was 1.87 ± 0.13 in SC5314, 1.07 ± 0.01 in N-2-1-6, and 1.93 ± 0.12 in P-4 (results are expressed as the mean of three independent experiments ± the standard error). The null mutant N-2-1-6, displayed considerably reduced ability to adhere, compared to the wild-type or revertant strains, which could explain, at least partly, for the loss of virulence. It is suggested that disruption of this pathway may have resulted in block in production of some adhesins, or since GlcNAc is an integral component of cell wall, the disruption has resulted in an altered cell wall structure, or a modified cell-surface, any of which could affect adherence, though adherence could be one of the many factors responsible for the loss of virulence.

Defects in Cell Walls

Among the putative virulence factors, the cell wall of *C. albicans* is one of the most important. To explore the relationship between cell wall metabolism and GlcNAc catabolic pathway, the wild-type strain SC5314, heterozygous mutant N-2, homozygous mutant N-2-1-6, and the revertant strain P-4, were checked for growth defect on YEPD plates supplemented with Congo red and calcofluor white. It was observed that only the wild-type strain was capable of growing on all four different concentrations of Congo red. Much lower concentrations of calcofluor white could support the growth of wild-type strain, which did not attain a comparable growth rate to that of control plate even at 25 μg/ml concentration, showing that the organism is more sensitive to calcofluor white, compared to its sensitivity to Congo red. The mutants N-2, N-2-1-6, and P-4 were highly susceptible to both the dyes known to interact with the fungal cell wall. The mutants also displayed an increased susceptibility to the antifungal agent nikkomycin Z as studied in the disc assay and microdilution assay results. At a concentration of 0.5 μM nikkomycin Z, the wild-type showed 90.72% growth of control conditions (without nikkomycin Z).
Z), N-2 showed 58.15% growth, N-2-1-6 showed 49.23% growth, and P-4 showed 55.75% growth. Such increased susceptibility of the mutant derivatives to nikkomycin Z could be because of altered permeability to certain compounds. Alterations in cell permeability is possible, though there is a strong indication towards defects in cell wall structure, as effects were obtained with dyes Congo red and calcofluor white, which show affinity for external cell wall polymers. Though the mutant derivatives were highly susceptible to nikkomycin Z, and this compound is a competitive inhibitor of chitin synthase, and GlcNAc is polymerized into chitin, chitin synthesis was not, at least significantly, impaired in our case, as the heterozygous mutant, null mutant, and revertant strains, could all grow at a rate comparable to that of wild-type, in presence of glucose pathways. However, the loss of virulence of the null mutant of GlcNAc catabolic pathway was not because of cell wall defect, as the heterozygous mutant N-2, and the revertant strain P-4, though tested positive for cell wall defects, retained the virulent phenotype. The effect on cell wall, to some extent, can account for the altered colony morphology and hyperfilamentous phenotype obtained with the GlcNAc null mutants.

**No Effect on Secretion of β-N-Acetylglucosaminidase**

Production of β-N-acetylglucosaminidase by *C. albicans* is induced by GlcNAc, and the enzyme is secreted into the culture medium during either yeast or mycelial growth in presence of GlcNAc (Sullivan et al., 1984). In *C. albicans*, among the different hydrolytic enzymes as determinants of virulence, β-N-acetylglucosaminidase encoded by *HEXJ* (Cannon et al., 1994), is reportedly one of them, as a mutant deficient in β-N-acetylglucosaminidase, ATCC 10261, was less pathogenic than the parental strain in a mouse infection model (Jenkins and Shepherd, 1987). Production of β-N-acetylglucosaminidase by the wild-type and mutant strains was assayed, and our results showed no effect on secretion of this enzyme in GlcNAc defective mutants. So, inactivation of the GlcNAc catabolic pathway did not inhibit induction of β-N-acetylglucosaminidase. This suggests independent mechanism of induction of GlcNAc-regulated genes and GlcNAc-induced germ tube formation.

**In vitro Germ Tube Induction in Serum**

Serum is still the magic potion to rapidly induce true hyphae in *C. albicans*, though the factor responsible for induction is yet to be identified. We wanted to check if the GlcNAc catabolic pathway has any contribution towards germ tube formation in serum. In YEPD medium supplemented with 20% calf bovine serum, induction of germ tubes was studied at 37°C, after 2
hours of induction. Both wild-type SC5314 and the homozygous mutant N-2-1-6, could form filaments, consisting of elongated cells with constrictions which looked like pseudohyphae. The heterozygous mutant N-2 and the revertant strain P-4, however failed to show any filament formation. The null mutant was not impaired in filamentation in serum both in vivo (demonstrated in the histopathological section of kidney) and in vitro, suggesting that GlcNAc signaling pathway was probably not involved in induction of filamentation in serum. Serum, moreover, triggers multiple signaling pathways in Candida, any one of which is capable of inducing yeast to hyphal conversion (Madhani and Fink, 1998b). Recently it has been shown that the RAS1 gene plays a key role in regulating the serum response in C. albicans (Feng et al., 1999).

**Morphogenesis on Solid Plates**

Since dimorphism is a long suspected mechanism of virulence (Corner and Magee, 1997; Kobayashi and Cutler, 1998), we investigated the effect of disruption of the GlcNAc catabolic pathway on the morphology of C. albicans, on established media like SLAD (Gimeno et al., 1992) and Spider (Liu et al., 1994). After induction of filamentous growth on SLAD plates for 10 days at 37°C, the homozygous mutant N-2-1-6 showed highly extensive filamentation compared to the wild-type. The heterozygous mutant N-2, and the revertant strain P-4, behaved in a similar fashion and formed restricted amount of filaments under the colonies with very few showing on the periphery.

On Spider medium observations were recorded after 7 days of incubation at 37°C. N-2 and P-4 were almost completely impaired in filamentation, and showed central smooth shiny colony surface with faint concentric rings. The homozygous mutant N-2-1-6, showed a dramatic change in colony phenotype, with extensive filamentation and highly increased fringe area, as compared to the wild-type. The colony surface showed an irregular-wrinkled morphology with an elevated mound of entangled hyphae, and the surface was not shiny either as in other cases.

Hyphal formation is a complex mechanism, in which both positive and negative signals play a role. It is quite possible that the GlcNAc signaling pathway is involved in derepression of a repressor, of some pathway that leads to filamentous growth, by interacting with components of the mating-hyphal MAP kinase pathway. It could also be a result of activation of repressors RBf1 (Ishii et al., 1997), or TUPl (Braun and Johnson, 1997), whose mutants generate excessive filamentation. Alternatively, this pathway could also interact with Efg1p, an essential regulator of hyphal formation in most inducing conditions (Stoldt et al., 1997; Lo et al., 1997). The signaling pathway comprising Efg1p is not yet known, but Efg1p has been proposed to act
downstream of the Ras-cAMP-Tpk2p pathway (Sonneborn et al., 2000). Given our current knowledge of different components involved in filamentation, we were tempted to find the targets of the GlcNAc signaling pathway, for which a detailed study was performed by creating combination mutants with different genes involved in filamentation. This part of the study has been discussed in the next section.

STUDY OF ROLE OF N-ACETYLGLUCOSAMINE INDUCIBLE (CATABOLIC) PATHWAY IN MORPHOGENESIS OF CANDIDA ALBICANS IN COORDINATION WITH OTHER SIGNALING PATHWAYS INVOLVED IN MORPHOGENESIS

Since the GlcNAc mutants gave rise to extensive filament formation on solid Spider and SLAD plates, we were interested to find the signaling pathway involved in this dimorphic transition, for which we created combination mutants with the MAP kinase pathway transcription factor gene ACPR, the cAMP-dependent kinase gene TPK2, and the morphogenetic regulator gene EFG1. The experiments delineate the relative roles of each gene in filamentous growth and also suggest the existence of an additional pathway of filamentous growth induction. GlcNAc catabolic pathway was disrupted in acpr, tpk2, and efg1 mutants of C. albicans using Ura-blaster technique. All the mutants created were studied for effect on morphogenesis. The strains checked were: SC5314 (DACINAGIHXK1/DACINAGIHXK1), N-2 (DACINAGIHXK1/daclnagIhxk1), N-2-1-6 (daclnagIhxk1/daclnagIhxk1), P-4 (daclnagIhxk1/daclnagIhxk1 + DACINAGIHXK1), N-2-1 + P31 (daclnagIhxk1/daclnagIhxk1), N-2-1-6-1 + P33 (daclnagIhxk1/DACINAGIhxk1), A-11-1-1 (acpr/acpr), AN-8-1-16 (acpr/acpr daclnagIhxk1/daclnagIhxk1), AS1 (tpk2/tpk2), AS1-3-1-8 (tpk2/tpk2 daclnagIhxk1/daclnagIhxk1, overexpressing TPK2), AS1-3-1-8-4 + pRC2312-PH (tpk2/tpk2 daclnagIhxk1/daclnagIhxk1, overexpressing EFG1), HLC67 (efgl/efgl), HLC67-16-1-9 (efgl/efgl daclnagIhxk1/daclnagIhxk1, overexpressing EFG1), HLC67-16-1-9-3 + pB1 (efgl/efgl daclnagIhxk1/daclnagIhxk1, overexpressing TPK2), and HLC67-16-1-9-3 + pRC2312-PH (efgl/efgl daclnagIhxk1/daclnagIhxk1, overexpressing EFG1).

Induction with 2.5 mM GlcNAc

Study of the mutants showed that the MAP Kinase pathway is not involved during GlcNAc induced germtube formation in Candida albicans and Efg1p is responsible for formation of germ tubes under GlcNAc induced conditions.
Induction with 20% Serum

Study of the mutants showed that the role of MAP kinase pathway, with serum as the inducer, appeared to be of little importance in filamentation. The pathway governed by Tpk2p and Efglp, plays an important role, as the single mutants of tpk2 and efgl were highly impaired in filamentation. But disruption of the GlcNAc inducible pathway in tpk2 and efgl background resulted in increased filamentation, indicating the involvement of a second pathway, under serum induced germ tube formation.

Induction in Liquid Spider Medium

The MAP kinase pathway clearly plays an important role in filamentation in Spider’s and signaling seems to be transmitted through both the MAP kinase pathway, and the cAMP-dependent EFG1-TPK2 pathway.

Morphogenesis on Serum Plates

ACPR does not seem to have a role in filamentation on serum plates. There was basically not much difference between single mutant of GlcNAc inducible pathway, single mutant of acpr, and the double mutant of both. The pathways therefore function separately in serum. Majority of the hyphae formed on serum plates is because of Efglp. Disruption of the GlcNAc inducible pathway in efgl background, showed highly elongated and branched hyphae, which indicates that EFG1, though important, is not indispensable in hyphae formation. The involvement of some other pathway is indicated.

Morphogenesis on SLAD plates

Under nitrogen-starvation conditions on SLAD plates we checked the mutants of the newly discovered GlcNAc kinase, to see if it has a role in morphogenesis. We observed that the dramatic effect on morphogenesis was because of disruption of hxl. The MAP kinase pathway however acted separately from the GlcNAc inducible pathway under such conditions. In the tpk2 mutant, when GlcNAc inducible pathway was deleted (AS1-3-1-8), it resulted in a dramatic increase in filamentation, producing highly branched thin hyaline hyphae which showed the largest fringe area, compared to other mutants. The additive effect on filamentation
pattern was a bit perplexing, as Tpk2p is known to play a positive role in filamentation. A possible explanation is the presence of the other PKA isoform, TPK1, identified in the genome of *C. albicans*, which resulted in hyperfilamentation. The *efg1* mutant HLC67 showed very less filamentation which slightly increased on disruption of the GlcNAc inducible pathway in HLC67-16-1-9, showing a fuzzy periphery. Overexpression of TPK2 and EFG1 further enhanced the filamentation to some extent, indicating that Efg1p is required under nitrogen starvation conditions.

**Morphogenesis on Spider Plates**

On Spider medium, apart from hyperfilamentation, a novel feature of change in colony-surface phenotype was noted in the mutants. The effect on morphology observed on disruption of GlcNAc inducible catabolic pathway, is because of the hexokinase, presumably the GlcNAc kinase and clearly showed that the MAP kinase pathway and the pathway regulating filamentation via GlcNAc kinase, function separately. The strain AS1-3-1-8, though formed extensive filaments like N-2-1-6, failed to show its typical colony surface morphology. A mound of distorted ripples was all it could form. On overexpression of TPK2, the ripples got partially restored, and a full restoration was noted on overexpression of EFG1. The strain HLC67-16-1-9, revealed no change in morphology, on deletion of the GlcNAc inducible pathway in *efg1* background. On overexpression of EFG1, some segregants showed filamentation, but the colony surface became wrinkled once again though the ripples were not exactly like the ones observed in N-2-1-6. But, it definitely indicates that, Efg1p plays a regulatory role in determining the colony surface phenotype.

**Conclusions Derived from the Study of Morphogenesis**

The results suggest that EFG1 represents the major activating pathway that forms filaments in a GlcNAc kinase mutant, on solid media like SLAD and Spider. As none of the combination mutants with different pathway components studied, showed a total filamentation compromised state, it indicates the presence of an additional pathway in filament formation under inducible conditions. Though the hyperfilamentation appeared to be a derepression event initially, and the repressor looked like TUP1 on many occasions, showing a ACPR/CPH1-independent and EFG1-dependent nature, on closer inspection TUP1 appears not the repressor regulated by the GlcNAc inducible pathway. If this is a repressor-mediated response, Efg1p has to interact with the repressor, which leads to hyperfilamentation, since in absence of Efg1p, most of the
hyperfilamentation disappears. Filamentation in absence of Efg1p is also noted, which could be because the derepressed repressor is also capable of interacting with some other component, which might belong to the unknown signaling pathway. The MAP kinase pathway plays no role in the regulation of filamentation in coordination with the GlcNAc inducible pathway. TUP1 pathway can either be totally independent, or could be involved in some cross talk with this pathway.

Based on our results, it is suggested that deletion of the GlcNAc kinase might have triggered an elevation of the intracellular cAMP level, resulting in stimulated filamentous growth, a theory that appears more probable. The induction of filamentous growth by elevated cAMP, could be the result of an activated Ras protein, a RAS gene required for morphogenesis has recently been identified in C. albicans (Feng et al., 1999). GlcNAc kinase therefore plays a dual role in metabolism of GlcNAc, and in morphogenesis of C. albicans. We propose that the kinase probably acts as a sensor molecule transmitting the signal via the cAMP regulated pathway, influencing filamentation. Recent studies in plants have shed light on a conserved sugar signaling pathway that uses hexokinase as a sugar sensor (reviewed by Jang and Sheen, 1997), and this case could be a similar one.

This also shows that, genes turned on during filamentous growth, do not respond to a central regulator, rather they respond individually to various pathways that regulate filamentous growth, suggesting strongly that a network of signaling pathways and transcriptional regulators extends down to target genes without an intervening central level of regulation.