CHAPTER 3

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

3.1. INTRODUCTION

Candida albicans is the most pathogenic of the medically important species of Candida. The most common infection in man is superficial candidiasis, characterized by infections limited to the lining surfaces such as skin, oropharynx, gastrointestinal tract, and upper and lower respiratory tracts. In this form, vascular invasion or deep organ involvement is not evident. C. albicans does not normally cause disease in immunocompetent hosts. It causes cutaneous, mucocutaneous, and life-threatening systemic disease in immunocompromised hosts such as leukemic, organ-transplanted, diabetic, and human immunodeficiency virus (HIV)-infected patients. The most frequently encountered invasive localized lesions of candidiasis are ulcerations of the intestinal, respiratory, or genitourinary tract. But, the most serious variety is systemic candidiasis, recognizable by intraparenchymal lesions usually involving the vital organs like heart, kidneys, liver, spleen, lungs, and brain. However, any organ can be infected in this process (Odds, 1979; Wey et al., 1989).

The fungus obviously possesses features that function as virulence attributes, though the total effect of these attributes seems to be insufficient to establish the fungus as a pathogen of the entirely uncompromised host. Among the commensals, C. albicans behaves as an alert opportunist, and is frequently able to initiate morbidity when given even the minimal chances. The interactions between fungus and host are in fact too complex, involving many molecular mechanisms of aggression and defense for any host debilitation to predispose inevitably to candida infection. The fact that the organism is diploid, has hindered the genetic study of potential virulence factors of this increasingly common cause of serious infections.

Recent advances in molecular genetic techniques have considerably enhanced the ability to identify and analyze factors that may contribute to the pathogenicity of C. albicans (Fonzi and Irwin, 1993). Though many cellular properties are proposed to contribute to the virulence of this organism, it has been possible to relate only a few factors directly to decreased virulence in vivo by using disruption of specific genes, or isolation of variants deficient in particular enzymes. Such putative pathogenicity factors include secreted aspartyl proteinases (Kwon-Chung et al., 1985; Macdonald and Odds, 1983; Schaller et al., 1998), chitin synthase III (Bulawa et al., 1998).
catalase (Wysong et al., 1998), phospholipase (reviewed by Ghannoum, 1998), pH of the growth environment (Saporito-Irwin et al., 1995; Muhlschlegel and Fonzi, 1997), and many more discussed in chapter 1. Among the major factors associated with virulence are adherence to host cells (Calderone, 1993a, b), and the transition from yeast to hyphal growth (reviewed by Soll, 1992).

Blastoconidia are usually found as members of the normal flora in healthy individuals. However, during infection, blastoconidia, pseudohyphae, and hyphae are frequently present in lesions. Strains of *C. albicans* that produce abundant hyphae are reported to be more pathogenic than those that grow predominantly as yeasts (Saltarelli et al., 1975), though the yeast form has also been shown to be more (Ianni et al., 1977), or equally (Shepherd, 1985), pathogenic.

Dimorphism is induced *in vitro* by many environmental conditions, including temperature, pH, starvation, presence of inducers like GlcNAc, proline, serum, and many yet unidentified stimulants. Genes involved in the dimorphic transition form yeast to hyphal phase have also been implicated to play a vital role in the virulence of *C. albicans*.

Based upon functional complementation, homologues of the MAP Kinase pathway similar to the pheromone response pathway of *Saccharomyces cerevisiae*, have been isolated from *C. albicans*. Mutants of *C. albicans* containing mutations in each of these genes are unable to undergo transition from yeast to hyphae on solid media, except when serum is included. Other key regulators involved in the morphogenesis process like the EFG1 (a PHD1 homolog) (Lo et al., 1997; Stoldt et al., 1997), CaTUP1 (a TUP1 homolog) (Braun and Johnson, 1997), and CaRSR1 (an RSR1 homolog) (Yaar et al., 1997) have also been isolated. The virulence of each of the single mutants was reduced in a murine model of hematogenously disseminated candidiasis (Csank et al., 1997, 1998; Leberer et al., 1996, 1997; Lo et al., 1997; Yaar et al., 1997). The function of *HWP1* has also been investigated, a gene that is expressed by hyphae and not blastospores of *C. albicans* (Sharkey et al., 1999). Deletion of both alleles of *HWP1* results in a conditional defect in hyphal formation *in vitro* and the *hwp1* mutants had markedly reduced virulence (Lo et al., 1997). The *C. albicans INT1* gene resembles *HWP1* in that, its product appears to be a surface protein that plays a role in hyphal formation (Gale et al., 1998). Like *hwp1* mutant, the *int1* mutant was less virulent in the murine model of systemic candidiasis. The *HOG1* gene of *C. albicans*, in addition to its role in cytokinesis and response to osmostress, also plays a role in morphogenesis and virulence of the organism (Alonso-Monge et al., 1999). In this regard, the two-component sensor histidine kinase *CHK1* of *C. albicans* (Calera et al., 1998), seems to regulate the expression of hyphal surface components (Calera and Calderone, 1999a) and perhaps also virulence factors, since a *chk1* null mutant is avirulent (Calera and Calderone, 1999b; Calera et al., 1999), while the *NIK1/COS1* gene (Nagahashi et
al., 1998; Srikantha et al., 1998) is required for hyphal development on solid media (Alex et al., 1998). The SSK1 homolog in C. albicans, CaSSK1, is functionally not related to S. cerevisiae SSK1, as it fails to complement the lack of SSK1 in S. cerevisiae, and was shown to be involved in morphogenesis and virulence of C. albicans (Calera et al., 2000).

The pathogenic strains of Candida possess a unique characteristic of utilization of the aminosugar N-acetylglucosamine (GlcNAc), as a carbon and nitrogen source for energy (Singh and Datta, 1979b), and mucin-rich areas of the body and most glycoconjugates (glycoproteins and glycolipids) of mammalian cell surfaces, contain aminosugars, like GlcNAc. It however looked improbable that this pathway was developed in pathogenic species in course of evolution to solely utilize aminosugars as a growth substrate during invasion and colonization, since glucose is available practically everywhere in the host system. Recently it has been reported that the gna1 null mutants of C. albicans were unable to grow on glucose, but were viable in presence of GlcNAc (Mio et al., 2000). The minimum concentration required was 10 μM, as the mutants failed to grow at a concentration less than that. It was also shown that 20% calf serum failed to suppress the growth defect, and the mutants were avirulent in a systemic mouse model of candidiasis, indicating that GlcNAc available in vivo is less than 10 μM.

GlcNAc is also polymerized into chitin, an integral component of the cell wall. GlcNAc can also act as a signaling molecule in triggering morphological transition in C. albicans. It has been hypothesized that the capability of GlcNAc to trigger hyphal development is somehow related to an activation of the synthesis of chitin (Braun and Calderone, 1978), the content of which markedly increases during germ tube formation (Chiew et al., 1980). In addition, chitin synthase was activated five-fold during the development (Chiew et al., 1980).

We suspected a possible correlation between GlcNAc utilization and virulence of C. albicans. The high degree of virulence possessed by this organism could be because GlcNAc acts as a signaling molecule and sets off a chain of reactions that confer particular virulence attributes to this pathogen. Before checking the prospective routes this pathway could be involved in, the first step was to study any effect of mutation of the GlcNAc catabolic pathway on virulence in a murine model of systemic candidiasis.
3.2. MATERIALS AND METHODS

3.2.1. Strains

*Candida albicans*

SC5314 (wild-type)
N-2 (heterozygous mutant)
N-2-1-6 (homozygous null mutant)
P-4 (revertant strain)

3.2.2. Media and Solutions

**YED-Chloramphenicol Plates**

Yeast Extract 1 %
Dextrose 2 %
Select Agar 2 %

Autoclaved at 15 lb/sq. inch pressure for 15 minutes. Allowed to cool down to 45°C-50°C.

Chloramphenicol added 5 µg/ml

**Chloramphenicol Stock Solution**

Chloramphenicol stock was made by dissolving chloramphenicol at a concentration of 34 mg/ml in sterile MQ-water. Stored at -20°C.

**Phosphate Buffer Saline (PBS)**

NaCl 8 g
KCl 0.2 g
Na₂HPO₄ 1.44 g
KH₂PO₄ 0.24 g

Dissolved in 800 ml MQ-water, and pH was adjusted to 7.4 with HCl. Water was added to make the volume 1 liter. Autoclaved at 15 lb/sq. inch pressure for 20 minutes, and stored at room temperature.

**Phosphate Buffer (0.02 M, pH 7.2)**

NaCl 0.02 M
Sodium phosphate 0.15 M

3.2.3. Culture of Strains

The strains SC5314, N-2, N-2-1-6 and P-4 were grown in rectangular patches on YEPD plates, at 30°C for 48 hours.

3.2.4. Preparation of Doses

1. The cells were resuspended in PBS and washed twice with PBS.
2. Dilutions were made and cells were counted with the help of a Haemocytometer. Cells/ml were calculated using the formula:

\[
\text{Cells/ml} = \text{Average count per square} \times \text{dilution factor} \times 10^4
\]

3. An aliquot of the dilutions of each strain was plated on YEPD plates and grown overnight at 30°C.

4. Colonies were counted with a manual Counter and was considered the final number to estimate the volume of cells required for preparation of different doses of injections.

5. A lethal dose of \(10^7\) CFU (viable cells), per 200 \(\mu\)l PBS, was made and a diluted aliquot of the same was plated on YEPD, grown at 30°C overnight, and checked for CFU again, for confirmation. A sublethal dose of \(10^6\) CFU per 200 \(\mu\)l PBS was made similarly, and checked on plates.

3.2.5. Murine Model

Female BALB/c mice (inbred) of 8-10 weeks old and weighing 18-20 g was obtained from CDRI (Central Drug Research Institute), Lucknow, India. The animals were kept in JNU Animal House. Normal feed and tap water \textit{ad libitum} was given. Animals were treated as per guided rules followed in India.

3.2.6. Intravenous Injection of Mice to Monitor Survival

1. Mouse was trapped in a restraining device, and the tail veins were heated with a wet cotton pad to a temperature of 50°C-55°C for 1 minute prior to injection.

2. The lateral tail vein was injected with the prepared doses of \(10^7\) and \(10^6\) CFU in 200 \(\mu\)l PBS, of the wild-type SC5314, heterozygous mutant N-2, null mutant N-2-1-6, and the revertant strain P-4. 1 ml insulin syringes (needle size 29) were used for injection.

3. The number of samples of mice taken was eight (n=8), per dose per strain.

4. Groups were made based on dosage and strains, and were kept in separate cages. Observations were recorded once in 24 hours for morbidity.

3.2.7. Intravenous Injection for Recovery of CFU from Vital Organs of Mice

1. Mouse was trapped in a restraining device as before, and the tail veins were heated with wet cotton pad to a temperature of 50°C-55°C for 1 minute prior to injection.
3. The dehydration was continued by incubating three times in 70% ethanol, 20 minutes each time at room temperature.

4. Incubated in 95% ethanol for 20 minutes at room temperature. Repeated twice.

5. Incubated in 100% ethanol for 20 minutes at room temperature. Repeated two more times.

6. 100% ethanol was replaced with xylene. Xylene was changed immediately with fresh xylene and incubated for 10 minutes. Two more xylene changes were given with 10 minutes of incubation after each change.

7. Xylene was poured off and 5 ml fresh xylene was added to each vial. An equal volume of molten wax was added using a hot glass pipet. The wax was transferred very quickly to avoid hardening.

8. It was mixed, and the samples were left at room temperature overnight. The wax/xylene mixture will harden, but enough paraffin was dissolved in xylene to start impregnation of the samples.

9. The samples were transferred to 60°C oven to melt the wax/xylene mixture.

10. A 60°C heating block was prepared and when the wax/xylene mixture was molten the vials were transferred to heating block.

11. The wax/xylene mixture was carefully poured out from one vial into a waste bottle not to lose the sample. Immediately fresh molten wax was added to the vial with hot glass pipet. The vial was put back into the heat block. The procedure was repeated with all vials.

12. The vials were incubated for 1 hour at 60°C oven.

13. The vials were again placed into 60°C heating block and steps 11 and 12 were repeated two more times for a total of 3 hour incubation.

3.2.9.3. Embedding Samples in Wax and Preparation of Blocks

1. Embedding molds (VWR Scientific) were made according to the manufacturer’s instructions, and one mold was filled with molten wax using hot glass pipet.

2. A sample was immediately transferred to the wax-filled mold using hot forceps, samples were also oriented within the mold using forceps.

3. An embedding ring was placed on the mold and filled with paraffin wax. The ring was labelled to facilitate future identification of samples.

4. The cast blocks were left at room temperature to harden completely.

5. The cast blocks were removed from embedding molds and stored in a dry place at room temperature.
3.2.9.4. Sectioning

1. The wax block was cut into a trapezoidal shape using a razor blade. The extra wax was carefully shaved off taking precaution not to cut too closely to the embedded sample.
2. The trapezoid block was attached to the holding clamp of a microtome and sections were made of 6 μm thickness.
3. A drop of 0.2X gelatin subbing solution was put on a gelatin-subbed glass slide. A ribbon of sections was transferred onto the drop of the subbed slide using fine brushes.
4. The slide was transferred onto a heating plate set between 45°C and 50°C.
5. After stretching was complete, the slide was removed from the plate and the remaining subbing solution was carefully removed using a Pasteur pipet.
6. All slides were prepared likewise, and dried at room temperature. Then the slides were incubated at 42°C for a day for firm attachment.

3.2.9.6. Staining with Hematoxylin-Eosin (HE)

1. Slides were stained for 20 to 30 seconds in hematoxylin.
2. Rinsed twice in water, 2 minutes each.
3. The slides were quickly dipped into 0.1% ammonium hydroxide and then rinsed with water.
4. Washed 5 minutes in water and then stained with eosin for 20 to 30 seconds.
5. The slides were dipped in ascending alcohol solutions of 50%, 70%, 80%, 95% twice, and then dipped twice in 100% ethanol. Fully dehydrated in 100% ethanol for 2 minutes.
6. Equilibrated thrice, 2 minutes each, in xylene.
7. Sections were mounted in DPX mountant.

3.2.9.7. Staining with Periodic Acid Schiff (PAS)

1. Sections were stained in Periodic acid taken in a staining dish for 10 minutes. Sections were rinsed carefully with several exchanges of deionized water.
2. Schiff reagent was added and stained for 5 minutes.
3. Carefully washed with three exchanges of deionized water.
4. Dehydrated in ascending alcohol solutions of 50%, 70%, 80%, 95% twice, and 100% twice.
5. Equilibrated thrice, 2 minutes each, in xylene.
6. Sections were mounted in DPX mountant.
3.2.10. Growth in YEPD at 30°C

1. An overnight culture of SC5314, N-2, N-2-1-6 and P-4 were grown in 30°C with shaking at 200 rpm.
2. The cells were diluted 1:100 into fresh YEPD medium, and \( A_{600} \) at zero time point were taken for all the samples.
3. The cells were grown at 30°C with shaking at 200 rpm and density of each culture was determined every hour (\( A_{600} \)).
4. Generation time (\( t_0 \)) was calculated using the formula:
\[
\ln \frac{2B_0}{B_1} = \alpha t, \quad \text{where } B_0 \text{ is the initial } A_{600} \text{ at zero hour, } B_1 \text{ is the final } A_{600} \text{ at } t \text{ time point and } \alpha \text{ being the instantaneous growth rate constant for that culture.}
\]

3.2.11. Growth in 2X YEPD at 37°C

The method followed was adopted from Lo et al., 1997.

1. An overnight culture of SC5314, N-2, N-2-1-6 and P-4 were grown in 37°C with shaking at 200 rpm.
2. The cells were diluted 1:500 into fresh 2X YEPD medium and \( A_{600} \) at zero time point were taken for all the samples.
3. The cells were grown at 37°C with shaking at 200 rpm and density of each culture was determined every hour (\( A_{600} \)).
4. Generation time (\( t_0 \)) was calculated using the formula:
\[
\ln \frac{2B_0}{B_1} = \alpha t, \quad \text{where } B_0 \text{ is the initial } A_{600} \text{ at zero hour, } B_1 \text{ is the final } A_{600} \text{ at } t \text{ time point and } \alpha \text{ being the instantaneous growth rate constant for that culture.}
\]

3.2.12. Growth in Serum at 37°C Measured by Consumption of Glucose in vitro

1. Cells of SC5314, N-2, N-2-1-6 and P-4 were patched in rectangular patches on YEPD plates and grown at 30°C for 48 hours.
2. The cells were suspended in PBS and washed twice with PBS. The final resuspension was also in PBS.
3. Cells were diluted and counted with the help of a Haemocytometer and an aliquot of the diluted cells were plated on YEPD plates to determine the CFU.
4. Cells were added in 10 ml calf serum bovine (SIGMA, cat. # C-6278) at a concentration of \( 10^7 \text{ CFU/ ml} \).
5. The cells were grown at 37°C with shaking at 200 rpm.

6. Every 2 hours 200 μl of each culture was taken and spun at 13,000 rpm for 5 minutes. 20 μl of each was taken for estimation of the quantity of glucose remaining in the serum (O.D. test). 20 μl of serum was estimated for the original amount of glucose present, which served as the control value. 20 μl of standard glucose provided in the kit was also estimated (O.D. std.). The glucose test kit used was from Span Diagnostics Ltd., India. O.D. of the working glucose reagent was taken as the blank value (O.D. blank). The O.D. values taken at 510 nm was converted into the amount of glucose in mg/100 ml at each time point for each culture using the formula: 

\[
\text{O.D. test} - \text{O.D. blank} \times 100
\]

O.D. std. – O.D. blank

3.2.13. Study of Effect on Adherence

The method followed was adopted from Bailey et al., 1995.

3.2.13.1. Growth of C. albicans Strains and Counting of Cells

1. The strains SC5314, N-2-1-6 and P-4 were patched on YEPD plates and grown at 30°C for 48 hours. The cells were then resuspended in 0.02 M PBS (pH 7.2).

2. The cells were diluted gradually and counted with the help of a Haemocytometer and the number of cells per ml was calculated.

3.2.13.2. Collection of Human Buccal Epithelial Cells (HBEC)

1. The buccal epithelial cells from three healthy male and female volunteers (aged 28-32 years) were scraped with clean spatulas and suspended in 0.02 M PBS.

2. The cells were washed twice with 0.02 M PBS, spun at 3,000 rpm for 5 minutes.

3. The cells were finally resuspended in 3 ml PBS of the same concentration, and counted using a Haemocytometer.

3.2.13.3. Incubation of Yeast Cells with HBEC

1. C. albicans cells (10^7) were incubated with HBEC (10^5) in 0.02 M PBS for 1 hour at 37°C.

2. After incubation, the cells were passed through 12 μm filters from SPI® Supplies (Lot # 1050725), and washed once with PBS.

3. The contents of the filter were then transferred to microscope slides and stained with Crystal Violet stain for 3-4 minutes.

4. Cells were counted at × 200 magnification under a Microscope.
3.2.13.4. Determining Percent of Adherence

1. The percent of HBEC with *Candida* cells adhering to it was determined from ten fields.
2. The total number of *Candida* cells adhering to 100 HBEC were counted.

The entire experiment was repeated thrice. Statistical analysis (Standard Error of Mean) was done with the help of GraphPad Prism 2.01 software.


3.2.14.1. Growth of *C. albicans* Strains and Cell Count

1. The strains SC5314, N-2, N-2-1-6 and P-4 were patched on SD plates and grown at 30°C for 24 hours.
2. The cells were scraped and suspended in sterile water. A serial dilution of each strain was made and cells were counted using a Haemocytometer.
3. A stock suspension of each strain was made which contained $10^5$ cells in 10 μl, and subsequent dilutions ten folds contained $10^4$, $10^3$, and $10^2$ cells in 10μl.

3.2.14.2. Preparation of YEPD Plates Supplemented with Congo Red and Calcofluor White

1. YEPD agar plates were supplemented with sterile dye Congo red (stock dissolved in water at 1 mg/ml concentration) at four different concentrations of 100 μg/ml, 125 μg/ml, 150 μg/ml and 200 μg/ml. Similarly calcofluor white (stock dissolved in water at 1 mg/ml concentration) was added at concentrations of 25 μg/ml, 50 μg/ml, 75 μg/ml, 100 μg/ml, 125 μg/ml, 150 μg/ml, and 200 μg/ml. The plates were made in duplicates for incubation at 30°C and 37°C.
2. The plates were dried thoroughly.

3.2.14.3. Spotting of Cells

1. Cells of SC5314, N-2, N-2-1-6 and P-4 of concentrations $10^5$, $10^4$, $10^3$ and $10^2$ in 10 μl water were spotted on two YEPD plates without any dye supplement (control plates). Cells were also spotted on Congo red and calcofluor white plates.
2. The spots were dried completely and then incubated at 30°C and 37°C for 48 hours.
3.2.15. Study of Effect of Nikkomycin Z on the Mutants

3.2.15.1. Disc Assay Method

3.2.15.1.1. Preparation of Lawn of *C. albicans* Cells

The strains SC5314, N-2, N-2-1-6 and P-4 were streaked on SD plates and grown at 30°C for a day. One loopful of cells was resuspended in 1 ml water and 150 μl cells was plated on SD plates to create a lawn.

3.2.15.1.2. Preparation of Nikkomycin Z Dilutions and Placement of Discs

Nikkomycin Z was dissolved in water to obtain a stock concentration of 5 mg/ml. Gradual dilutions of 500 μg/ml, 250 μg/ml, 100 μg/ml, 50 μg/ml, 10 μg/ml, 5 μg/ml, 2.5 μg/ml, and 1 μg/ml were made. Sterile 3 mm Whatman® discs were soaked with 7 μl solution of each concentration and the eight discs of gradually decreasing concentrations were placed on the lawn of each strain. Incubated at 30°C and 37°C for 24 hours.

3.2.15.2. Microdilution Assay

3.2.15.2.1. Growth of *C. albicans* Strains and Cell Count

1. The strains SC5314, N-2, N-2-1-6 and P-4 were patched on SD plates and grown at 30°C overnight.
2. The cells were then scraped and suspended in sterile water. A serial dilution was made of each strain and cells were counted using a Haemocytometer.
3. A stock suspension of each strain was made which contained 2 x 10^5 cells/ml in 2X SD.

3.2.15.2.2. Nikkomycin Z Dilutions

Nikkomycin Z (Sigma) was dissolved in sterile water (the stock concentration was 10,000 μM). Serial dilutions of 2000 μM, 1500 μM, 1000 μM, 500 μM, 200 μM, 150 μM, 100 μM, 50 μM, 20 μM, 10 μM, 2 μM, 1 μM concentrations were made.

3.2.15.2.3. Growth in Microtiter Plates

1. 75 μl of each of the twelve nikkomycin Z concentration was put in the slots for each strain and to it 75 μl cells of the respective strains were added. The final concentration of nikkomycin Z was therefore in 1X SD as 1000 μM, 750 μM, 500 μM, 250 μM, 100 μM, 75 μM, 50 μM, 25
μM, 10 μM, 5 μM, 1 μM, 0.5 μM respectively. Control sets without nikkomycin Z were kept in triplicates for each strain.
2. Incubated at 30°C for 24 hours.
3. OD was measured at 600 nm.

3.2.16. Study of Effect on Secretion of β-N-Acetylglucosaminidase

1. The wild-type C. albicans SC5314, the heterozygous mutant N-2, the homozygous mutant N-2-1-6 and the revertant strain P-4 were grown on YEPD plates at 30°C, overnight.
2. Cells from the overnight culture of each strain were scraped and suspended in sterile water. Incubated at 30°C for 8 hours for starvation.
3. The cells were then streaked on SD agar plates (1% Dextrose) supplemented with 10 mM GlcNAc and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUAG) (Sigma) at a concentration of 0.15 mg/ml.
4. Incubated at 30°C for 48 hours.
5. Fluorescent halo was visualized by exposure to UV light (304 nm).
6. Photographs were taken with a Polaroid camera.

3.2.17. Induction of Germ Tubes with 20% Serum

Induction procedure was as described in section 2.2.19. Cells (OD₆₆₀ =0.5) were induced with 20% Calf Serum Bovine in YEPD. Calf Serum Bovine was obtained from Sigma (C-6278). Cells were induced at 37°C for 2 hours with 200 rpm shaking.

3.2.18. Morphogenesis on Solid Plates

3.2.18.1. Study of Hyphae Formation on SLAD Plates

This method was adopted from Gimeno et al., 1992.

3.2.18.1.1. Preparation of SLAD Plates

1. Bacto-Agar (DIFCO) was weighed out at 2 % concentration and poured into a graduated medium bottle.
2. Milli-Q water was added and agar was swirled to wash thoroughly with water and allowed to settle for sometime. Water was discarded by decantation without disturbing the agar. The residual water was discarded by pipetting out with a glass pipette.
3. Washing with water was repeated four times.
4. Water was then added to agar at a concentration of 2X.
5. Yeast Nitrogen Base (0.17%) (w/o amino acids and ammonium sulfate) and Dextrose (2%) were dissolved in a separate graduated medium bottle and then poured into the bottle containing washed agar to obtain a 1X SLAD medium.
6. Autoclaved at 15 lb/sq. inch pressure for 15 minutes. Ammonium sulfate was added to a final concentration of 50 μM, swirled to mix and plates were poured.

3.2.18.1.2. Induction

1. Cells were counted and plated at a concentration of 80-100 cells per plate as before.
2. Incubated at 30°C for 10 days.

3.2.18.2. Study of Hyphae Formation on Spider Plates

1. Cells were grown in 2 ml Spider medium at 30°C for 5 days to obtain a saturated culture.
2. Cells were counted using a Haemocytometer. The formula used to calculate the number of cells per ml was: average number of cells per square x dilution factor x 10^4.
3. An aliquot of the diluted cells was plated on YEPD plates to count the CFU.
4. Cells were then plated on Spider plates at a concentration of 80-100 cells per plate.
5. The plates were incubated at 37°C for 7 days.

3.2.19. Microscopy

Photographs of germ tubes were taken in Leica DMIL Inverted Microscope with an automated camera attached to the microscope. 40X objective was used to obtain a x400 magnification for photographing germ tubes. Histopathology sections were photographed with 5X, 10X and 40X objectives. Petriplates were scanned directly in a UMAX Scanner. Photographs of colonies on SLAD or Spider plates were taken in Leica MPS32 Stereomicroscope using 1X objective. Magnification was adjusted to 0.63, 1.6, 2, as mentioned on the photographic plates.
3.3. RESULTS AND DISCUSSION

3.3.1. The Null Mutant of GlcNAc Catabolic Pathway is Avirulent

3.3.1.1. Lethal dose of *Candida albicans* (10^7 CFU)

Much recent research on the pathogenesis of disseminated *C. albicans* infections has focused on genes encoding putative virulence factors. The criterion for virulence is usually measurement of survival times of intravenously infected mice (Calera *et al.*, 1999, 2000; Csank *et al.*, 1998; Alonso-Monge *et al.*, 1999; Yamada-Okabe *et al.*, 1999). In order to investigate whether GlcNAc catabolic pathway plays any role in conferring virulence to this organism, hematogenously disseminated candidiasis of the wild-type and mutant strains, was studied in murine model. A group of eight female BALB/c mice (n=8), aged between 8-10 weeks, was injected with SC5314 (wild-type), N-2 (heterozygous mutant), N-2-1-6 (homozygous null mutant), and P-4 (revertant strain), with two lethal doses of 10^7 and 10^6 cells, administered into the lateral tail vein of mice. The animals were monitored every 24 hours for morbidity.

In case of lethal dose of 10^7 (Fig.3.1.A.), 87.5% of the mice was noted surviving at the end of the first day in case of infection with the wild-type SC5314. On the second day, only 50% of the mice was alive. On the third day, all the animals were noted dead. No death was recorded for the same dose in case of the homozygous mutant strain N-2-1-6, for the first four days. On the fifth day 87.5% of the mice was noted alive, which dropped to 75% on the tenth day, 62.5% on the sixteenth day, 50% on the nineteenth day, 37.5% on the twenty-seventh day, 25% on the thirty-third day, and 12.5% on the forty-third day. The percentage of survival did not drop after that, observed until day seventy-five. For the heterozygous mutant N-2, no death of animals was recorded on the first day, though some were visibly very sick. On the second day, 50% of the mice survived, which was 25% on the third day, and all animals were dead on day four. In case of the revertant strain P-4, 37.5% of the mice died on the first day, leaving 62.5% surviving. On the second day, 50% of the mice survived, and on the third day all animals were noted dead.

The lethal dose of 10^7 killed all the mice in just three days in case of wild-type *C. 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, with such high dose of inoculum, the homozygous null mutant showed a prolonged period of survival. Statistical analysis was performed to confirm the significance of the results obtained. By using the GraphPad Prism 2.01 statistical program, survival data was analyzed to find a significant correlation. The two-tailed P values were 0.0058 for SC5314, 0.0173 for N-2, 0.0007 for N-2-1-6 and 0.0232 for P-4.
which were all statistically significant. An additional observation was that, the homozygous mutant did not result in sick animals immediately after infection, and the mice appeared quite healthy and showed no sickness initially. This was strikingly different from other groups like that of wild-type, heterozygous mutant, and revertant strains, all of which resulted in very sick animals within a few hours of injection.

3.3.1.2. Sublethal dose of *Candida albicans* (10^6CFU)

For sublethal dose of 10^6 cells (Fig.3.1.B.), mice infected with the wild-type SC5314 showed a drop in the percent of survival from 100% to 62.5% on the fourth day. On the sixth day, 37.5% of the mice survived, 25% on the seventh day, 12.5% on the eighth day, and all the animals were dead on day eleven. The null mutant, N-2-1-6, on the other hand continued to show healthy animals. The first death was recorded on day twenty-eight, showing a drop in 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 heterozygous mutant N-2, showed 87.5% survival on the eighth day, 75% on the eleventh day, 62.5% on the thirteenth day, 50% on the fourteenth day, 37.5% on the fifteenth day, and 25% on the seventeenth day. After that there was no further drop in the percent of survival, and 25% of the samples continued to survive even on day seventy-five. For the revertant strain P-4, 87.5% of the population survived on the tenth day, 75% on the eleventh day, 62.5% on the thirteenth day, 50% on the seventeenth day, 37.5% on the twentieth day, and 25% on day twenty-one. Like the heterozygous mutant 25% of the samples continued to survive as noted even on day seventy-five. Statistical analysis was performed as before. Two-tailed P values determined for SC5314 (0.0003), N-2 (0.0014), and P-4 (0.0001), were all statistically significant.

Among the many avirulent strains of *C. albicans* reported, it could be mentioned that the double mutant of *cphl efgI*, failed to kill mice with inocula of 10^6, or less, observed for a period of fifteen days, and with an inoculum of 10^7, half of the mice survived after fifteen days (Lo et al., 1997). Another avirulent strain, the mutant of two-component response regulator *sskl*, was reported to result in no death even after a period of three weeks with a dose of 10^6 (Calera et al., 2000). In the avirulent histidine kinase mutant *cahkl*, with a dose of 10^6, no death was reported even after twenty-two days (Calera et al., 1999). 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*. We would try to unravel in this study how this pathway affects virulence of the organism.
Fig. 3.1. Survival Curves Showing Prolonged Survival in Mice with Lethal Doses of Candida albicans Null Mutant Cells.

A. Mice injected with a dose of $10^7$ cells of C. albicans cells of SC5314 (wild-type), N-2 (heterozygous mutant), N-2-1-6 (homozygous mutant), and P-4 (revertant strain). The entire population of wild-type died in 3 days. In case of homozygous mutant 50% of the population survived even after 25 days.

B. Mice injected with a dose of $10^6$ cells of C. albicans cells of SC5314, N-2, N-2-1-6 and P-4. Homozygous mutant showed healthy population with 100% survival even after 25 days.
3.3.2. Clearance of the Homozygous Mutant from Vital Organs

*C. albicans* in systemic infection is known to infect the vital organs. In the first few hours after intravenous inoculation of *C. albicans*, the highest fungal counts are found in lungs and liver, with lower concentrations in the spleen, kidneys and other organs. When an organism penetrates an epithelial surface, it encounters phagocytic cells of the reticuloendothelial system, which could be the kidney mesangial phagocytes, or the liver Kupffer cells, or splenic macrophages, which engulfs and destroys the infectious agents. With time, kidney becomes the most conspicuously affected organ, and counts in the lungs, liver and spleen are reported to decline. The high predilection of *C. albicans* for kidney remains unexplained (Odds, 1988).

Mice were injected in groups of four, per strain, per time point. Because of constraint of animals, the study was performed with three groups, consisting of wild-type, mutant, and revertant strains of *C. albicans*. The fungus was recovered from liver, spleen and one kidney. The other kidney was preserved for histopathological sections. 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. As seen in Table 3.1., 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 values have been expressed in terms of $\log_{10}$. 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 (Table 3.1.). In case of liver and spleen, the difference between wild-type and mutant derivatives, was not so pronounced on the first day. Kidneys are considered to be the organ in which *C. albicans* preferentially colonizes, though the reason for doing so is not clear. 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 mice after a period of 75 days (Table 3.1.), but showed total clearance from liver and spleen.

A functional GlcNAc catabolic pathway is therefore essential for maintenance of infection in these anatomic sites. The inability of *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. It could also be because it is unable to form germ tubes. Germ tubes are believed to adhere and invade tissues better than the yeast form. In systemic infection, vascular endothelium is reported to play a
Indicates clearance from some or most animals

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>DAYS</th>
<th>CFU FROM VITAL ORGANS SHOWN AS $\log_{10}$ VALUES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KIDNEY</td>
</tr>
<tr>
<td>SC5314</td>
<td>1</td>
<td>$5.93 \pm 0.07$</td>
</tr>
<tr>
<td>(wild-type)</td>
<td>2</td>
<td>$6.10 \pm 0.16$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$6.46 \pm 0.06$</td>
</tr>
<tr>
<td>N-2-1-6</td>
<td>1</td>
<td>$3.71 \pm 0.13$</td>
</tr>
<tr>
<td>(null mutant)</td>
<td>2</td>
<td>$3.99 \pm 0.28$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$4.50 \pm 0.19$</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>$2.91 \pm 0.13^*$</td>
</tr>
<tr>
<td>P-4</td>
<td>1</td>
<td>$5.75 \pm 0.33$</td>
</tr>
<tr>
<td>(revertant)</td>
<td>2</td>
<td>$5.96 \pm 0.07$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$6.18 \pm 0.13$</td>
</tr>
</tbody>
</table>

* Indicates clearance from some or most animals

**Table 3.I. Clearing of Fungal Load from Vital Organs.**
The fungal burden was very high in the kidneys infected with wild-type and revertant strains on day 1. In contrast, the null mutant showed very low fungal burden in the kidneys. Colonization of the null mutant progressed gradually in the kidneys in the initial few days, but was extremely low in the kidneys of mice that survived for a prolonged period (75 days), and ~66% mice showed total clearance after such a long period. Colonization in liver and spleen was also low in the null mutant compared to the wild-type and revertant strains. Clearance from liver and spleen was noted in some mice on day 3 in case of infection with null mutant.
critical role, since blood-borne Candida species likely adhere to and penetrate through the endothelial cell lining of the blood vessels, to gain access to the tissue parenchyma. A potential method to increase host defense against the pathogen is blocking its escape from the intravascular compartment. Virulence among Candida species is closely correlated with the ability of the organism to adhere to cells in vitro (Calderone et al., 1984; Douglas, 1987; King et al., 1980; Rotrosen et al., 1986). 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 in vivo, resulting in rapid clearance from the blood.

### 3.3.3. Highly Reduced Foci of Infection and Inflammatory Reactions in Kidney

To find out more about the behavior of the GlcNAc mutants in vivo histopathological studies were conducted. The histology sections stained with periodic acid-Schiff, revealed huge focal collections of Candida hyphal and pseudohyphal cells, with severe host inflammatory reactions in case of wild-type and revertant strains on the first day (24 hours) (Fig.3.2.A and C). In contrast, the null mutant showed a very small area of infection, though the ability to form hyphae remained unaltered (Fig.3.2.B). After three days, no hyphae were seen in the heavily infested necrotic areas of host tissues in case of wild-type and revertant strains (Fig.3.3.A and C). This observation agrees with the theory that hyphae are formed only in the initial stages of infection. The kidneys in case of infection with wild-type and revertant strains were fully infested with Candida showing large necrotic areas, and damage of the entire cortical region of kidney (Fig.3.4.A and C.). Though the lower magnification plate of the null mutant appeared to be clear of infection (Fig.3.4.B), the null mutant showed an increased area of colonization after three days, as seen in Fig.3.3.B., which was also consistent with the gradual increase in CFU values (Table 3.1.). It could be mentioned in this context that, the disruption of mannosyl transferase gene CaMNTI in C. albicans, also led to a strong attenuation of virulence, and although 50% of the mice were alive after fourteen days, 80% of these animals had their kidneys fully colonized (Buurman et al., 1998). Similar findings have also been reported with avirulent Caches3 null mutants of C. albicans, where colonization of the kidneys seemed normal, but progress of the infection, and subsequent death of animals, were prevented for reasons unknown (Bulawa et al., 1995). The hematoxylin-eosin stained sections, after three days of infection (72 hours), showed massive infiltrates of neutrophils in wild-type and revertant strains (Fig.3.5.A. and C.). Leucocytes, particularly neutrophil polymorphs, and to a lesser extent macrophages, migrate out of the capillaries and into the surrounding tissue in response to inflammatory reaction, and once in the tissue, they migrate towards the site of infection by
Fig. 3.2. Histopathology Sections of Kidneys Showing Colonization of Wild-type, Null Mutant, and Revertant Strains of *Candida albicans* 24 Hours Post Infection. The wild-type and revertant strains showed huge focal collections of *Candida* hyphal and pseudohyphal cells, whereas the homozygous mutant showed a very small area colonized by *Candida*. Hyphal formation in N-2-1-6 was not affected. The sections were stained with PAS. Magnification ×400.
Fig. 3.3. Histopathology Sections of Kidneys Showing Colonization of Wild-type, Null Mutant, and Revertant Strains of *Candida albicans* 72 Hours Post Infection.

The wild-type and revertant strains showed huge areas of necrosis of the host tissues. Kidney infected with N-2-1-6 showed a small area of infection as seen in plate B. The sections were stained with PAS. Magnification ×400.
Fig. 3.4. Sections Showing Necrotic Lesions in the Host Kidneys Infected with Wild-type, Null Mutant, and Revertant *Candida albicans* 72 Hours Post Infection. Necrotic lesions spread throughout the kidneys can be seen in hosts infected with wild-type and revertant strains. Areas of infection and necrotic tissues were not detected in the host kidney infected with homozygous mutant (plate B). The sections were stained with PAS. Magnification ×100.
Fig. 3.5. Kidney Sections Showing Inflammatory Response in the Hosts Infected with Wild-type, Null Mutant, and Revertant *Candida albicans* 72 Hours Post Infection. The wild-type and revertant strains showed massive infiltrates of neutrophils (stained as dark blue spots in plates A and C), a response to inflammatory reactions. No such accumulation of neutrophils could be detected in hosts infected with the homozygous null mutant. The sections were stained with HE. Magnification \( \times50 \).
chemotactic movement. Infiltration of neutrophils could not be detected in case of null mutant (Fig.3.5.B). 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 very low initial count in the kidneys infected with homozygous mutant could be partly responsible for the loss of virulence of the pathogen. It was also speculated that a reduced capacity of the mutant to adhere to vascular endothelial lining in vivo could result in less number of Candida reaching the organs, and subsequently showed less colonization.

3.3.4. 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 in vivo. The wild-type strain SC5314, and the mutants N-2, N-2-1-6 and P-4, were grown in YEPD, and growth was monitored by reading the cell OD every hour, over a period of 9 hours, till growth was into the stationary phase. The generation time of each was calculated from the logarithmic phase of growth. Rate of growth in 1X YEPD at 30°C was studied because the strains are normally grown in this medium. There was almost no difference in generation time among the wild-type and mutants (Table 3.II.).

The doubling times of Candida strains were also determined in 2X YEPD at 37°C, which is close to the human body temperature. Strains grown in 2X YEPD at 37°C remain in the yeast form (Lo et al., 1997). The generation time of each was calculated as in the above case from the logarithmic phase of growth (Table 3.II.), and generation times of wild-type and mutant derivatives were highly similar. Growth of Candida cells in serum at 37°C results in filamentation, and it was therefore not possible to monitor growth rate by measuring the cell OD. But, it was important to know if the wild-type and the mutant derivatives had significant differences in growth rates in this environment. So, growth in serum was monitored in vitro by quantitating the consumption of glucose present in serum, over a period of time (Fig.3.6.), which served as the growth indicator (Garrigues et al., 1994). The wild-type showed slightly faster rate of glucose consumption, consuming the entire glucose in 9 hours, compared to the mutants N-2, N-2-1-6, and P-4. Glucose was consumed in 10 hours by these mutants, which was in fact a very negligible difference (Fig.3.6.). The slight difference in growth rate displayed by the wild-type and
Table 3.II. Generation Time of Wild-type and Mutant Derivatives in Hours.
From the table it could be seen that there was no significant difference in generation time of the wild-type SC5314, heterozygous mutant N-2, null mutant N-2-1-6, and revertant strain P-4, under the conditions tested.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>GENERATION TIME IN YEPD AT 30°C</th>
<th>GENERATION TIME IN 2X YEPD AT 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314 (Wild-type)</td>
<td>2.61 ± 0.028</td>
<td>1.82 ± 0.067</td>
</tr>
<tr>
<td>N-2 (Heterozygous Mutant)</td>
<td>2.58 ± 0.014</td>
<td>1.79 ± 0.042</td>
</tr>
<tr>
<td>N-2-1-6 (Null Mutant)</td>
<td>2.64 ± 0.028</td>
<td>1.85 ± 0.106</td>
</tr>
<tr>
<td>P-4 (Revertant Strain)</td>
<td>2.63 ± 0.035</td>
<td>1.81 ± 0.134</td>
</tr>
</tbody>
</table>
Fig. 3.6. Rate of Consumption of Glucose in Serum at $37^\circ$C.
mutants was not responsible for avirulence, since the heterozygous mutant N-2 and the revertant strain P-4, showed a growth rate similar to the null mutant N-2-1-6, but was as virulent as the wild-type.

3.3.5. Reduced Adherence to HBEC \textit{in vitro}

As discussed in section 3.3.2., 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 \textit{in vivo}. It has been observed that a hierarchy among \textit{Candida} species exists, such as those which exhibit adherence \textit{in vitro} are among the most pathogenic ones (Calderone, 1993a, b). Mutant strains of \textit{C. albicans} with reduced ability to adhere \textit{in vitro} are reported to be less virulent in animal models of vaginitis (Lehrer \textit{et al.}, 1986) and endocarditis (Calderone \textit{et al.}, 1985). To account for the non-pathogenicity displayed by the null mutant, we examined the ability of wild-type \textit{C. albicans} SC5314, null mutant N-2-1-6, and the revertant strain P-4, to adhere to human buccal epithelial cells (HBEC) \textit{in vitro}, using a visual assay of stained cell preparations. We chose HBEC as the host cell to study adherence, as adherence to HBEC has been extensively studied. Recognition by \textit{C. albicans} is known to occur through a cell surface mannoprotein and a fucosyl glycoside of HBEC (Brassart \textit{et al.}, 1991; Tosh and Douglas, 1992), but a variety of other candidal cell wall components such as glucans, lipids, and chitin have also been investigated as possible adhesins. Recently, three genes \textit{ALAI}, \textit{ALSI}, and \textit{HWPI} encoding proteins with adherent properties have been identified (reviewed by Sundstrom, 1999). It is highly probable that \textit{C. albicans} expresses more than one adhesin, and different adhesins probably mediate the attachment of the organism to different host cells (Hostetter, 1994). Also, blastospores may use different adhesins to bind to host cells from those used by germinated organisms (Edwards \textit{et al.}, 1992).

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 \textit{C. albicans} that adhered per BEC was also studied 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 results are presented in a tabular form in Table 3. III. 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 possible that, because of this reason few \textit{Candida} cells were able to cross the barrier of vascular
Table 3. III. Reduced Adherence of Null Mutant of GlcNAc Catabolic Pathway to Human Buccal Epithelial Cells *In Vitro*.

The null mutant N-2-1-6 displayed reduced ability to adhere to HBEC, considering the percentage of adherence, as well as the average number of cells that adhered per HBEC.
epithelium and reach target organs for colonization. A functional GlcNAc catabolic pathway was therefore required for adherence. 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.

3.3.6. Defects in Cell Wall Structure

Among the putative virulence factors, the cell wall of *C. albicans* is certainly one of the most important. The cell wall provides rigidity, as well as protection against osmotic lysis, and promotes infection by supporting the interaction of *C. albicans* adhesins and host cell receptors (Calderone and Sturtevant, 1994). With considerable variation among different species, the gross macromolecular components of most fungi include chitin, a homopolymer of β-1,4-N-acetylglucosamine, β- or α-linked glucans, and a variety of mannoproteins. Chitin is synthesized by the enzyme chitin synthase, utilizing nucleotide sugar, uridine diphospho-N-acetylglucosamine (UDP-GlcNAc), as substrate. Uncoordinated increases in chitin deposition also result from treatment with a range of antifungal compounds such as Congo red and calcofluor white, interfering with chitin microfibril assembly (Munro *et al.*, 1998; Alonso-Monge *et al.*, 1999). Current investigational systemic antifungal agents directed against, or involving major constituents of the fungal cell wall include the nucleoside-peptide antifungal agent nikkomycin Z, a competitive inhibitor of chitin synthase (Cabib, 1991; Gaughran *et al.*, 1994; Walsh *et al.*, 2000).

To explore the relationship between cell wall metabolism and GlcNAc catabolic pathway (Fig.3.7.), 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. The highest and the lowest concentration plates (Fig.3.9) of Congo red showed only the growth of SC5314, and the mutant derivatives were all unable to grow at 37°C. On calcofluor plates, similar observations were noted (Fig.3.8.) at the same temperature. Much lower concentrations of calcofluor white was observed to 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 (data not shown). It showed that *C. albicans* is more sensitive to calcofluor white, compared to its sensitivity to Congo red. The
Fig. 3.7. Major pathways of metabolism of chitin. The enzymes involved (RHS) and the antibiotics inhibiting these enzymes (LHS) are shown.
Fig. 3.8. Study of Cell Wall Defects on YEPD-Calcofluor White Plate.
A. YEPD plate without dye supplement on which the four strains were spotted. This served as the control plate.
B. YEPD-Calcofluor white plate (100 μg/ml) on which cells were spotted. Incubated at 37°C for 48 hours.
Fig 3.9. Study of Cell Wall Defects on YEPD-Congo Red Plates.
Congo red plates showing no growth of mutant derivatives after incubation at 37°C for 48 hours. Number of cells spotted are shown on top of the plates. The strains are on the left of the plates. SC5314 (wild-type), N-2 (heterozygous mutant), N-2-1-6 (homozygous mutant), P-4 (revertant strain).
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. Susceptibility in all mutant strains was more pronounced at 37°C than at 30°C in case of Congo red, while in case of calcofluor white even the wild-type was unable to grow at 30°C.

The mutants also displayed an increased susceptibility to the antifungal agent nikkomycin Z (Fig.3.10. and Fig.3.11.), as seen in the disc assay and microdilution assay results. The disc assay was performed at first, which showed an increase in susceptibility of the mutants N-2, N-2-1-6, and P-4, to nikkomycin Z. The same concentration of nikkomycin Z did not inhibit growth of the wild-type, but inhibited the growth of N-2, N-2-1-6, and P-4, evidenced by no-growth zone diameters, which increased with the rise in concentration of nikkomycin Z (Fig.3.10.A. and B.).

It was followed by microdilution assay to determine the actual concentration of nikkomycin Z that inhibited growth (Fig.3.11. and Table 3.IV). The graph shows that at a concentration of 0.5 μM nikkomycin Z, the wild-type showed 90.72% growth of control conditions (without nikkomycin 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.

Nikkomycin Z is imported inside the cell through a peptide transport system (McCarthy et al., 1985; Shallow et al., 1991; Yadan et al., 1984), therefore, 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, which is a competitive inhibitor of chitin synthase, and GlcNAc is polymerized into chitin (Fig.3.7.), chitin synthesis was not, at least significantly impaired, 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. The C. albicans hog1 mutant, on phenotypic characterization, showed defects in cytokinesis and cell wall biogenesis, conclusively showing a link between cell wall metabolism and the HOG pathway. The effect produced by mutation of GlcNAc catabolic pathway was quite similar to the effect of HOG1 mutation. In addition, the hog1 mutant showed reduced virulence in systemic infection in murine model, and affected morphogenesis of C. albicans. It may indicate that there exists a cross talk between these two 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
Fig. 3.10.A. Sensitivity to cell wall inhibitor NikkomycinZ checked by Disc Assay Method.
On a lawn of Candida cells on SD plates, Whatman® discs (1 mm thick) soaked in eight different concentrations of NikkomycinZ were placed, and incubated at 37°C for 24 hours. The stock concentrations labelled on the discs as 1 to 8 were: 500 μg/ml (1), 250 μg/ml (2), 100 μg/ml (3), 50 μg/ml (4), 10 μg/ml (5), 5 μg/ml (6), 2.5 μg/ml (7) and 1 μg/ml (8).
Fig. 3.10.B. Sensitivity to cell wall inhibitor NikkomycinZ checked by Disc Assay Method.

C1 and C2: Homozygous mutant N-2-1-6. D1 and D2: Reintroduced strain P-4.

On a lawn of Candida cells on SD plates, Whatman® discs (1 mm thick) soaked in eight different concentrations of NikkomycinZ were placed, and incubated at 37°C for 24 hours. The stock concentrations labelled on the discs as 1 to 8 were: 500 µg/ml (1), 250 µg/ml (2), 100 µg/ml (3), 50 µg/ml (4), 10 µg/ml (5), 5 µg/ml (6), 2.5 µg/ml (7) and 1 µg/ml (8).
Table 3.IV. Growth of *Candida albicans* Wild-type and Mutant Strains in YNB with Different Concentrations of Nikkomycin Z.

<table>
<thead>
<tr>
<th>NIKKOMYCIN Z CONC. IN μM</th>
<th>O.D OF CELLS AT 600 NM AFTER 24 HOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC5314</td>
</tr>
<tr>
<td>0.0</td>
<td>0.798</td>
</tr>
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<td>0.724</td>
</tr>
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<td>1.0</td>
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</tr>
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<td>0.016</td>
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<tr>
<td>250.0</td>
<td>0.011</td>
</tr>
<tr>
<td>500.0</td>
<td>0.012</td>
</tr>
<tr>
<td>750.0</td>
<td>0.009</td>
</tr>
<tr>
<td>1000.0</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Fig. 3.11. Increase in Susceptibility of the Mutant Derivatives to Nikkomycin Z.
account for the altered colony morphology and hyperfilamentous phenotype obtained with the GlcNAc null mutants.

3.3.7. 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). This enzyme hydrolyzes the dimers and trimers of β-N-acetyl-D-glucosamine (GlcNAc) releasing GlcNAc from the substrates (Niimi et al., 1997). β-N-acetylglucosaminidase was also reported from E. coli (Yem and Wu, 1976), and NagZp has been reported to be the sole β-N-acetylglucosaminidase expressed in E. coli (Cheng et al., 2000). This enzyme completely cleaves GlcNAc from anhydro-muropeptides and also readily cleaves GlcNAc linked β-1,4 to MurNAc-peptides. This enzyme is an integral component of the murein tripeptide recycling pathway in bacteria (Cheng et al., 2000). 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 (Jenkinson and Shepherd, 1987).

Production of β-N-acetylglucosaminidase by the wild-type and mutant strains was assayed by initially culturing them on SD agar plates containing 0.15 mg/ml 4-methylumbelliferyl-N-acetyl-β-D-glicosaminide (MUAG). The substrate on breakdown by β-N-acetylglucosaminidase formed the product methylumbelliferone, which was visualized as a fluorescent halo on exposure to UV light (Fig.3.12.). Our results showed no effect on secretion of this enzyme in GlcNAc defective mutants, at least in visual estimation, where the halo could be seen in the wild-type as well as the mutants, without any visible difference. 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.

3.3.8. Study of Effect on Morphogenesis

3.3.8.1. 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. It is not albumin, since albumin-free serum from a rat mutant was as efficient at promoting hyphae as normal serum (Feng et al., 1999). It was assumed that the two known inducers of hyphae formation, GlcNAc and proline, may
Fig. 3.12. No Effect on β-N-acetylglucosaminidase Production in the Mutants.
A. SC5314 (wild-type). B. N-2 (heterozygous mutant).
C. N-2-1-6 (homozygous mutant). D. P-4 (revertant strain).
Strains of \textit{C. albicans} streaked on SD agar plates supplemented with 0.15 mg/ml
4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUAG). The substrate on breakdown by β-N-acetylglucosaminidase formed methylumbelliferone which fluoresces on exposure to UV light. No difference observed among wild-type and mutant derivatives.
contribute to the serum effect, since they are generated by degradation of serum (glyco-) proteins (reviewed by Ernst, 2000). 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 (Fig.3.13.A. and C.), consisting of elongated cells with constrictions which looked like pseudohyphae. The heterozygous mutant N-2 and the revertant strain P-4 (Fig.3.13.B. and D.), however failed to show any filament formation. The null mutant was therefore not impaired in filamentation in serum both in vivo (demonstrated in the histopathological section of kidney), and in vitro, suggesting that GlcNAc signaling pathway is 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).

3.3.8.2. 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). Hyphae formation in C albicans stimulated by severe nitrogen starvation on solid SLAD medium, also stimulates pseudohyphal development in S. cerevisiae (Gimeno et al., 1992). After induction of filamentous growth on SLAD plates for 10 days at 37°C, the wild-type strain SC5314 had colonies with smooth central regions of non-filamentous cells, surrounded by a filamentous fringe (Fig.3.14.A.). The homozygous mutant N-2-1-6 showed highly extensive filamentation compared to the wild-type (Fig.3.14.C.). The filaments were highly branched, ramified in different directions, and appeared to consist mostly of true hyphae. The heterozygous mutant N-2, and the revertant strain P-4, on the other hand, did not show an intermediate phenotype. The mutants N-2 and P-4 (Fig.3.14.B. and D.) behaved in a similar fashion and formed restricted amount of filaments under the colonies, with very few showing on the periphery.

On Spider medium (Liu et al., 1994) containing mannitol as the carbon source, observations were recorded after 7 days of incubation at 37°C (Fig.3.15.). Wild-type SC5314 formed extreme-jagged colonies consisting of hyphal and pseudohyphal cells, emanating from a central smooth and shiny region with ridges (Fig.3.15.A.). N-2 and P-4 were almost completely
impaired in filamentation, and showed central smooth shiny colony surface with faint concentric rings. Peripheral hyphae were highly restricted (Fig.3.15.B. and D.). The homozygous mutant N-2-1-6 (Fig.3.15.C.), 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 as seen in Fig.3.15.C. The surface was not shiny as in other cases.

Hyphal formation is a complex mechanism, in which both positive and negative signals play a role, presented in a model by P.T. Magee in 1997. 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 TUP1 (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 chapter.
Fig. 3.13. Germ Tube Induction in 20% Calf Bovine Serum at 37°C for 2 Hours.
A. SC5314 (wild-type).
B. N-2 (heterozygous mutant).
C. N-2-1-6 (homozygous strain).
D. P-4 (revertant strain).
Fig. 3.14. Morphogenesis on SLAD Plates at 37°C After 10 Days.
A. SC5314 (wild-type).
B. N-2 (heterozygous mutant).
C. N-2-1-6 (homozygous strain).
D. P-4 (revertant strain).

Hyperfilamentation noted in the null mutant N-2-1-6.
Fig. 3.15. Morphogenesis on Spider Plates at 37°C After 7 Days.

A. SC5314 (wild-type).
B. N-2 (heterozygous mutant).
C. N-2-1-6 (homozygous strain).
D. P-4 (revertant strain).

Hyperfilamentation and change in morphology of colony surface observed in N-2-1-6.