CHAPTER 2

DISRUPTION OF N-ACETYLGLUCOSAMINE CATABOLIC PATHWAY IN CANDIDA ALBICANS AND CHARACTERIZATION OF THE MUTANTS

2.1. INTRODUCTION

The pathogenic fungus Candida albicans can utilize the aminosugar N-acetyl-D-glucosamine (GlcNAc) as a source of carbon and nitrogen for growth. GlcNAc is first transported into the cells by GlcNAc permease, and then by the sequential action of GlcNAc kinase, GlcNAc-6-phosphate deacetylase, and glucosamine-6-phosphate deaminase, GlcNAc is converted to fructose-6-phosphate, which is then metabolized through glycolysis (Singh and Datta, 1979a). Apart from the role in providing energy for growth, GlcNAc is also capable of inducing change in cellular morphology from the yeast form to the mycelial form (Shepherd et al., 1980; Gopal et al., 1982; Simonetti et al., 1974).

C. albicans can also utilize glucosamine (GlcN), another aminosugar, which is converted to glucosamine-6-phosphate after transportation (Corner et al., 1986), and is the substrate for glucosamine-6-phosphate deaminase (Fig. 2.1.). Aminosugars catabolized by this route have been reported in organisms like Escherichia coli (Peri et al., 1990; Rogers et al., 1988) and Klebsiella pneumoniae (Vogler and Lengeler, 1989). For utilization of another aminosugar N-acetylmannosamine (ManNAc) by C. albicans, it was presumed that ManNAc first gets converted to GlcNAc by the action of N-acetylmannosamine epimerase, and is subsequently catabolized by enzymes of the GlcNAc catabolic pathway (Biswa et al., 1979). N-acetylmannosamine was later reported to be not a growth substrate for C. albicans, but was capable of inducing germ tube formation and GlcNAc catabolic pathway enzymes (Sullivan and Shepherd, 1982).

The purification, characterization, cDNA cloning, genomic DNA isolation, and sequencing of glucosamine-6-phosphate deaminase (NAG1) from C. albicans was reported from our laboratory (Natarajan and Datta, 1993; Kumar et al., 2000). This enzyme catalyzes the reversible conversion of glucosamine-6-phosphate to fructose-6-phosphate (Fig. 2.1.). The forward reaction involves an aldo-keto isomerization coupled with an amination-deamination process. In the backward reaction, glucosamine-6-phosphate is produced from fructose-6-phosphate and ammonia. However, in E. coli and in C. albicans, the backward reaction requires
Fig. 2.1. The GlcNAc Catabolic Pathway in *Candida albicans*.
GlcNAc is transported into the cell by GlcNAc permease A. GlcNAc is then utilized by the sequential action of the following enzymes—B, GlcNAc kinase (*HXK1*); C, GlcNAc-6-phosphate deacetylase (*DAC1*); D, GlcN-6-phosphate deaminase (*NAG1*), to generate fructose-6-phosphate that enters the glycolytic pathway. Another aminosugar GlcN enters the cell via a general sugar permease E, and gets phosphorylated to GlcN-6-phosphate by GlcNAc kinase, further action of deaminase converts it to fructose-6-phosphate.
very high substrate concentrations, suggesting that it may not occur under physiological conditions. Deaminase is induced more than 100-fold when GlcNAc is used as the carbon source. The deaminase was purified from crude extract of YNB-GlcNAc grown SC5314 cells. Polyclonal antibody against purified deaminase was developed in rabbit, and a λ gt11 cDNA expression library was prepared using oligo (dT) primers and poly (A)+ RNA from GlcNAc-grown C. albicans. Using the deaminase-antibody, cDNA clones were isolated by immunoscreening of the λ gt11 cDNA library. The clones were digested with EcoRI, Southern blotted, and cross-hybridized to study the relatedness among them. This is how NAG1 was isolated (Natarajan and Datta, 1993). To isolate the NAG1 genomic DNA clone, a λEMBL3 library containing partial Sau3A I fragments (average size of 7 to 16 Kb) of C. albicans genomic DNA, was screened with NAG1 cDNA. Considering the size of the haploid genome in C. albicans to be ~1.3 to 1.8 x 10^4 Kb, approximately 30,000 colonies were screened, and, after several rounds of screening, six positive phages were identified by selecting the plaques with the strongest signals. A preliminary restriction mapping was done by digesting with Sal I. All the phages contained common bands of ~4 Kb and ~1 Kb. The clone λED14, with the largest insert of 16.3 Kb, was chosen for further analysis. To identify the region of λED14 containing NAG1, a Southern analysis was performed. Based on Southern hybridization results, the 4 Kb Sal I fragment was chosen for subcloning into pBluescript II KS+. The plasmid housing the NAG1 gene was named pED4 (Kumar et al., 2000). The sequence of NAG1 genomic clone revealed 3915 base pairs. It is an AT-rich sequence, which is typical of C. albicans, with an AT content of 57.2%. From the sequencing data, it was established that, pED4 has a 1.725 Kb upstream region, the 0.747 Kb NAG1 ORF, and a 1.442 Kb downstream region (Fig. 2.2.). Analysis of the sequence revealed that the genomic clone lacked a consensus TATA element, but a TATA-like sequence 5'CCATAAAAAGGCC3', at position -59 with respect to ATG, was present. A putative Cap signal 5'CCAATTTTC3', and the polyadenylation sequence 5'AATAAA3' at 475 nucleotides downstream of the NAG1 stop codon at position +1222, were also identified. In addition, sequence analysis revealed multiple putative binding sites for transcription factors NF-E1 (sequence WGATAMS) at positions -996, -816, -66, and for CTF/NF1 (sequence GCCAAT) at positions -1311, -853, -600 with respect to ATG. However, it is unclear whether homologues of these transcription factors exist in C. albicans. In the NAG1 promoter (240 base pairs), there exists a polyA-rich sequence 5'GGAGCAAAAAAATGT3' at position -164 to -150, called boxA. This sequence is quite similar to the one found in E. coli NagC binding region, box G15'TCCATTTCCACGCATGAAAAATG3' (Plumbridge and Kolb,
1991, 1995). *NagC* is a repressor in the GlcNAc catabolic pathway in *E. coli*, and this envisages that a protein like *NagC* may be involved in the recognition of the site, and thus affect regulation of *NAG1*. The sequence immediately upstream of the *NAG1* start site contains at least two more regions similar to boxA (-113 to -102) 5'AAAAAAATGC3' and (-91 to -80) 5'AAAAAAAATGT3', which could represent additional binding sites for the protein(s).

The multifarious role of GlcNAc inducible pathway in *C. albicans* was studied in the current and following chapters, for which the catabolic pathway was disrupted in *C. albicans*. The last gene specific to this pathway, *NAG1*, was disrupted by the “ura-blaster” technique. This is currently the most successful and widely used technique, to disrupt genes in *C. albicans*. The chitin synthase *CHS2* (Gow *et al.*, 1994), phosphatase *CPP1* (Csank *et al.*, 1997), catalase *CAT1* (Wysong *et al.*, 1998), mannosyl transferase *CamNT1* (Buurman *et al.*, 1998), and *RAS1* (Feng *et al.*, 1999), are few among the many genes disrupted in *C. albicans* by the ura-blaster technique. In the process of *NAG1* disruption, a deacetylase (*DAC1*) and a hexokinase (*HXK1*) gene were discovered, present in a cluster. 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).
2.2. MATERIALS AND METHODS

1. All percent shown here are on a-w/v basis unless mentioned otherwise.
2. All media and solutions were prepared in MilliRO and MilliQ-water respectively.
3. All solutions and media were sterilized by autoclaving at 15 lb/sq.inch for 15 minutes, or filter sterilized by passing through a 0.22 μM Millipore filter.
4. Media used were from GIBCOBRL or DIFCO unless mentioned otherwise.
5. Chemicals used were of analytical grade (Glaxo or MERCK) or molecular biology grade (SIGMA, GIBCOBRL or USB) unless mentioned otherwise.
6. Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs and Boehringer Mannheim. Buffers provided with the enzymes were used.

2.2.1. Strains and Plasmids

<table>
<thead>
<tr>
<th>Strains</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>DH5α</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>CAF3-1</td>
</tr>
</tbody>
</table>

| Plasmids | pED4, pCUB6 |

2.2.2. Media and Solutions

<table>
<thead>
<tr>
<th>Terrific Broth (TB) Source GIBCOBRL, medium was supplemented with 0.4% v/v Glycerol.</th>
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<tbody>
<tr>
<td>Terrific Broth (TB) Source GIBCOBRL, medium was supplemented with 0.4% v/v Glycerol.</td>
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<tr>
<td>Luria Broth Base (Miller's LB) Source GIBCOBRL</td>
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<tr>
<td>2XL Broth 2% Bacto Tryptone 1% Yeast Extract 0.1% NaCl 0.2% Dextrose</td>
</tr>
<tr>
<td>Media with Antibiotics LB/TB with 50μg/ml Ampicillin LB agar with 75 μg/ml Ampicillin</td>
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| YEPD 1% Yeast Extract 2% Peptone 2% Dextrose |
| YEPD Agar YEPD with 2% Agar |
| YEPD Uridine YEPD with 25μg/ml Uridine |
| Trituration Buffer 40 mM NaOAc 100 mM CaCl₂ 70 mM MgCl₂ |
10X TE (pH 8.0)
100 mM Tris-HCl, pH 8.0
10 mM EDTA, pH 8.0

Solution I
25 mM Tris-HCl, pH 8.0
10 mM EDTA
50 mM Dextrose

Solution II
1% SDS
0.2 N NaOH

Solution III
KOAc for alkaline lysis. 3M with respect to Potassium and 5M with respect to Acetate, as per Sambrook et al., 1989.

Phenol
Glaxo Laboratories Exel AR Grade.
Redistilled at 180°C and stored at -20°C in small aliquots.

TE-saturated Phenol
Prepared as per Ausubel et al., 1994.

Phenol: Chloroform: Isoamylalcohol
25 parts of TE saturated Phenol, 24 parts of Chloroform, 1 part Isoamylalcohol.

50X TAE Buffer
242 g Tris base
57.1 ml Glacial Acetic Acid
100 ml 0.5 M EDTA, pH 8.0

10X EndoR
20% Ficoll 400
0.1 M EDTA, pH 8.0
1% SDS
0.25% Bromophenol Blue

Spider (pH 7.2, after autoclaving)
1% Nutrient Broth
1% Mannitol
0.2% K$_2$HP0$_4$, 1.35% Bactoagar (for plates)

STM
1 M Sorbitol
10 mM Tris-HCl, pH 7.5
40 mM β-mercaptoethanol

STC
1 M Sorbitol
10 mM Tris-HCl, pH 7.5
10 mM CaCl$_2$

SOS
1 M Sorbitol
0.35% Yeast Extract
0.7% BactoPeptone
6.5 mM CaCl$_2$

Top Agar
1 M Sorbitol
0.5% Agar

Synthetic Minimal Media (SD)
0.67% Yeast Nitrogen Base (w/o amino acids)
2% Dextrose

SD Agar
SD with 2% Agar

SD Sorbitol
SD Agar with 1 M Sorbitol

5-FOA Plate
0.67% Yeast Nitrogen Base
2% Dextrose
25 μg/ml Uridine
1 mg/ml 5-Fluoroorotic acid (5-FOA)
2% Agar

20X SSC (1 liter)
175.3 g NaCl
88.2 g Sodium citrate

Prehybridization Buffer
1% SDS
2X SSC
10% Dextran Sulphate, Na salt
50% Deionized Formamide
2X YEPD
2% Yeast Extract
4% Peptone
4% Dextrose

SLAD
0.17% Yeast Nitrogen Base w/o amino acids and (NH₄)₂SO₄
2% Dextrose
2% Bacto Agar (DIFCO)
50 μM (NH₄)₂SO₄

2.2.3. Storage of Escherichia coli

For long term storage, overnight grown cultures of Escherichia coli in 1ml TB ampicillin, were stored as glycerol stocks with a final concentration of 15% glycerol, at −80°C. Cultures on plates were stored at 4°C for 1-2 months.

2.2.4. Growth and Maintenance of Candida albicans

Strains of Candida albicans were regularly grown and maintained on YEPD plates or YEPD plates supplemented with uridine, in case of uracil auxotrophs. For long term storage, liquid cultures were grown overnight, and stored at −80°C with 15% glycerol.

2.2.5. Transformation of Escherichia coli

2.2.5.1. Preparation of Chemically Competent DH5α Cells

Method was adopted from Hanahan et al., 1991.

1. DH5α was streaked for single colonies on an LB plate and grown overnight at 37°C.
2. A single colony was inoculated in 10 ml 2XL and grown for 16 hours at 30°C with shaking at 200 rpm.
3. 1% inoculum was added to 90 ml 2XL and grown at 30°C with shaking at 200 rpm for 3-4 hours until it reached an OD₆₀₀ of 0.45-0.50.
4. Cells were chilled on ice for 2 hours.
5. Harvested at 5,000 rpm for 15 minutes at 4°C. Supernatant was discarded.
6. Cells were resuspended in 45 ml (0.5 volume of culture) chilled trituration buffer and incubated in ice-cold water for 45 minutes.
7. Harvested at 4,000 rpm for 10 minutes at 4°C. Supernatant was discarded.
8. Cells were resuspended in chilled 10 ml trituration buffer with 15% glycerol and stored at −80°C in aliquots of 200 μl.
2.2.5.2. Transformation of Chemically Competent DH5α Cells with pED4 and pCUB6 Plasmid DNA

Method was adopted from Hanahan et al., 1991.
1. Competent cells from -80°C were thawed on ice.
2. 1 µl DNA (5-10 ng) of each plasmid was taken in microfuge tubes and 49 µl 1X TE (pH 8.0) was added in each to make the volume 50µl.
3. 100 µl thawed competent cells was added in each tube and incubated on ice for 45 minutes with frequent tapping.
4. Heat shock at 37°C for 5 minutes was given followed by immediate chilling on ice for 2 minutes.
5. 1 ml 2XL was added to the cells, and allowed to outgrow for 1 hour at 37°C with gentle shaking, for expression of the antibiotic resistant gene.

2.2.5.3. Plating of the Transformation Mix

An aliquot of the transformation mix was plated on LB agar plates with ampicillin. The plates were incubated at 37°C until the colonies were apparent (12-16 hours).

2.2.6. Inoculation for Medium Scale Isolation of Plasmid DNA

One transformant of each was inoculated in 35 ml TB with ampicillin. Grown at 37°C for 18 hours with shaking at 200 rpm.

2.2.6.1. Medium Scale Plasmid DNA Isolation (Midiprep)

Plasmid isolation method was adopted from Sambrook et al., 1989.
1. Cells were harvested at 10,000 rpm for 5 minutes at room temperature in SS34 tubes, and the supernatant was removed by aspiration.
2. Pellets were resuspended in 2 ml Solution I by vortexing.
3. 0.5 ml lysozyme (20 mg/ml in Solution I) was added, mixed well, and incubated at room temperature for 5 minutes.
4. 5 ml Solution II (SDS: NaOH) was added, mixed gently by inverting the tube 3-4 times and incubated at room temperature for 10 minutes.
5. 3.75 ml ice-cold Solution III was added, mixed thoroughly, and incubated on ice for 15 minutes.
6. Spun at 10,000 rpm for 10 minutes at 4°C. Supernatant of each sample was carefully transferred to new SS34 tubes, avoiding cell debris.
7. 6.75 mL (0.6-volume)-propan-2-ol was added to each, mixed thoroughly, and incubated at room temperature for 1 hour.
8. Spun at 10,000 rpm for 10 minutes at room temperature. Supernatants were discarded.
9. Pellets were washed with 70% ethanol, air-dried, resuspended in 500 μl 1X TE (pH 8.0), and transferred to microcentrifuge tubes.
10. 10 μl RNaseA (10 mg/ml) was added, mixed well, and incubated at 37°C for 30 minutes.
11. 125 μl 4M NaCl and 625 μl 13% PEG 8000 was added, mixed well, and incubated on ice for 1 hour.
12. Spun at 10,000 rpm for 10 minutes at 4°C. Supernatants were discarded.
13. Pellets were washed with 70% ethanol, air-dried and dissolved in 200 μl 1X TE (pH 8.0).
14. 200 μl TE-saturated phenol: chloroform: isoamylalcohol mix was added, mixed thoroughly with the DNA solution, and spun at 10,000 rpm for 10 minutes.
15. The aqueous phase was transferred to a new microcentrifuge tube and the process was repeated.
16. To the aqueous phase 200 μl chloroform: isoamylalcohol was added, mixed well, and spun at 10,000 rpm for 10 minutes. The aqueous phase was transferred to a new tube.
17. DNA was precipitated with 0.3 M sodium acetate and 2.5 volumes of absolute alcohol at −20°C for 1 hour.
18. Spun at 10,000 rpm for 10 minutes at 4°C, supernatants were discarded.
19. Pellets were washed with 70% ethanol, air-dried, and dissolved in 100 μl 1X TE (pH 8.0).
20. DNAs of pED4 and pCUB6 were quantitated in a Spectrophotometer at Abs260.

2.2.7. Restriction Analysis of Plasmid Clones

1. 2 μg DNA of pED4 was digested with Sal I in 20 μl reaction volume containing 1X reaction buffer, 1X BSA, and 8 units of Sal I. The reaction was incubated at 37°C for 4 hours.
2. 2 μg DNA of pED4 was digested with Nco I in 20 μl reaction volume containing 1X reaction buffer, and 4 units of Nco I. The reaction was incubated at 37°C for 4 hours.
3. 2 μg DNA of pCUB6 was digested with BamH I and Bgl II in 20 μl reaction volume containing 1X compatible reaction buffer, 1X BSA and 2 units of each BamH I and Bgl II. The reaction was incubated at 37°C for 4 hours.
4. 2 μg DNA of pCUB6 was digested with BamH I, Bgl II and Pvu II in 20 μl reaction volume containing 1X compatible reaction buffer, 1X BSA and 2 units of BamH I, Bgl II and Pvu II. The reaction was incubated at 37°C for 4 hours.

5. The digestions were stopped by adding 1X EndoR, resolved on a 0.8% agarose gel with 1 Kb DNA ladder as the marker. These were the test digestions preformed before going in for cloning.

6. Gel photographs were taken with a Polaroid Camera.

2.2.7.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis was done as described in Sambrook et al., 1989. Routinely 1X TAE buffer was used. Ethidium bromide was added to 0.5 μg/ml concentration both in gel and in the buffer. For most purposes 0.8% agarose gels were used.

2.2.8. Cloning of the hisG-URA3-hisG Fragment into pED4

2.2.8.1. Preparation of Vector

1. 3 μg DNA of pED4 was digested with Nco I in 50 μl reaction volume containing 1X reaction buffer, and 15 units of Nco I. The reaction was incubated at 37°C for 4 hours.

2. 4 μl of the reaction mix was run on a 0.8% agarose gel to check for completion of digestion. The digestion was loaded on a preparative gel after checking.

3. The vector backbone was cut out with a sterile surgical blade and gel purified using Geneclean II (BIO 101) kit.

4. An end-fill-in reaction of the Nco I digested pED4 vector backbone was done in 15 μl reaction volume containing 1X Klenow buffer, 33 μM of each dNTP, and 1 unit Klenow. Incubated at 25°C for 15 minutes. Enzyme was heat inactivated at 75°C for 20 minutes.

5. DNA was extracted with phenol: chloroform: isoamylalcohol once, followed by chloroform: isoamylalcohol extraction. Precipitated with sodium acetate and absolute alcohol. Pellet was washed with 70% ethanol, air-dried, and resuspended in 20 μl sterile MilliQ-water. 1 μl DNA was loaded and estimated on a fresh gel.

2.2.8.2. Preparation of Insert

1. 10 μg pCUB6 DNA was digested with BamH I, Bgl II and Pvu II in 50 μl reaction volume containing 1X BamH I buffer, 1X BSA and 10 units of Bgl II, Pvu II, and 20 units of BamH I. The reaction was incubated at 37°C for 4 hours.
2. 2 µl of the reaction mix was loaded with 1X EndoR and run on a 0.8% agarose gel to check for completion of digestion. The digestion was loaded on a preparative gel after checking.

3. The 4 Kb band was cut out with a sterile surgical blade, and gel purified using GeneClean II (BIO 101) kit.

4. An end-fill-in reaction of the 4 Kb fragment was done in 10 µl reaction volume containing 1X Klenow buffer, 33 µM of each dNTP, and 1 unit Klenow. Incubated at 25°C for 15 minutes. Enzyme was heat inactivated at 75°C for 20 minutes.

5. DNA was extracted with phenol: chloroform: isoamyl alcohol once, followed by chloroform: isoamyl alcohol extraction. Precipitated with sodium acetate and absolute alcohol. Pellet was washed with 70% ethanol, air-dried, and resuspended in 10 µl sterile MilliQ-water. 1 µl DNA was loaded and estimated on a fresh gel.

2.2.8.3. Ligation

Ligation reaction was set up using 25 ng vector, at a vector: insert ratio of 1:3 nanomole ends, in 25 µl reaction volume, containing 1X ligase buffer, 15% PEG 4000, and 1 µl T4 ligase (Boehringer Mannheim). Incubated at 25°C for 16 hours. Ligase was inactivated at 65°C for 10 minutes.

2.2.8.4. Transformation

The entire ligation mix was transformed, and the entire transformation mix was plated on LB ampicillin plates. Incubated at 37°C for 16 hours.

2.2.8.5. Screening for Recombinants

All the transformants were inoculated for minipreps in 500 µl TB ampicillin.

2.2.8.5.1. Small Scale Plasmid DNA Isolation (Miniprep)

Plasmids were isolated by alkaline lysis method adopted from Sambrook et al., 1989.

1. Cells were harvested at 13,000 rpm for 2 minutes, and the supernatant was removed by aspiration.

2. Pellets were resuspended in 50 µl Solution I by vortexing.

3. 100 µl Solution II (SDS:NaOH) was added in each tube and mixed by inverting the tube 3-4 times.
4. 75 μl ice-cold Solution III was added, mixed thoroughly by tapping, and incubated on ice for 5 minutes.
5. Spun at 13,000 rpm for 5 minutes at room temperature.
6. The cell debris was carefully removed using sterile toothpicks.
7. 150 μl (0.6 volume) propan-2-ol was added to each tube, mixed thoroughly, and incubated at room temperature for 1 hour.
8. Spun at 13,000 rpm for 5 minutes, supernatant was discarded from each tube.
9. Pellets were washed with 70% ethanol, air-dried, and resuspended in 25 μl 1X TE (pH 8.0).

2.2.8.5.2. Identification of Recombinants

1. Plasmids isolated were electrophoresed on a 0.8% agarose gel with pED4 DNA as the marker.
2. 2 μg DNA of clones with retarded migration was digested with Sal I in 20 μl reaction volume, containing 1X reaction buffer, 1X BSA, 10 μg/ml RNaseA and 5 units of Sal I. The reactions were incubated at 37°C for 4 hours.
3. The reactions were loaded with 1X EndoR and run on a 0.8% agarose gel with 1 Kb DNA ladder as the marker.
4. 2 μg DNA of the putative positive clones were digested separately with EcoR I and Pvu II in 20 μl reaction volumes containing 1X respective reaction buffer, 10 μg/ml RNaseA and 4 units of EcoR I or Pvu II. The reactions were incubated at 37°C for 4 hours.
5. Reactions were loaded with 1X EndoR and run on a 0.8% agarose gel with 1Kb DNA ladder as marker.

2.2.8.6. Medium Scale Plasmid Isolation of the Recombinant Clone

The selected clone pED4-11 was inoculated in 35 ml TB ampicillin and DNA isolated by the method described in section 2.2.6.1.

2.2.9. Transformation of CAF3-1 for Disruption of the First Allele of NAGJ

2.2.9.1. Sal I Digestion of pED4-11 to Release the Cassette

1. 10 μg of pED4-11 was digested with Sal I in 50 μl reaction volume containing 1X reaction buffer, 1X BSA, and 80 units of Sal I. The reaction was incubated at 37°C overnight.
2. 2 μl of the reaction was loaded with 1X EndoR and run on a 0.8% agarose gel with 1 Kb DNA ladder as the marker to check for completion of digestion.

3. The enzyme was inactivated at 65°C for 20 minutes.

2.2.9.2. Transformation by the Spheroplast Method

1. A preculture of CAF3-1 was grown overnight in 10 ml YEPD supplemented with uridine at 30°C with shaking at 200 rpm.
2. 1 x 10^6 cells of the preculture was added to 20 ml of YEPD supplemented with uridine and grown at 30°C with shaking at 200 rpm to a density of 1 x 10^8 cells/ml.
3. Cells were induced with 0.5% GlcNAc at 30°C for 30 minutes, with shaking at 200 rpm.
4. Cells were harvested at 5,000 rpm for 5 minutes at room temperature. Supernatant was discarded.
5. Cells were washed twice with STM and resuspended in 5 ml STM. 30 μl Zymolyase (10 mg/ml) was added to it. Incubated at 30°C for 45 minutes.
6. Spheroplasts were harvested at 3,000 rpm for 5 minutes 4°C. Resuspended in 400 μl STC.
7. Sal I digested pED4-11 (10 μg) was mixed with 50 μg salmon sperm DNA and the volume was made up to 50 μl with STC. 100 μl cells was added to it and tapped gently to mix well.
8. 1 ml mix of 20% PEG 4000 and 10 mM CaCl$_2$ was added, and tapped gently to mix. Incubated at 30°C for 30 minutes with frequent gentle tapping.
9. Spun briefly at 3,000 rpm, supernatant was discarded. Cells were resuspended in 1 ml SOS and incubated at 30°C for 40 minutes.
10. The transformation mix was mixed with 5 ml Top agar and poured on an SD Sorbitol plate.
11. Agar was allowed to solidify and the plate was incubated at 30°C until the transformants came up (2-3 days).

2.2.10. Checking the Transformants on SD Medium

16 transformants were patched on an SD plate and incubated at 30°C for a day.

2.2.11. Genomic DNA Isolation of Ura Positive Transformants

1. The parent strain CAF3-1, and 16 Ura positive transformants, were inoculated in 10 ml YEPD and grown at 30°C with shaking at 200 rpm, till saturation.
2. The cells were collected by centrifugation at 5,000 rpm for 5 minutes, supernatants were discarded.
3. The cells were resuspended in 0.5 ml distilled water and transferred to microcentrifuge tubes.
4. The cells were collected by giving a brief spin at 5,000 rpm for 2 minutes. The supernatants were decanted; and cells were resuspended in the residual liquid by vortexing.
5. A 0.2 ml mixture of 2% TritonX-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA was added to each tube. To it 0.2 ml phenol: chloroform:isoamylalcohol was added. 0.3 g acid-washed glass beads were then added to each tube. The glass beads were prepared by soaking in concentrated HNO₃ for 1 hour, then washed extensively with water till the pH was similar to that of water. The beads were then baked until dry.
6. Vortexed at full speed for 4 minutes. 0.2 ml 1X TE (pH 8.0) was added.
7. Spun at 10,000 rpm for 5 minutes in a microcentrifuge. The aqueous layer was transferred to a new tube and 0.6 volume propan-2-ol was added to each, and mixed gently.
8. Spun at 10,000 rpm for 2 minutes. The supernatants were discarded. Pellets were washed with 70% ethanol, air-dried, and resuspended in 0.4 ml 1X TE (pH 8.0).
9. 30 µg RNaseA was added and incubated at 37°C for 15 minutes.
10. 10 µl 4M ammonium acetate plus 1ml absolute alcohol was added to each and mixed gently by inverting the tubes.
11. Spun at 10,000 rpm for 2 minutes. Pellets were washed with 70% ethanol, air-dried, and resuspended in 50 µl 1X TE (pH 8.0).
12. 2 µl of each sample was loaded and checked on a gel, DNA quantity was estimated visually.

2.2.12. Checking Integration of the Cassette by Southern Hybridization Technique

This technique was developed by Southern (1975).

2.2.12.1. Restriction Digestion of Genomic DNA

1. 2 µg genomic DNA of CAF3-1 and 16 Ura positives (N-1 to N-16) were digested with Sal I in 20µl reaction volume containing 1X reaction buffer, 1X BSA, and 8 units of Sal I. The reactions were incubated at 37°C overnight.
2. 2 µl of each reaction was loaded with 1X EndoR and run on a 0.8% agarose gel, to check for completion of digestion.
2.2.12.2. Agarose Gel Electrophoresis

1. The digestions, along with 1 Kb DNA ladder as marker, were loaded on a 12 cm x 14 cm 0.8% agarose gel, containing 0.5 µg/ml ethidium bromide. The gel was run as usual with 1X TAE buffer at 80 V constant, until the dye reached the bottom of the gel.
2. After electrophoresis, a tracing of the gel, and photograph, were taken to mark the positions of the bands.

2.2.12.3. Southern Transfer

1. The gel was rinsed with MQ-water and depurination of the DNA was done by immersing the gel in 150 ml 0.25 N HCl for 10 minutes in a baking dish, with mild shaking on gyratory platform shaker.
2. The solution was discarded, the gel was rinsed with MQ-water to wash off excess HCl, and treated with 150 ml denaturing solution (0.6 M NaCl and 0.4 N NaOH) for 30 minutes, under similar conditions.
3. The solution was then exchanged with 150 ml neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5) for 30 minutes, under similar conditions.
4. Gene Screen Plus® membrane and 3 mm Whatman® paper were pre-wet for few seconds in MQ-water and equilibrated in 10X SSC for 15 minutes.
5. Gel was aligned on top of the Whatman paper and the Gene Screen Plus® membrane, and the wells were marked on the membrane with a needle.
6. DNA was transferred onto the positively charged nylon membrane by pressure blotting method, using PosiBlot Pressure Blotter (Stratagene) at 80 mm Hg pressure for 90 minutes.
7. After transfer, the membrane was agitated in 0.4 N NaOH for 1 minute. This step was performed to denature the DNA. The membrane was then neutralized in 0.2 M Tris-HCl (pH 7.5)/1X SSC for 1 minute.
8. UV-crosslinking of nucleic acids to the membrane was done in Stratalinker.

2.2.12.4. Prehybridization

1. The membrane was pre-wet in 2X SSC for 1 minute.
2. The membrane was then placed in a hybridization bottle and prehybridized overnight, with 50 µl prehybridization buffer per cm² membrane (~9 ml for 12 cm x 14 cm membrane), at 42°C.
2.2.12.5. Hybridization

2.2.12.5.1. Preparation of Probe

2.2.12.5.1.1. Preparation of DNA

1. 2 μg DNA of pED4 was digested with Sal I in 50 μl reaction volume containing 1X reaction buffer, and 20 units of Sal I. The reaction was incubated at 37°C overnight.
2. The digestion was loaded on a 0.8% preparative agarose gel, with 1Kb DNA ladder as the marker.
3. The 3.91 Kb band was cut out with a sterile surgical blade and gel purified using GeneClean II (BIO 101) kit.
4. An aliquot of the eluted DNA was checked on gel for visual estimation.

2.2.12.5.1.2. Random Priming Reaction

Labeling was done according to Feinberg and Vogelstein (1983).

1. 25 ng template DNA of 3.91 Kb pED4 Sal I fragment was taken in a volume of 33 μl sterile MQ-water.
2. Denatured in boiling water bath for 5 minutes and quickly placed in ice for 5 minutes.
3. Centrifuged briefly in the cold.
4. The following reagents were added to the DNA in the order listed:

   5 μl 10X Labeling Buffer which includes Random Octadeoxyribonucleotides
   6 μl dNTP mixture (2μl of dATP, dTTP, and dGTP)
   5 μl α 32P dCTP (3,000 ci/mmol, 50 μCi)
   1 μl DNA Polymerase I-Klenow fragment (5 units)

Total reaction volume was 50 μl.
5. Incubated at 37°C for 1 hour.
6. The reaction was terminated by adding 5 μl of 0.2 M EDTA.

2.2.12.5.1.3. Determination of % Incorporation

1. 1 μl of the reaction sample was diluted to 100 μl with 0.2 M EDTA.
2. 5 μl of the diluted sample was spotted onto each of two DE-81 filters. One of the filters was used to measure total radioactivity in the reaction and was not subsequently washed. The other filter was used to measure radioactivity incorporated.
3. The filters were dried under a heat lamp for 10 minutes.
4. One filter was added to a beaker containing 100 ml of 0.5 M Na₂HPO₄ and washed for 5 minutes, with mild agitation. Washing was repeated twice using fresh 0.5 M Na₂HPO₄.

5. The filter was then washed twice with MQ-water and twice with ethanol.

6. The filter was dried under a heat lamp for 15 minutes and placed in a scintillation vial. The cerenkov counts of the filters were read and the ratio of washed to unwashed counts was calculated which gave the % incorporation.

2.2.12.5.1.4. Purification of Labeled Probes

Probes synthesized were separated from unincorporated nucleotides by filtration through Sephadex® G-50 spin column. The reaction volume was increased to 200 µl with 1X TE (pH 8.0) before purification.

2.2.12.5.2. Preparation of Probe Solution for Hybridization

1. For 9 ml prehybridization buffer, 900 µl probe solution was prepared. To the 200 µl probe solution, salmon sperm DNA was added at a concentration of 0.5 mg/ml, and the volume was made upto 900 µl with sterile MQ-water.

2. The probe solution was denatured by heating for 10 minutes in a boiling water bath and chilled on ice for 15 minutes before adding to the prehybridization buffer.

2.2.12.5.3. Hybridization

Hybridization was carried out in hybridization bottles, at 42°C, for 16 hours.

2.2.12.5.4. Post-hybridization Washing

1. The membrane was washed with 2X SSC at room temperature for 10 minutes.

2. The second washing was with 2X SSC/1% SDS at 42°C for 20 minutes. The washing was repeated.

3. The membrane was then washed with 0.2X SSC/1% SDS at 42°C for 20 minutes. The washing was repeated.

4. After each wash (steps 2 and 3) the background count was monitored with a Hand Monitor to avoid washing off specifically bound signal.

2.2.12.5.5. Exposing and Developing of Film

1. The membrane was wrapped securely in a saran wrap, and marked with fluorescent ink at different positions to align the blot on the film later.
2. The blot was exposed to Kodak X-Omat™ film in a film cassette, and incubated at --80°C for 2 hours.

3. The film was developed, aligned with the blot and tracing, to mark the positions of the bands.

2.2.13. Curing of URA3 Marker

1. Two of the disruptants, N-2 and N-14, were streaked on YNB-dextrose-uridine-FOA plate for single colonies, and grown at 30°C for 2 days.
2. The single colonies were patched simultaneously on SD and SD-uridine plates, and grown at 30°C for a day.

2.2.13.1. Checking by Southern

Eight single colonies of N-2 (N-2-1 to N-2-8) and N-14 (N-14-1 to N-14-8), were inoculated in 10 ml YEPD medium supplemented with uridine. The cultures were grown till saturation, and genomic DNA isolation was followed by Southern, done exactly as described in sections 2.2.11. and 2.2.12.

2.2.14. Disruption of the Second Allele of NAG1

1. The Ura cured disruptants N-2-1 and N-14-1, were selected for disruption of the second allele.
2. Transformation with SalI fragment of pED4-11 was done in the same way as done in the case of first allele disruption.
3. Eight Ura positive transformants of each (N-2-1-1 to N-2-1-8 and N-14-1-1 to N-14-1-8) were checked for disruption by Southern.
4. One selected disruptant (N-2-1-6) was streaked on on YNB-dextrose-uridine-FOA plate for single colonies and grown at 30°C for 2 days. The single colonies were patched simultaneously on SD and SD-uridine plates, and grown at 30°C for a day.
5. Eleven single colonies were checked by Southern, and the final Ura cured disruptant selected was N-2-1-6-1.

2.2.15. Growth of Mutants on Aminosugars GlcNAc and GlcN

The wild-type strain SC5314 (NAG1/NAG1, URA3/URA3), the heterozygous mutant N-2 (NAG1/nag1, URA3/ura3) and the homozygous mutant N-2-1-6 (nag1/nag1, URA3/ura3) were
streaked on YNB-2% Glucose, YNB-2% N-acetylglucosamine, and YNB-2% Glucosamine plates, in duplicates. Incubated at 30°C and 37°C, for 2 days.

2.2.16. Reintroduction of a single copy of NAG1 in N-2-1-6-1

2.2.16.1. Preparation of NAG1: hisG-URA3-hisG Construct

2.2.16.1.1. Preparation of Vector

1. 4 µg DNA of pED4 was partially digested with Neo I in 80 µl reaction volume containing 1X reaction buffer, and 10 units of Neo I. The reaction was incubated at 37°C and checked every 10 minutes until the linear band was obtained.

2. The digestion was loaded on a 0.8% preparative agarose gel, with 1 Kb DNA ladder as the marker.

3. The 7 Kb linear band was cut out with a sterile surgical blade, and purified using Geneclean II (BIO 101) kit.

4. An end-fill-in reaction was done in 15 µl reaction volume containing 1X Klenow buffer, 33 µM of each dNTP, and 1 unit Klenow. Incubated at 25°C for 15 minutes. Enzyme was heat inactivated at 75°C for 20 minutes.

5. DNA was extracted with phenol: chloroform: isoamylalcohol once, followed by chloroform: isoamylalcohol extraction. Precipitated with sodium acetate and absolute alcohol. Pellet was washed with 70% ethanol, air-dried, and resuspended in 20 µl sterile MilliQ-water. 1 µl DNA was loaded and estimated on a fresh gel.

6. A dephosphorylation reaction was done in 50 µl reaction volume containing 1X dephosphorylation buffer and 1 µl Alkaline Phosphatase (Boehringer Mannheim). Incubated at 37°C for 1 hour. Enzyme inactivated at 65°C for 10 minutes.

7. DNA was extracted with phenol: chloroform: isoamylalcohol once, followed by chloroform: isoamylalcohol extraction. Precipitated with sodium acetate and absolute alcohol. Pellet was washed with 70% ethanol, air-dried, and resuspended in 10 µl sterile MilliQ-water. 1 µl DNA was loaded and estimated on a fresh gel.

2.2.16.1.2. Preparation of Insert

The end-filled 4 Kb fragment of pCUB6 (section 2.2.8.2.) was used.
2.2.16.1.3. Ligation

Ligation reaction was set up using 50 ng vector, at a vector: insert ratio of 1:3 nanomole ends, in 15 μl reaction volume containing 1X ligase buffer, 15% PEG 4000, and 1 μl T4 ligase (NEB). Incubated at 20°C for 16 hours. Ligase was inactivated at 65°C for 10 minutes.

2.2.16.1.4. Transformation

Transformation of DH5α was done with the entire ligation mix, and the entire transformation mix was plated on LB ampicillin plates.

2.2.16.1.5. Screening for Recombinants

1. Fifty-three transformants were inoculated for minipreps.
2. Plasmids isolated were run on a 0.8% agarose gel with pED4 -11 as the marker.
3. 2 μg DNA of clones with retarded migration was digested with Sal I in 20 μl reaction volume containing 1X reaction buffer, 1X BSA, 10 μg/ml RNaseA and 8 units of Sal I. The reactions were incubated at 37°C for 4 hours.
4. The digestions were run on a 0.8% agarose gel, with 1 Kb DNA ladder as the marker.
5. 2 μg DNA of selected clones giving the expected bands were digested with Xba I in 20 μl reaction volume containing 1X respective reaction buffer, 1X BSA, 10 μg/ml RNaseA and 6 units of Xba I. The reactions were incubated at 37°C for 4 hours.
6. Reactions were loaded with 1X EndoR and run on a 0.8% agarose gel with 1 Kb DNA ladder as marker.

2.2.16.1.6. Medium Scale Plasmid Isolation of the Recombinant

The selected clone P33 was inoculated in 35ml TB ampicillin and DNA isolated by the method described earlier.

2.2.16.2. Transformation of N-2-1-6-1 with P33

2.2.16.2.1. Sal I Digestion of P33 to Release the Cassette

1. 10 μg of P-33 was digested with Sal I in 25 μl reaction volume containing 1X reaction buffer, 1X BSA, and 80 units of Sal I. The reaction was incubated at 37°C overnight.
2. 2 μl of the digestion was run on a 0.8% agarose gel, with 1 Kb DNA ladder as the marker, to check for completion of digestion.
2.2.19. Induction of Germ Tubes with 2.5 mM GlcNAc

This protocol was adopted from Delbruck and Ernst, 1993.

1. Cells of SC5314, N-2; N-2-1-6 and P-4 were patched on YEPD plates and grown at 30°C overnight.

2. Culture of each was prepared by inoculating the strains in 10 ml YEPD, and grown at 30°C with shaking at 200 rpm, to the logarithmic growth phase.

3. Cells were harvested by centrifuging at 5,000 rpm for 5 minutes, were washed twice with sterile MQ-water, and resuspended in 5 ml MQ-water.

4. Cells were starved overnight at 30°C, with shaking at 120 rpm.

5. The OD of each was taken and cells (OD600 =0.5) were induced with 2.5 mM GlcNAc in salt base, containing 0.45% NaCl and 0.335% Yeast Nitrogen Base w/o amino acids. Cells were induced at 37°C for 4 hours with 200 rpm shaking.

6. Cells were fixed with 4% Formaldehyde, and germ tube formation was examined under a light microscope.

2.2.20. Microscopy

All gel photographs were taken with a polaroid camera. Southern blots were directly scanned in a UMAX Scanner (Astra1220P) at 300 dpi. Petriplates were photographed and scanned, or directly scanned. Photographs of germ tubes were taken in Leica DMIL Inverted Microscope with an automated camera attached to the microscope. Photographs were taken with 40X objective to obtain a ×400 magnification.
2.3. RESULTS AND DISCUSSION

2.3.1. Disruption of \( NAG1 \) by \( \text{Ura-blaster} \) Technique

The strategy adopted for disruption of \( NAG1 \) 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 \textit{et al.}, 1981). The replacement event can even be monitored, by including an appropriate selection marker on the incoming molecule (Scherer and Davis, 1979), but in many cases it is not easy to remove the marker and thus the same selection cannot be used for subsequent gene replacements.

The \( \text{hisG-URA3-hisG} \) construct was developed to make repeated use of a convenient selectable marker, in which the \( \text{URA3} \) gene of \( \text{C. albicans} \) is flanked by direct repeats of the bacterial \( \text{Salmonella typhimurium} \) sequence \( \text{hisG} \) (Alani \textit{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 \( \text{hisG} \) repeats to eliminate the \( \text{URA3} \) gene, and leave behind a single copy of the \( \text{hisG} \) 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 \textit{et al.}, 1984). The process has been schematically explained in Fig.2.5. The \( \text{URA3} \) gene encodes orotidine-5'-phosphate decarboxylase, an enzyme required for the biosynthesis of uracil. Selection of \( \text{ura3} \) cells (cells that have lost \( \text{URA3} \)) is accomplished by growing the cells on media containing 5-FOA. This compound is apparently converted to a toxic product, 5-fluorouracil by the action of the decarboxylase, killing the \( \text{URA3} \) cells, but \( \text{ura3} \) cells are resistant to 5-FOA and can grow on 5-FOA plates. 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.

2.3.2. Disruption of \( NAG1 \) in \( \text{C. albicans} \)

\textbf{2.3.2.1. Construction of Cassette for Disruption of \( NAG1 \) in \( \text{C. albicans} \)}

The clone pED4 was used for creating the deletion insertion mutants of \( \text{nag1} \) gene. pED4 is the 3.915 Kb \( \text{Sal I} \) fragment from \( \text{C. albicans} \) SC5314, cloned into pBSKS (Kumar \textit{et al.}, 2000), housing the glucosamine-6-phosphate deaminase (\( NAG1 \)) gene. Sequence analysis revealed that it contains the 747 bp \( NAG1 \) ORF, with a 1.725 Kb upstream region, and a 1.442 Kb downstream region. The \( NAG1 \) ORF is without introns, and encodes a protein of 248 amino acids. pED4 was used for the construction of disruption cassette (Fig.2.2.A.). Plasmid pCUB6
A. pED4 digested with Neo I

B. 4.9 Kb backbone end-filled

C. The 31 bp and 346 bp boxes represent parts of derivative plasmids.

D. 4 Kb BamHI-Bgl II fragment of pCUB6 end-filled

E. Neo I deleted pED4 was ligated to 4Kb fragment of pCUB6 to obtain the clone pED4-11 which contains the cassette for disruption of NAG1. Digestion with Sal I released the 5.97 Kb disruption cassette.

Fig. 2.2. Schematic Diagram Showing Construction of the Cassette for Disruption of NAG1.
was obtained from Dr. W. Fonzi (Fonzi and Irwin, 1993). pED4 was digested with Sal I, to check for the release of the 3.915 Kb band, and the 2.96 Kb pBSKS backbone (Fig.2.3.A.). The restriction enzyme Neo I was chosen, as it could remove the entire NAGl ORF (Fig.2.2.B.), and the fact that Neo I could be conveniently used since pBSKS lacks an Neo I site. The strategy adopted for disruption of NAGl has been schematically shown in Fig.2.2. The positions of the Neo I sites with respect to NAGl ORF, has been shown schematically (Fig.2.2.A.).

The plasmid pCUB6 is a derivative of pNKY50 (Fonzi and Irwin, 1993), which contains the C. albicans URA3 gene flanked by direct repeats of S. typhimurium hisG sequences. The construct has been shown schematically (Fig.2.2.C.), in which sizes of the respective sequences are given. On digestion with BamH I and Bgl II, pCUB6 released a 4 Kb fragment containing the hisG-URA3-hisG cassette (Fig.2.2.D.), but an overlapping fragment from the vector backbone was also released. To enhance the efficiency of cloning with the fragment of our interest, the backbone was first broken with Pvu II, which generated a linear band. After complete digest with Pvu II, further digest with Bam H I and Bgl II released the 4 Kb hisG-URA3-hisG fragment, free of the overlapping band. Bands of 4 Kb, 2.3 Kb and 1.7 Kb were obtained, as expected (Fig 2.3.B.). Both Neo I deleted pED4 vector, and BamH I-Bgl II fragment of pCUB6, were made flush with Klenow polymerase and ligated.

To screen for recombinants, the higher migrating clones of minipreps were first digested with Sal I, which released the 5.97 Kb cassette of nagl: hisG-URA3-hisG: nagl, along with the pBSKS backbone (Fig.2.4.A.). Single digest with Pvu II also released the cassette, and showed a 6.3 Kb band housing the disruption cassette, and a 2.5 Kb band from pBSKS backbone (Fig.2.4.B.). Further digest with EcoR V was done to confirm the presence of hisG-URA3-hisG cassette, since each of the hisG sequences has an EcoR V site, positions shown in Fig.2.2.D. The bands obtained were of 5.37 Kb, 2.44 Kb, 723 bp, and 450 bp sizes, (Fig.2.4.B.). The digest also showed the orientation in which the cassette got ligated, schematically shown in Fig.2.2.E. The recombinant selected was pED4-11.

2.3.2.2. Disruption of the First Allele of NAG1 in C. albicans

The Southern blot (Fig.2.6.B.) shows the first allele disruptants of NAG1. The genomic DNAs were digested with Sal I (Fig.2.6.A.) which on hybridization with the 3.91 Kb Sal I fragment of pED4 showed bands of 3.91 Kb and 5.97 Kb in the disrupted transformants. The 3.91 Kb Sal I fragment of the genomic DNA represents the undisrupted allele of NAG1 and the 5.97 Kb Sal I fragment represents the disrupted NAG1 allele in which the disruption cassette nagl: hisG-
Fig. 2.3. Restriction Digestions of the Parent Clones pED4 and pCUB6.
A. 2 μg DNA of pED4 digested with *Sal* I, showed bands of 3.915 Kb that houses the *NAG1* gene, and the 2.96 Kb pBSKS backbone.
B. 2 μg DNA of pCUB6 digested with *BamHI*, *BglII*, and *PvuII*. The 4 Kb *BamHI-BglII* band contains the *hisG-URA3-hisG* cassette. Digestion with *PvuII* was to get rid of the overlapping 4 Kb fragment of vector backbone, which breaks into 2.3 Kb and 1.7 Kb bands. Digests were run on 0.8% agarose gels with 1 Kb DNA ladder as the mark.
Fig. 2.4. The Disruption Cassette of NAG1.

A. 2 µg DNA of pED4-11 digested with Sal I showed a 5.97 Kb band containing the nag1:hisG-URA3-hisG:nagI cassette, and the 2.96 Kb pBSKS backbone.

B. 2 µg DNA of pED4-11 digested with Pvu II released the nag1:hisG-URA3-hisG:nagI cassette in the 6.3 Kb band, and the 2.5 Kb band from pBSKS backbone. Digestion with EcoRV released bands of 5.37 Kb, 2.44 Kb, 723 bp and 450 bp, as the hisG sequences each had an EcoRV site. Digestions were run on a 0.8% agarose gels with 1 Kb DNA ladder as the marker.
A. *C. albicans* transformed with the 5.97 Kb *Sal I* fragment from pED4-11

B. Homologous recombination and excision of *URA3* with one *hisG* sequence.

C. Selection on 5-FOA for *ura3* cells. Transformation and selection on 5-FOA repeated for disruption of the second allele.

D. Both alleles disrupted, one *hisG* sequence remains.

**Fig. 2.5.** Schematic Diagram Showing Disruption of *NAG1* by “Ura-blaster” Technique.
Fig. 2.6. Disruption of the First Allele of NAG1 Showing Insertion of the Disruption Cassette at NAG1 Locus.

A. Genomic DNA (1.5 µg to 2 µg), of fifteen Ura+ transformants (transformed with NAG1 disruption cassette) of CAF3-1, digested with SalI. Digestions run on 0.8% agarose gel with 1 Kb DNA ladder as the marker.

B. Southern blot: the 3.91 Kb SalI fragment represents the undisturbed allele of NAG1. The 5.97 Kb band represents insertion of the disruption cassette nag1:hisG-URA3-hisG:nag1 into the other allele. N-2 and N-14 marked with asterisks were selected.
URA3-hisG; nagl got integrated. As shown in Fig.2.6.B., ~50% of the transformants checked were disruptants.

2.3.2.3. Curing of URA3 from the First Allele Disruptants

N-2 and N-14, marked with asterisks in Fig.2.6.B., were selected and streaked on YNB-dextrose-uridine-5-FOA plates to select for the ura3 segregants. The colonies which grew on 5-FOA plates were streaked simultaneously on SD and SD-uridine plates to ensure that they could grow only in presence of uridine, and the loss of URA3 gene was then confirmed by Southern (Fig.2.7.B.). The ura3 strains showed a 3 Kb Sal I fragment, the undisrupted NAG1 allele however showed a 3.91 Kb Sal I band as before. The reduction in size of the disrupted allele was because of excision of URA3 along with one hisG sequence, shown schematically in Fig.2.5.C. Colonies selected from 5-FOA plates showed curing with almost 100% efficiency (Fig.2.7.B.). The selected Ura cured strains were N-2-1 and N-14-1 (Fig.2.7.B.).

2.3.2.4. Disruption of the Second Allele of NAG1

Disruption of the second allele of NAG1 was done in a similar manner in the Ura cured strains N-2-1 and N-14-1. Integration of the cassette is shown in the Southern blot (Fig.2.8.B.). The 3.91 Kb Sal I band was no longer present in the disruptants, because the disruption cassette got integrated into the remaining allele of NAG1, and showed a 5.97 Kb Sal I band. The first disrupted and Ura cured allele showed a 3 Kb band, seen in the blot (Fig.2.8.B.). N-2-1-6 was the selected strain, marked with an asterisk in the figure.

2.3.2.5. Curing of URA3 from the Second Allele Disruptant N-2-1-6

N-2-1-6 was streaked on 5-FOA plate to select for ura3 segregants, and was confirmed by Southern as before (Fig.2.9.B.). The ura3 mutant, N-2-1-6-1 (Fig.2.9.B.), showed two 3 Kb Sal I bands as expected. Sequential disruption of NAG1 is shown in Fig.2.10.

2.3.3. Inability of the Null Mutant to Utilize N-acetylglucosamine and Glucosamine for Growth

Since 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
Fig.2.7. Curing of URA3 from the Selected First Allele Disruptants (N-2 and N-14) of NAG1.

A. Genomic DNA (1.5 μg to 2 μg) of eight transformants of each N-2 and N-14, from 5-FOA plates, digested with SalI. Run on a 0.8% agarose gel with 1 Kb DNA ladder as marker.

B. Southern blot: the Ura cured strains showed a band of 3 Kb size. The 3.91 Kb band represents the undisrupted second allele. Almost all the transformants seen in the blot is cured of Ura. The selected strains N-2-1 and N-14-1 are marked with asterisks.
Fig. 2.8. Disruption of the Second Allele of NAG1.

A. Genomic DNA (1.5 μg to 2 μg) of eight selected Ura+ transformants of each N-2-1 and N-14-1 transformed with the disruption cassette were digested with SalI and run on 0.8% agarose gel with 1 Kb DNA ladder as the marker.

B. Southern blot: the cassette on integration at the NAG1 locus shows a 5.97 Kb band. The other band of 3 Kb size represents the first disrupted and Ura cured allele of NAG1. N-2-1-6, marked with asterisk, was selected.
Fig. 2.9. Curing of URA3 from the Second Allele Disruptants of NAG1.
A. Genomic DNA (1.5 μg to 2 μg) of eleven transformants of N-2-1-6 from 5-FOA plate were digested with SaI I and run on 0.8% agarose gel with 1 Kb DNA ladder as the marker. B. Southern blot: transformants cured of Ura showed two 3 Kb bands. N-2-1-6-1, marked with asterisk, was selected.
Fig. 2.10. Disruption of *NAG1* in *Candida albicans* Shown in a Sequential Manner. Disruption of both alleles of *NAG1* shown in a serial manner in the Southern blot (B). The corresponding gel is shown in (A). SC5314 (wild-type, *URA3/URA3*), N-2 (1st allele mutant, *URA3/ura3*), N-2-1 (1st allele mutant, *ura3/ura3*), N-2-1-6 (both alleles mutant, *URA3/ura3*), N-2-1-6-1 (both alleles mutant, *ura3/ura3*).
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. Only the Ura positive strains were considered for functional studies, because disruption of URA3 has been reported to significantly decrease the in vivo growth rate and pathogenicity of C. albicans (Kirsch and Whitney, 1991). Therefore, strains tested for studying mutation effect must have a functional URA3 gene. It was observed that the null mutant N-2-1-6 could not utilize GlcNAc (Fig.2.11.B.). The NAGI/nagI 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 (Fig.2.11.A.).

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 NAGI (Fig.2.1.). The homozygous mutant N-2-1-6 was impaired in utilization GlcN, and the single allele mutant N-2 displayed a slow growth (Fig.2.11.C.). Overall, a slower rate of utilization of GlcN by C. albicans was observed, as compared to the rate of utilization of GlcNAc by this organism. Glucosamine-6-phosphate is the common intermediate in GlcNAc and GlcN metabolism. The next product fructose-6-phosphate (Fig.2.1.), is not specific to this pathway. So on disruption of the glucosamine-6-phosphate deaminase gene, it was expected that the mutant would be unable to utilize glucosamine, which was subsequently confirmed.

The aminosugar utilization pathway in C. albicans is very similar to the GlcNAc catabolic pathway of E. coli (Plumbridge, 1990), in which both the aminosugars GlcNAc and GlcN can induce the Nag regulon. GlcNAc is also a good carbon source for E. coli, producing growth rates comparable to those of glucose, and results in high level of induction of both NagE (encodes the transporter GlcNAc-specific EII of the PTS) and NagB (encodes glucosamine-6-phosphate deaminase) genes (Peri and Waygood, 1988; Rogers et al., 1988). Like in C. albicans, E. coli shows a very slow growth on GlcN, the induction of regulon being also less. The reason for slow growth on GlcN plates could be because of the low affinity of the GlcNAc transporter for GlcN. In E. coli it has been shown that there exists a common PTS transport system for these two aminosugars (Plumbridge, 1990).
Fig. 2.11. The Homozygous Mutant of GlcNAc Catabolic Pathway was Unable to Grow on Aminosugars.

SC5314 (wild-type), N-2 (heterozygous mutant), N-2-1-6 (homozygous mutant).
A. Growth on YNB-2% Glucose plates (SD).
B. Growth on YNB-2% N-acetylglucosamine (GlcNAc).
C. Growth on YNB-2% Glucosamine (GlcN).
2.3.4. Identification of Genomic Cluster Containing GlcNAc Catabolic Pathway Genes

2.3.4.1. Generation of NAG1 Revertant

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 NAG1 was introduced at the NAG1 locus in the null mutant N-2-1-6-1, to create a revertant strain. To select for the transformants the hisG-URA3-hisG cassette was inserted into pED4. This insertion cassette can revert the transformant back to ura3, and can be used repeatedly for genetic manipulations, which is a great advantage, as the selection markers are limited in C. albicans.

The construction of NAG1: hisG-URA3-hisG cassette for introduction of NAG1 has been shown schematically in Fig.2.12. Because of the unavailability of any suitable restriction site outside the NAG1 ORF, a partial digest of pED4 was done with Neo I. The first Neo I site lies in the promoter region of NAG1, and would disturb the function of NAG1, but the second site falling outside the NAG1 ORF, would not disturb the function of the gene (Fig.2.12.A.). The target clone was in which the hisG-URA3-hisG sequence would get ligated at the second Neo I site (Fig.2.12.B.). For cloning purpose the Neo I ends were polished and ligated to the polished BamHI/BglII ends of the 4 Kb hisG-URA3-hisG fragment of pCUB6. The recombinants were first selected by digesting the higher migrating miniprep clones with SalI (Fig.2.13.A.), which showed a 7.9 Kb band housing the hisG-URA3-hisG cassette within the 3.91 Kb SalI fragment of pED4, and the pBSKS backbone of 2.9 Kb.

A second screening was done to identify the clones in which the cassette got inserted at the second Neo I site, by digesting with XbaI. The positions of XbaI can be seen in Fig.2.12.C. Analysis of XbaI digestion showed clones in which the cassette was inserted at the second Neo I site, and also showed the orientation in which it was present, though orientation was not of any importance. The clone P33 produced the expected bands of 5.38 Kb, 3.53 Kb, and 1.99 Kb on XbaI digestion (Fig.2.13.B.). The SalI fragment of this clone was the vehicle that reintroduced NAG1 back into the genome of the null mutant, to create the NAG1 revertant strain. The Southern blot (Fig.2.13.C.) confirmed integration of the NAG1: hisG-URA3-hisG cassette into the genome at NAG1 locus. It showed a 3 Kb SalI band, as seen in N-2-1-6-1, and the other 3 Kb fragment in which the cassette got integrated showed a 7.9 Kb SalI band (Fig.2.13.C.). As seen in the figure, N-2-1-6-1 + P33 - 2, 34, and 37 showed the right sized bands. N-2-1-6-1 + P33 - 34 was chosen as the revertant, and was renamed N-2-1-6-1 + P33 for convenience.
A. pED4 with two Nco I sites

B. pED4 partial digested with Nco I, end-filled

C. hisG-URA3-hisG cassette ligated to the second Nco I site without disturbing NAG1 ORF was the desired construct.

D. B. hisG-URA3-hisG cassette ligated to the first Nco I site disturbed the NAG1 ORF, and was not the desired construct.

Fig. 2.12. Schematic Diagram Showing the Strategy for Reintroduction of One Copy of NAG1 into the Genome of the Null Mutant.
Fig. 2.13. A Hexokinase (HXK1) Gene Discovered in the Process of Reintroduction of NAG1 in the Mutant.

A and B show the gel photographs of digests of P31 and P33. P31 represents the clone with the reintroduction cassette at the first Neo I site and P33 represents the clone with the cassette at the second Neo I site.

C. Southern blot: integration of the cassette created a strain with functional HXK1 (N-2-1 + P31) and a hxxl mutant strain (N-2-1-6-1 + P33). Both the integrations showed bands of 7.9 Kb and 3 Kb, selected ones marked with asterisk.
2.3.4.2. Inability of NAG1 to Complement Function in the ‘Revertant’ Strain

The strain with one copy of NAG1 was expected to behave like the heterozygous mutant N-2, and grow on GlcNAc, but the strain failed to restore growth (Fig.2.16.) 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 NAG1 to restore function came as a total surprise. Southern was performed to ensure integration at the right position. When Southern (Fig.2.13.C.) showed integration at the right position, there was only one possibility: that some gene adjacent to NAG1 was disturbed while constructing the cassette for reintroduction of 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 (Fig.2.14.A.). Clustal W analysis of the hexokinase (Fig.2.14.C.) 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. There is a high degree of homology (97%) of this stretch containing the DAC1 coding region to a deacetylase sequence published on the web by the C. albicans genome project (http://alces.med.umn.edu/candida.html, and http://candida.stanford.edu/). The promoter regulating NAG1 gene is a bidirectional one, also responsible for regulation of DAC1. So, while creating NAG1 mutant, DAC1 was also functionally impaired, as the first Neo I site lies in the promoter.

The positions of DAC1, NAG1 and HXXK1 have been shown schematically in Fig.2.14.A. The BLAST search results (Fig.2.14.B.) show the positions of the three genes present in the cluster. N-2-1-6-1 + P-33 therefore created a dac1nag1hxkl/DAC1NAG1hxkl strain, which would be considered the hexokinase mutant in further functional assays, since one copy of NAG1 was sufficient for function, as observed earlier. To examine the effect of GlcNAc on transcription of the genes responsible for catabolism, a Northern analysis of the GlcNAc induced RNA was performed. DNA fragments corresponding to DAC1, NAG1, and HXXK1 were radiolabeled and
Fig. 2.14.A. Schematic Diagram Showing the Positions of DAC1 NAG1 HXK1 in the Cluster.

Fig. 2.14.B. A Graphical Representation of the Output of a Nonredundant BLAST Search. The search was done on the GenBank database with pED4 sequence at the NCBI web site. The results show that the sequence has high homology with three genes: GlcN-6-P-deaminase (NAG1), GlcNAc-Deacetylase (DAC1) and a Hexokinase (HXK1). Each bar represents a homologous region found in a sequence from the GenBank database. The gradation of homology: red bars are highly homologous, followed by pink, green, blue and black, in a decreasing order for homology. The homology is scored on the occurrence of identical amino acid residues, followed by a lesser score for similar residues, on a three frame translation of the query sequence. A weak homology is observed with many membrane associated proteins at the 3' side of the sequence.
The HXX1 coding region available in the clone pED4 has been aligned to the sequences available in the database and the alignment shows that the gene is indeed a hexokinase, which shows reasons to be called the GlcNAc phosphorylating enzyme. The amino acid sequence from *Candida* (CAHXK) has been aligned to Hexokinase sequences from *Saccharomyces* (HXKBY, and HXKAY), *Kluyveromyces lactis* (KLULA), Rat (HXKRA), Mouse (MOUSE), Human (HXHUM), *Xenopus laevis* (XLEVS), *Aspergillus niger* (ASPNG). The identical residues (red) are indicated by an asterisk (*), residues with high similarity (green) by a colon (:), and those of low similarity (blue) by a dot (.), dashes indicate gaps introduced to show best alignment. The numbers on the right indicate the position in the amino acid sequence. The portion with the best identity is shown. The sugar binding residues are indicated by a red dot (•).
used as probes. The results showed that the genes \textit{NAGJ}, \textit{DACJ}, and \textit{HXKJ} are transcribed in response to GlcNAc induction, and remain uninduced when grown in glucose (Kumar et al., 2000). The hexokinase mutant when checked on YNB-2\% GlcN for growth, was unable to utilize GlcN (Fig.2.16.B.) for growth. This was unexpected, since a different kinase was believed responsible for the phosphorylation of GlcN, so far. Therefore a functional deaminase (\textit{NAGJ}) was assumed capable of glucosamine catabolism. But, the results showed otherwise. The indispensability of \textit{HXKJ} for function positively indicates its being the GlcNAc kinase responsible for phosphorylation of both aminosugars GlcNAc and GlcN.

\textbf{2.3.5. Functional Defect 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. Thirteen transformants were able to grow on GlcNAc, and integration was further confirmed by Southern. On digestion of genomic DNAs with Sal I (Fig.2.15.A.), the 3.91 Kb band could be seen in most of the transformants. Three positive transformants (marked with asterisks in Fig.2.15.A.) were confirmed for integration in the right orientation by digesting DNAs with \textit{Xba I} and \textit{Sca I} (Fig.2.15.B.). Integration in the right orientation was important in this case for restoration of function, though the fact that it could grow on GlcNAc and restore the functional defect, was also confirmatory. Among the strains that showed bands similar to wild-type pattern, N-2-1-6 + P4-9 was selected, and renamed P-4. This strain would be referred as the revertant strain in future sections. Growth of P-4 on GlcNAc and GlcN has been shown in Fig.2.16. This also confirmed that the growth defect of the null mutant on GlcNAc and GlcN, was solely because of disruption of the GlcNAc catabolic pathway genes.

\textbf{2.3.6. Generation of a Strain with a Functional HXK1 in dac1nag1 background}

While cloning for insertion of \textit{hisG-URA3-hisG} at the second \textit{Neo} I site, a clone P31 was obtained, in which the cassette got inserted at the first \textit{Neo} I site (Fig.2.12.D.). Since that disturbed the \textit{NAGJ} function, being located in the promoter (it is to be noted that presence of \textit{DAC1} in the cluster was not known then), the clone appeared of no importance, and P33 was used for introducing \textit{NAG1} in the null mutant N-2-1-6-1 (section 2.3.4.1.). After \textit{HXK1} was
Fig. 2.15. Reintroduction of NAG1 in the Homozygous Mutant Using the 3.91 Kb Sal I Fragment of pED4.

A. Southern blot: genomic DNA of all the transformants which could grow on GlcNAc digested with Sal I to confirm integration. Three selected ones marked with asterisks were further confirmed for site of integration.

B. Southern blot: site of integration was confirmed by digesting genomic DNA with Xba I and Sca I which gave bands of sizes similar to the parent strain. Xba I and Sca I have sites located on regions flanking the 3.915 Kb Sal I fragment, besides the 3.915 Kb fragment also has one Xba I and one Sca I site.
Fig. 2.16. Growth of Wild-type and Mutants on Aminosugars.

A. Growth on YNB-2% N-acetylglucosamine. The numbers on the plates indicate the strains:

B. Growth on YNB-2% Glucosamine.

The presence of a copy of $HXK1$ in N-2-1 + P31, or a copy of $DAC1NAG1$ in N-2-1-6-1 + P33 was not sufficient to restore growth on aminosugars. The revertant strain P-4 with a copy of $DAC1NAG1HXK1$ could restore growth, seen in A and B.
discovered, we were interested to create a strain with one copy of \textit{HXK1} in the \textit{dac1nag1} mutant to study its function. For that, the 7.9 Kb \textit{Sal I} fragment of P31 was integrated into the genome of N-2-1 (Fig.2.13.B.). The integration disrupted one remaining copy of \textit{DAC1NAG1} in N-2-1, as P31 was a \textit{dac1nag1HXK1} construct, and created a \textit{dac1nag1HXK1/dac1nag1hxkl} strain, with a single copy of \textit{HXK1}. This mutant when checked for growth on aminosugars was unable to utilize GlcNAc and GlcN, which shows that the aminosugars were not catabolized even if phosphorylated on entry, because of absence of downstream genes, and that this pathway is indispensable for catabolism of aminosugars.

2.3.7. Growth of the Mutants on Other Aminosugars

The growth of \textit{C. albicans} and the mutant derivatives was also checked on mannosamine, \textit{N-acetylmannosamine (ManNAc)}, and \textit{N-acetylneuraminic acid (data not shown)}. The wild-type \textit{C. albicans} (SC5314), was unable to grow on these aminosugars even after incubation at 30°C for 10 days. It confirmed that ManNAc is not a growth substrate for \textit{C. albicans} (Sullivan and Shepherd, 1982), and does not agree with the report (Biswas et al., 1979), that \textit{C. albicans} can utilize ManNAc. The results showed that unlike \textit{E. coli} (Piumbridge and Vimr, 1999), \textit{C. albicans} did not have a varied choice for aminosugars as carbon source, but has a convergent pathway for utilization of the two aminosugars GlcNAc and GlcN. Existence of a convergent pathway for utilization of different aminosugars in \textit{E.coli} has been discussed in Chapter 1.

2.3.8. Null Mutant Impaired in Germ Tube Formation on Induction with GlcNAc

GlcNAc is known to induce cellular morphogenesis in \textit{C. albicans}. The yeast cells start producing germ tubes on induction with GlcNAc at 37°C (Shepherd et al., 1980). It was reported that immobilized GlcNAc bound covalently to agarose beads was capable of inducing germ tubes (Shepherd and Sullivan, 1983) in \textit{C. albicans}. Analysis of agarose-GlcNAc before and after germ-tube formation showed that the covalently bound GlcNAc was not removed during incubation with cells. It indicated that GlcNAc was an effective signaling molecule that probably triggered germ tube formation through a cell-surface receptor. GlcNAc therefore can function as a carbon and nitrogen source, and additionally as a signaling molecule for dimorphic transition in \textit{C. albicans}. 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 (Fig. 2.17.C.), while the heterozygous mutant N-2 (Fig. 2.17.B.) and the revertant strain P-4 (Fig. 2.17.D.) formed germ tubes as efficiently as the wild-type strain SC5314 (Fig. 2.17.A.). Germ tube formation is always accompanied by clumping of cells, which can be observed visually (Shepherd et al., 1980). As seen in Fig. 2.18., there was heavy clumping in the wild-type SC5314, heterozygous mutant N-2, and revertant strain P-4, which showed highly elongated germ tubes on examination under the microscope. N-2-1-6, which showed no clumping visually, remained locked in the yeast form when examined under the microscope.

To determine any role of the discovered GlcNAc kinase gene in signaling under GlcNAc inducible conditions, we checked the germ tube induction pattern of N-2-1 + P31 (dac1nag1hxkl/dac1nag1HXK1) and N-2-1-6-1 + P33 (dac1nag1hxkl/DAC1NAG1 hxk1), under similar inducible conditions with 2.5 mM GlcNAc (Fig. 2.17.E and F.). The strains remained locked in the yeast form, and showed no germ tube formation. The results suggested that the cascade of genes of the GlcNAc catabolic pathway was required for induction of germ tubes, and deaminase or kinase gene individually could not restore function. 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.
Fig. 2.17. Induction of Germ Tubes with 2.5 mM GlcNAc at 37°C for 4 hours.
A. SC5314 (wild-type). B. N-2 (heterozygous mutant). C. N-2-1-6 (homozygous mutant)
D. P-4 (revertant strain). E. N-2-1+P31 (HXX1 + strain). F. N-2-1-6-1+ P33 (hxxl mutant)
Fig. 2.18. Flocculation of Cells on Germ Tube Induction with 2.5 mM GlcNAc at 37°C for 4 hours.
A. SC5314 (wild-type).
B. N-2 (heterozygous mutant).
C. N-2-1-6 (homozygous mutant).
D. P-4 (revertant strain).
N-2-1-6, which failed to form germ tubes, showed no flocculation.