CELL SURFACE ANALYSIS OF THE BASIDIOMYCETE YEAST *CRYPTOCOCCUS NEOFORMANS*

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Cell surface analysis of the basidiomycete yeast

Cryptococcus neoformans

by

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Thesis Summary

Cell surface properties of the basidiomycete yeast *Cryptococcus neoformans* were investigated with a combination of novel and well proven approaches. Non-specific cell adhesion forces, as well as exposed carbohydrate and protein moieties potentially associated with specific cellular interaction, were analysed. Experimentation and analysis employed cryptococcal cells of different strains, capsular status and culture age.

Investigation of cellular charge by particulate microelectrophoresis revealed encapsulated yeast forms of *C. neoformans* manifest a distinctive negative charge regardless of the age of cells involved; in turn, the neutral charge of acapsulate yeasts confirmed that the polysaccharide capsule, and not the cell wall, was responsible for this occurrence. Hydrophobicity was measured by MATH and HIC techniques, as well as by the attachment of polystyrene microspheres. All three techniques, where applicable, found *C. neoformans* yeast to be consistently hydrophilic; this state varied little regardless of strain and culture age.

Cell surface carbohydrate and protein were investigated with novel fluorescent tagging protocols, flow cytometry and confocal microscopy. Cell surface carbohydrate was identified by controlled oxidation in association with biotin hydrazide and fluorescein-streptavidin tagging. Marked amounts of carbohydrate were measured and observed on the cell wall surface of cryptococcal yeasts. Furthermore, tagging of carbohydrates with selective fluorescent lectins supported the identification, measurement and observation of substantial amounts of mannose, glucose and N-acetyl-glucosamine. Cryptococcal cell surface protein was identified using sulfo-NHS-biotin with fluorescein-streptavidin, and then readily quantified by flow cytometry. Confocal imaging of surface exposed carbohydrate and protein revealed common localised areas of vivid fluorescence associated with buds, bud scars and nascent daughter cells. Carbohydrate and protein fluorescence often varied between strains, culture age and capsule status of cells examined. Finally, extension of protein tagging techniques resulted in the isolation and extraction of two biotinylated proteins from the yeast cell wall surface of an acapsulate strain of *C. neoformans*.

Key words: hydrophobicity, charge, lectin, biotin, SDS-PAGE

For my parents

Michael and Marjorie Foster

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Abbreviations

AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
API	analytical profile index
ATCC	American type culture collection
CGB	canavanine-glycine-bromthymol blue (agar)
CNS	central nervous system
Con A	Concanavalin A (*Canavalia ensiformis* agglutinin)
CSF	cerebrospinal fluid
CSH	cell surface hydrophobicity
DMSO	dimethyl sulphoxide
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
Gal	galactose
GalXM	galactoxylomannan
GlcA	glucuronic acid
GNL	*Galanthus nivalis* lectin
GXM	glucuronoxylomannan
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HIC	hydrophobicity interaction chromatography
HIV	human immunodeficiency virus
HMA	hydrophobic microsphere attachment
Hn	Hill coefficient
LCA	*Lens culinaris* agglutinin
LDV	laser Doppler velocimetry
Log	logarithmic (growth phase)
LSD	least significant difference
LTL	*Lotus tetragonolobus* lectin
Man	mannose
MATH	microbial adherence to hydrocarbons
MBL	mannose binding lectin

MercPMSF	see Appendix D
OD	optical density
PBS	phosphate buffered saline
PBS C/M	PBS with 1mM CaCl2 and 1 mM MgCl2 pH 7.4
Phosphate+ buffer	see Appendix E
PSA	*Pisum sativum* agglutinin
psi	pounds per square inch
PUM	phosphate urea magnesium (buffer), see Appendix B
SBA	soybean agglutinin
SDS	sodium dodecyl sulphate
SDSMerc	0.1 % SDS - 1 % v/v 2-mercaptoethanol
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Stat	stationary (growth phase)
sulfo-NHS-biotin	sulfo-N-hydroxysuccinimidobiotin
TEMED	N,N,N'N'-tetramethylethylenediamine
TIFF	tagged image file format
UEA I	*Ulex europaeus* agglutinin I
v/v	volume for volume
w/v	weight for volume
WGA	wheat germ agglutinin
Xyl	xylose

CHAPTER 1 – Introduction

1.1 The basidiomycete *Cryptococcus neoformans*

1.1.1 Discovery and classification

Cryptococcus neoformans (sexual state *Filobasidiella neoformans*) is one of 38 or so species which make up the genus *Cryptococcus*. It was discovered, both as a human pathogen and as an environmental organism, in 1894. Two German physicians, Otto Busse and Abraham Bushke, isolated the organism from the tibial lesion of a 31 year old woman and named it *Saccharomyces hominis* (referred to in Benham, 1935). In the same year, an Italian by the name of Sanfelice isolated the organism from fermenting peach juice and named it *Saccharomyces neoformans*. From this time until the 1950s, various different names were used to describe the organism, which then became more universally known as *C. neoformans* (Benham, 1935; Barnett *et al.*, 1983; Casadevall & Perfect, 1998).

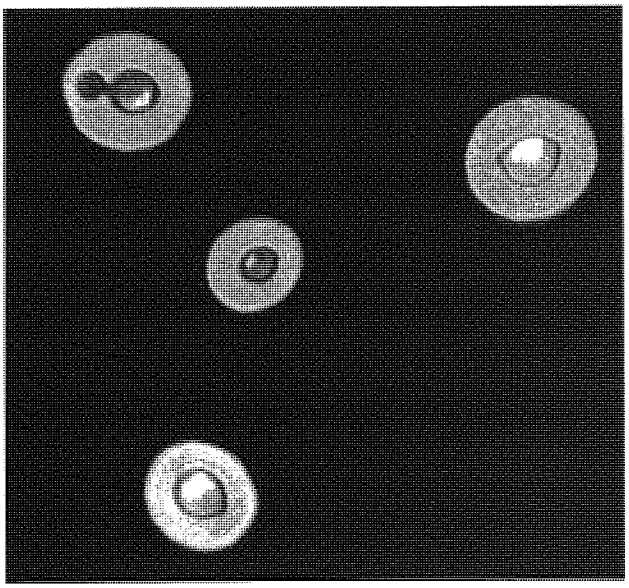


Figure 1.1.1. Encapsulated *Cryptococcus neoformans* viewed in India ink under light microscopy.

Further investigation into the species *C. neoformans* was undertaken by Benham (1935) and continued by other researchers up until the 1960s (Evans, 1950; Littman, 1959; Wilson *et al.*, 1968). These investigations established classification of the organism and revealed the presence of four serotypes, A, B, C and D. In 1985 another potential serotype, AD, was also documented, strains composing this group exhibit the characteristics of both serotype A and serotype D yeast (Ikeda *et al.*, 1985). In the 1970s, *C. neoformans* was found to produce clamp connections and hypha suggesting that rather than being a blastomycete, it was more likely to be a basidiomycete. Mating studies between the serotypes in the 1970s culminated in the acceptance of two mating types and varieties of *C. neoformans*: *C. neoformans* variant *neoformans* (serotypes A, D & AD) and *C. neoformans* variant *gattii* (serotypes B & C) (Casadevall & Perfect, 1998). The discovery of sexuality, and production of basidiospores, resulted in the classification of the sexual form as *Filobasidiella neoformans* (Kwon-Chung, 1975, 1976a, b). Recently, Franzot *et al.* (1999) proposed that *C. neoformans* serotype A be reclassified as variant *grubii*. This new naming convention is not as yet commonly accepted and, although is becoming more so with time, is not used in the following report.

1.1.2 Characteristics

As noted, the genus *Cryptococcus* is populated by 38 or so species; each of the species shares several common characteristics: they are generally considered to be non-fermenting yeast, capable of assimilating inositol and producing urease. Many have been found in extreme climates ranging from the low temperatures of Antarctica to high saline conditions of salt waters. Like other members of the genus, *C. neoformans* yeast are capable of surviving to environmental extremes, this includes toleration of a pH range from 4 – 7, and extended viability in a desiccated environment, in some cases of more than a year. The one characteristic, above all others, that separates *C. neoformans* from other members of the genus *Cryptococcus*, is the ability to survive and multiply at temperatures in excess of 37 °C. It is this

characteristic which is thought to be the major contributor towards making *C. neoformans* the only member of the genus *Cryptococcus* capable of readily colonising mammals and birds. *C. neoformans* yeast are capable of growth in many different types of media, from simple chemically defined broths, to the more complex preparations commonly used for culturing fungi. Such a lack of fastidiousness is due to an ability to create, from simple raw materials, most, if not all, amino acids, sugars, lipids and vitamins necessary for survival. Despite this enhanced ability to survive and grow in simple media, *C. neoformans* isolates do not thrive without an adequate oxygen supply (Barnett *et al.*, 1983; Casadevall & Perfect, 1998).

1.1.3 Lifecycle

C. neoformans is an opportunistic pathogen, it is free-living and capable of surviving in various ecological niches. It is thought unlikely to be a human commensal as it is rarely isolated from healthy individuals (Casadevall & Perfect, 1998). Of the two varieties of *C. neoformans*, variant *gattii* is found in tropical and sub-tropical climates associated with a particular species of eucalyptus tree (*Eucalyptus camaldulensis*). Contrastingly, variant *neoformans* is found throughout the world and is generally isolated in, or around, guano of pigeons and other birds (Ellis & Pfeiffer, 1990).

The exact lifecycle of *C. neoformans* has yet to be fully elucidated, however the discovery of an association between eucalyptus trees, koala bears and *C. neoformans* variant *gattii* has led to the proposal of a lifecycle for both varieties, this lifecycle is thought to revolve around a host plant and animal carrier (Ellis & Pfeiffer, 1990). Ellis & Pfeiffer (1990) suggested that infectious basidiospores are spread from host plants, either through the air in which case they may be inhaled, or via the gut of certain animal hosts. At some point, cells become deposited in a new environment; those in a sheltered environment will remain viable for several years as desiccated yeast awaiting further dissemination. As already stated, the plant host of variant *gattii* is thought to be eucalyptus, the animal host is thought to be koala bears. In the case of variant *neoformans*, the animal carrier was postulated to be primarily pigeons; no strong evidence has been found for a plant host, but it may be a grass or cereal

ingested by pigeons and other birds. Ellis & Pfeiffer (1990) did not attempt to explain at which point in this lifecycle sexual reproduction might occur. In addition, it is widely assumed that the colonisation and infection of the human body is not associated with a completed lifecycle; this assumption is arrived at through the knowledge that human to human transmission is extremely rare (Casadevall & Perfect, 1998).

1.1.4 The cell wall and capsule of *C. neoformans*

The yeast or 'imperfect' state of *C. neoformans* is the form in which this microbe is most readily found, in fact thus far the 'perfect' state has only been described under specific laboratory conditions (Kwon-Chung, 1975, 1976a, b). Three layers generally separate the cytoplasmic contents of *C. neoformans* yeast cells from the environment: the cell membrane, cell wall and the capsule. Of the three, the capsule is best researched and documented, whereas the cell membrane and cell wall have received only limited attention (Casadevall & Perfect, 1998).

The capsule of *C. neoformans* is an important virulence factor, capsular material is commonly found associated with yeast observed in patient samples, however Bergman (1965) suggested capsule manifestation and extent is less apparent than might be expected amongst the majority of cell lines grown *in vitro*. The capsule itself can vary in size from minute, to 50, or even 100 μm in diameter (Cruickshank *et al.*, 1973). Structurally the capsule is composed of interwoven polysaccharide fibres thought to be non-covalently linked to the cell wall (Casadevall & Perfect, 1998; Doering, 2000). As well as various other excreted enzymes and proteins, there are three major components of the capsule, glucuronoxylomannan (GXM), galactoxylomannan (GalXM), and mannoprotein (Casadevall & Perfect, 1998). GXM is in greatest abundance, making up approximately 90 % of the total capsule content. It is composed of a 1-3 linked repeating mannose backbone with glucuronic acid and xylose side chains. Figure 1.1.4a describes how the number and positioning of the side chains varies amongst the four main serotypes.

Figure 1.1.4a. The four main serotype specific repeating structures of glucuronoxylomannan (GXM). Man = Mannose; GlcA = Glucuronic acid; Xyl = Xylose. Arrows originate at C1 (Doering, 2000).

In contrast to GXM, GalXM is a minor component of the capsule which can also be found in the cell wall, and may be secreted into culture supernatant. GalXM, in comparison to GXM, is less well studied, however it is known to be 275 ± 24 kDa in size (Casadevall *et al.*, 1998). The structure of pure samples of GalXM, isolated from the culture supernatant of a GXM-free strain, has been deduced (Vaishnav *et al.*, 1998), and is presented in Figure 1.1.4b.

Of the three main components of the capsule, mannoprotein is both present in least abundance, and least well characterised (Casadevall *et al.*, 1998). Unlike both GXM and GalXM, mannoprotein is known to bind Concanavalin A (Con A), a lectin which specifically binds α-mannose and α-glucose moieties (Cherniak & Sundstrom, 1994). Mannoprotein is thought to vary in size, implying varying forms; one such form was analysed and found to contain 20.6 % protein, of which the predominant amino acids were serine, threonine and alanine (Turner *et al.*, 1984).

Figure 1.1.4b. The repeating structure of galactoxylomannan (GalXM) isolated from culture supernatant of a GXM-free strain of *C. neoformans*. Man = Mannose; Gal = Galactose; Xyl = Xylose. Arrows originate at C1 (Vaishnav *et al.*, 1998).

As with all yeast, *C. neoformans* has, outside the cell membrane, a cell wall. The cell wall is in place to maintain cell shape and reduce the various stress conveying attributes of the external environment, such as osmotic variation. Along with the capsule, the cell wall is a major defensive barrier and shield against environmental disruption and other entities such as the various forces of the vertebrate immune system, and indeed predators such as phagocytic amoeba. In their extensive review Casadevall & Perfect (1998) describe the cell wall as being made up of cross-linked polysaccharides which become denser as they approach the inner cell. Studies on acapsular cells have indicated that the entire cell wall is composed of 86 % glucose, 7.3 % hexosamine, 2.2 % nitrogen and 0.3 % phosphate; of particular note is the apparent lack of mannose as a structural component (James *et al.*, 1990). Research has also isolated a mannoprotein in the inner cell wall (Vartivarian *et al.*, 1989), as well as melanin (Wang *et al.*, 1995). Finally, there is some evidence that chitin may also be present. The overall view of the *C. neoformans* cell wall is that it varies markedly from those of *S. cerevisiae* and *C. albicans* (Casadevall & Perfect, 1998).

The cell membrane of *C. neoformans* has not been studied in any detailed manner. Limited research exists to date in which *C. neoformans* cell membranes were found to contain iron reducing enzymes (Nyhus *et al.*, 1997). Also of note is that cryptococcal membranes are susceptible to agents which disrupt the production of ergosterol (Richardson *et al.*, 2001). This was studied in more depth by various

researchers (Joseph-Horne *et al.*, 1995; Lamb *et al.*, 1995; Venkateswarlu *et al.*, 1997), who in separate studies found high levels of ergosterol amongst isolates of *C. neoformans*, indicating that the cell membranes of these yeast were composed, in part, of this sterol.

1.2 *C. neoformans* as a human pathogen

1.2.1 Background and serotype variation

As a consequence of its status as an opportunistic fungal pathogen capable of infecting a broad spectrum of mammalian hosts, *C. neoformans* is researched more than any other member of the *Cryptococcus* genus. This spectrum of mammalian hosts includes cats, dogs, cows, horses, koala bears, mice and even dolphins (Casadevall & Perfect, 1998). The first reported *C. neoformans* infection was discovered in a 32 year old human female in 1894 (referred to in Benham, 1935), since this time the epidemiology of infection has varied greatly. In the first half of the 20th century, as few as 300 cases of human infection due to *C. neoformans* were reported, of which only a small minority survived. A combination of more accurate laboratory diagnosis and increased use of immunocompromising medical treatments led to an increase in reported infection from the 1950s onwards, particularly in the USA. In the fifteen or twenty years after 1980, developed nations, as a direct result of the AIDS epidemic, saw a dramatic rise in the numbers of patients presenting with cryptococcosis. In their extensive review of the subject, Casadevall & Perfect (1998) concluded that throughout the late 1980s and early 1990s, between 5 and 10 % of HIV infected patients in the USA, were in turn infected with *C. neoformans*. They also extended their review and concluded that worldwide, in the same time period, infection rates were estimated at around 5 % (although African countries may have had a higher prevalence). In recent years the discovery and increased use of anti-retroviral drugs, along with prophylactic use of fluconazole, has reduced the number of HIV patients presenting with cryptococcosis. In the USA the availability of new treatments allows patients, that are able to afford healthcare, to avoid cryptococcosis whilst such

treatments continue to be effective (Mirza *et al.*, 2003). Contrastingly, in parts of the developing world infection rates remain high, particularly in Africa; a recent report from Zimbabwe stated that 45 % of patients suffering meningitis were infected with *C. neoformans* (Gumbo *et al.*, 2001).

Although declining in the developed world, *C. neoformans* infection is not fully under control. Developing drug resistance, both for anti-HIV drugs (Brenner *et al.*, 2002), and anti-fungals (Cuenca-Estrella *et al.*, 2001), as well as increasing levels of infection in the developing world, indicate that an increase in infection worldwide is likely. Meanwhile, in the developed world *C. neoformans* infection is becoming increasingly prevalent amongst organ transplant recipients, with 2.8 % of recipients suffering infection and 42 % of the infected dying (Husain *et al.*, 2001). Since the 1950s, the western world has increasingly employed immunocompromising treatments and it appears that this trend has the effect of continually producing new potential hosts for this opportunistic pathogen.

C. neoformans is not solely implicated in infection amongst immunocompromised hosts; in fact it is also capable of infecting immunocompetent individuals. Immunocompetent infection is most often associated with variant *gattii* and therefore is rarely reported outside of tropical and sub-tropical areas such as Australia, New Zealand and Papua New Guinea. In rural Australia immunocompetent hosts are more likely to be male aborigines suffering infection mediated by variant *gattii*; this suggests a lifestyle or genetic susceptibility. Indeed, it may be that *C. neoformans* variant *gattii* and male aborigines come in contact through mutual association with the eucalyptus tree, a known environmental host of this *C. neoformans* serotype (De Wytt *et al.*, 1982; Chen *et al.*, 2000).

1.2.2 Forms of infection

The exact route of infection *C. neoformans* takes has yet to be fully established. However, the most commonly held view is that the *C. neoformans* infectious agent, in the form of small non-encapsulated yeast cells (Farhi *et al.*, 1970) or basidiospores, are inhaled and produce a primary pulmonary lymph node complex. This initial infection is often asymptomatic and the yeast can remain dormant in this

state until conditions are favourable for further infection. Infection can take many forms, examples of which are listed in Table 1.2.2.

Table 1.2.2. Examples of sites and types of cryptococcal infection (collated from Casadevall & Perfect, 1998).

Central Nervous System	Bone and Joints
Acute Meningitis Chronic Meningitis Dementia	Chronic Osteomyelitis Acute Arthritis Chronic Arthritis
Lung	Gastrointestinal Tract
Adult Respiratory Distress Syndrome Nodules Concomitant Opportunistic Infection	Hepatitis Pancreatitis Peritonitis
Skin	Eye
Papules Ulcers Abscess	Keratitis Optic Nerve Atrophy Endophthalmitis
Genitourinary Tract	Heart
Prostatitis Genital Lesions Pyelonephritis	Cryptococcemia Endocarditis Myocarditis

Of the various different sites of infection two predominate, the lungs and the central nervous system (CNS). The common occurrence of pulmonary infection provides evidence that the lungs are a likely site of initial colonisation. More interestingly, the propensity of *C. neoformans* cells for the CNS is both unique and yet to be fully explained; it occurs regardless of the immunocompetency of the individual involved (Casadevall & Perfect, 1998).

1.2.3 Diagnosis, treatment and prognosis

Due to the wide range of infection targets and vast array of symptoms reported, it is difficult to define a set of general symptoms for cryptococcosis. Pulmonary cryptococcosis, as already stated, can be asymptomatic and when symptoms do occur they vary between immunocompetent and immunosuppressed patients. Immunocompetent patients may present with, among others, the following symptoms: a cough, chest pain, weight loss and fever. Immunosuppressed patients, who are not suffering from the onset of AIDS, can suffer pulmonary infection in the form of pneumonia. AIDS patients uncommonly present with pulmonary infection alone. Symptoms of pulmonary infection amongst immunosuppressed patients include: fever, dyspnoea, weight loss and headache. Cryptococcal meningitis, the most commonly treated infection caused by *C. neoformans*, presents in varying forms between patients groups and tends to be more chronic amongst immunocompetent individuals. Patients often present with headache, fever, lethargy, nausea, personality changes, memory loss and coma. The time of onset is over 2 to 4 weeks and can even be months, however time of onset is variable and immunocompromised patients in particular may experience worsening of the disease in a far shorter time period (Richardson & Warnock, 1997; Casadevall & Perfect, 1998).

If symptoms and patient history indicate cryptococcosis, laboratory diagnosis is used to confirm infection. The most common form of infection, cryptococcal meningitis, can often be preliminarily diagnosed in the laboratory using a simple India ink stain. Essentially, patient cerebrospinal fluid (CSF) is mixed with a black colloidal medium (India ink) and viewed using light microscopy. Encapsulated yeast cells are identified amongst un-encapsulated lymphocytes. This method is more effective

amongst AIDS patients than other groups. Another basic diagnostic test is culture from samples, followed by strain identification using either a battery of laboratory based tests (Barnett *et al.*, 1983), or a commercial identification system such as analytical profile index (API) reference strips (Bio Merieux UK, Basingstoke, UK). Although laboratory based culture and isolate identification effectively supports disease diagnosis, unfortunately this can take up to two weeks, a period during which an infected individual can deteriorate markedly. A faster diagnosis can be achieved through the use of antibody or antigen testing. Antigen testing is both more useful and successful as it has a good success rate in immunocompromised patients. Essentially, cryptococcal polysaccharide antigen is detected in body fluids using the latex agglutination method. Diagnosis via the detection of antigen is generally successful, however, false negatives can occur (Berlin & Pincus, 1989; Taelman *et al.*, 1994).

Once diagnosed, treatment of *C. neoformans* infection varies depending on the type of patient and site of infection. Pulmonary infection is not normally treated in immunocompetent individuals unless it becomes symptomatic, in which case fluconazole is administered for an extended period (3 – 6 months). Progressive pulmonary infection as well as pulmonary infection diagnosed in AIDS patients, is treated with amphotericin B and flucytosine initially. Extra-pulmonary non-meningeal infection is initially treated with amphotericin B and relevant triazole anti-fungals. Meningitis is treated with amphotericin B in high dose initially, along with flucytosine for several weeks. AIDS patients with meningitis may be treated with liposomal amphotericin B. The majority of seriously infected patients are also put on a relevant triazole based treatment for 6 to 12 months post infection, this helps to reduce the likelihood of relapse. AIDS patients are generally given fluconazole for life (Casadevall & Perfect, 1998; Evans *et al.*, 2001; Richardson *et al.*, 2001).

Until the 1950s, 86 % of patients suffering meningitis died from the infection within a year, 70 % within three months of the onset of symptoms (Carton, 1952). Despite the increase in patient numbers brought about by novel anti-immune treatments, this death rate was reduced as a direct result of the introduction of novel antifungal agents and their therapeutic use. In the period from 1963 until 1998 several studies investigated the success of treatment in patients suffering cryptococcal meningitis (reviewed in Casadevall & Perfect, 1998). Success rates, measured by a number of criteria including: length of survival, levels of drug toxicity and underlying

disease, varied between 36 and 100 %. Death occurred in 10 to 25 % of patients, either during treatment or in the follow up period.

1.3 Host immune response

During colonisation and infection by microorganisms, the vertebrate immune system acts to reduce and remove all invading elements in a number of different ways. In turn, *C. neoformans* cells must employ a wide range of structural and enzymatic defensive and offensive components in order to exploit host resources and evade vertebrate-immunity in all its forms. The various cryptococcal virulence factors available are discussed later, however, immediately following material considers host responses with reference to their efficacy against *C. neoformans*.

1.3.1 Generalised non-specific immunity

Elements within this category of most relevance to *C. neoformans* are host body temperature, pH, physical barriers, collectins and complement. Although *C. neoformans* cells grow well at 37 °C, they grow poorly or not at all at greater temperatures indicating that elevated body temperature, due to fever, will reduce colonisation and subsequent infection by *C. neoformans* (Casadevall & Perfect, 1998). Similarly, research has indicated that the pH of human serum (pH 7.4) may curtail colonisation and infection (Howard, 1961). Physical barriers, such as the skin and nasal passage linings, prevent microbes from entering the body. With respect to *C. neoformans*, the most important element of this sort is probably ciliary action of pulmonary tissue and the associated clearance of infectious propagules from the lungs.

In addition to structural entities and associated functional elements, chemical components are also widely associated with cryptococcal suppression. Collectins are opsonins, found in lung surfactants and serum, which bind to microbes and promote phagocytosis. Among the collectins, pulmonary surfactant proteins SP-A and SP-D, along with Mannose Binding Lectin (MBL) and collectin CL-43, have all been shown

to bind *C. neoformans* cells. Pulmonary surfactant protein SP-D was shown to agglutinate acapsular *C. neoformans* cells, and therefore may fulfil a significant role in clearance of *C. neoformans* from the lungs (Schelenz *et al.*, 1995).

Complement, a major immune contributor, provides a complex system in which various proteins interact with microbes and other elements of the immune system, including MBL, and then promote phagocytosis. Complement is known to bind both acapsulate and encapsulated *C. neoformans* cells. Acapsulate *C. neoformans* cells will bind complement protein C3 in similar amounts to other pathogenic fungi, contrastingly, encapsulated cells bind somewhere in the region of 25 times more (Kozel, 1996). It is clear that the complement system seems to play a significant role in defence against infecting *C. neoformans* cells; however, as is discussed later (section 1.4.1), increased binding of complement to encapsulated forms of *C. neoformans* may not necessarily lead to a host-favourable outcome.

1.3.2 Cellular non-specific immunity

Research using polymorphonuclear (Diamond *et al.*, 1972) and natural killer cells (Murphy & McDaniel, 1982), has indicated that they are both involved in an immune response to *C. neoformans*. However, it is macrophages that play the foremost role in destruction of *C. neoformans* yeast cells. Indeed, destruction and compromisation of mouse macrophages mediates an increase in susceptibility to cryptococcal infection (Monga, 1981). Macrophages are stimulated to phagocytose and destroy *C. neoformans* cells by both complement (Levitz & Tabuni, 1991), and antibody deposition (Mukherjee *et al.*, 1995). In addition, macrophages have the ability to bind and phagocytose cryptococcal cells in the absence of such serum factors; this process is initiated by a mannose receptor (Bolanos & Mitchell, 1989). Indeed, macrophages have been shown both to bind and phagocytose capsule free cells via the use of receptors with affinity for mannose and β-glucan (Cross & Bancroft, 1995).

1.3.3 Specific immunity

Specific immunity can be divided into that mediated by T-cells, and that by antibody production. In their extensive review of the subject Casadevall & Perfect (1998) concluded that humans commonly retain viable antibodies against cryptococcal capsule polysaccharide, regardless of their current state of *C. neoformans* exposure. They remarked that anti-capsule polysaccharide antibodies had been heavily studied, whereas, in contrast, little investigation had followed the discovery of antibodies raised against protein antigen. Casadevall & Perfect (1998) concluded that capsule polysaccharide is poorly immunogenic and, in addition, produces a tolerogenic effect, thus leading to antibody unresponsiveness.

Cell mediated specific immunity is of particular significance with regards to *C. neoformans* incidence and infection frequency, as this response is decimated after the onset of AIDS. Casadevall & Perfect (1998) have reviewed this subject in detail, and in particular the research which establishes the importance of T-cell immunity in protecting mice against *C. neoformans* infection. Reviewed material indicates that protection from cryptococcal infection is provided by both CD4+ and CD8+ T-cells, and that they act to recruit and police macrophages, as well as actively inhibiting the growth of *C. neoformans* cells. Interestingly, there is also evidence that antibody response is dependent upon T-cell function.

1.4 Virulence factors

C. neoformans cells infect with the aid of several key virulence factors, these include capsule production (Chang & Kwon-Chung, 1994), melanin synthesis (Salas *et al.*, 1996), pyrimidine and purine metabolism (Varma *et al.*, 1992; Perfect *et al.*, 1993), urease production (Cox *et al.*, 2000), growth at 37 °C (Odom *et al.*, 1997) and phospholipase production (Cox *et al.*, 2001). As well as these known virulence factors, several other possible virulence factors of *C. neoformans* are being investigated, these include: mannitol synthesis (Wong *et al.*, 1990; Chaturvedi *et al.*, 1996) and proteinase secretion (Steenbergen & Casadevall, 2003). Of these virulence-

conferring attributes, the capsule of *C. neoformans* has probably received most attention.

1.4.1 The polysaccharide capsule

Many authorities consider that the capsule of *C. neoformans* evolved in the greater environment thereby affording protection from amoeboid phagocytic attack and possibly desiccation. While such functions may still be attributed to the cryptococcal capsule, it also successfully protects cells from disruption by elements of the vertebrate immune system (Casadevall *et al.*, 2003). Some of the extensive range of benefits encapsulation brings to cells of *C. neoformans* during infection are outlined in Table 1.4.1.

Table 1.4.1. Mammalian immune related protective effects of capsule production in *C. neoformans* (collated from Casadevall & Perfect, 1998).

Effect	Reference
Antiphagocytosis	(Kozel & Gotschlich, 1982; Kozel *et al.*, 1988)
Complement Depletion	(Macher *et al.*, 1978)
Antibody Unresponsiveness	(Murphy & Cozad, 1972; Kozel *et al.*, 1977)
Interferes with Antigen Presentation	(Collins & Bancroft, 1991)
High Negative Charge of Cells	(Nosanchuk & Casadevall, 1997)

1.4.2 Melanin synthesis

Melanin synthesis occurs through the copper dependant enzyme laccase. Although documented that the ability of cells to produce this enzyme affords extra virulence (Salas *et al.*, 1996), the exact mechanism is unknown. A general perception exists that melanin production may take advantage of several basic-components found in the CSF, including: adrenalin, noradrenalin and dopamine. This may in turn explain the predilection of *C. neoformans* for the CNS. The various explanations so far put forward for melanin production and use are presented in Table 1.4.2.

Table 1.4.2. Possible scenarios for melanin production (collated from Williamson, 1997).

- Free radical scavenger during oxidative attack by macrophages.
- Incorporation into cell walls to increase negative charge and thus repel macrophages.
- Incorporation into cell walls increasing stability against antifungals.
- Interference of TNF-α production thus reducing lymphoproliferation.

Similarly to capsule production, it is thought that laccase based melanin synthesis probably evolved in the greater environment. Melanin may protect *C. neoformans* cells from temperature and U.V. damage associated with direct sunlight; laccase may also be useful in breaking down rotting wood (Williamson, 1997), a fact that may in part explain the common association of *C. neoformans* variant *gattii* and eucalyptus trees.

1.4.3 Growth at 37 °C and auxotrophy

The vast majority of fungi do not grow at temperatures as high as 37 °C and, as a result, are incapable of surviving at mammalian body temperature. Through production of calcineurin, a protein phosphatase, *C. neoformans* cells are capable of surviving and proliferating at temperatures of 37 °C, thus enabling them to successfully infect mammals (Odom *et al.*, 1997).

A lack of fastidiousness amongst *C. neoformans* cells is well known. Their capacity to proliferate in the most basic media (Littman, 1958), suggests that *C. neoformans* cells are autotrophic for the majority of complex components, this overall quality is most likely to enhance cryptococcal virulence and survival. Indeed, investigation into production of pyrimidine and purine components by *C. neoformans*, via the manipulation of auxotrophic strains, confirmed that this attribute alone confers virulence (Varma *et al.*, 1992; Perfect *et al.*, 1993). It is likely that investigation into amino acid production may also identify additional virulence enhancing mechanisms.

1.4.4 Further enzyme based virulence factors

As well as melanin-synthesising laccase and temperature-desensitising calcineurin, two other enzymes are proven virulence factors, urease and phospholipase. Urease hydrolyses urea to ammonia and carbamate, and is a known virulence factor of certain bacteria including *Helicobacter pylori* and *Proteus mirabilis*. Although not thought to be essential for infection, urease is produced by the vast majority of *C. neoformans* isolates in substantial amounts, and has been proven to enhance infection. The mechanism of urease induced protection is currently unknown, however, it is thought that urease interferes with phagocytosis by altering the pH of the microenvironment around cells (Cox *et al.*, 2000). Phospholipase, similarly to urease, is known to be produced by *C. neoformans* cells. Mutant *C. neoformans* cells with reduced phospholipase activity have been found to be less virulent in animal models of cryptococcal infection than parent strains, thus indicating that phospholipase production is indeed a virulence factor. Again, similarly to urease

production, the reason phospholipase increases *C. neoformans* virulence is as yet undetermined; however, evidence suggests that this enzyme may work intracellularly in phagocytes destroying phagolysosome membranes, as well as extracellularly, disrupting tissues and surfactant (Cox *et al.*, 2001).

Of various other possible virulence factors reported, mannitol synthesis and proteinase production are of interest. Mannitol production may protect cells from disruption, by elements of the mammalian immune system, as it has been reported that *C. neoformans* secretes mannitol into the CSF of animal models (Wong *et al.*, 1990). Indeed, research has also indicated that mannitol may protect cells from oxidative killing by the reactive oxygen intermediates produced by polymorphonuclear neutrophils (Chaturvedi *et al.*, 1996). Like mannitol, secreted proteinases have been detected from *C. neoformans* strains. Proteinases may increase virulence by degrading host tissues thus supporting nutrition, compromising tissue integrity and reducing host immune response (Steenbergen & Casadevall, 2003).

1.5 Cell surface investigation

Cell surfaces are the dynamic interface between microorganisms and their surrounding environment; in turn, due to the size of microorganisms the surface to volume ratio is relatively enormous. Such surfaces must act as a shield reducing ingress of potentially damaging entities, and as gateways for the intake of nutrients and removal of waste. With respect to pathogenic microorganisms, cell surfaces are of particular interest as virulence is often, if not always, dependant in some way upon cell surface attributes (Marshall, 1991). The three main areas where cell surfaces of mammalian pathogens may support increased virulence are adhesion, resistance and nutrient gathering.

1.5.1 Microorganism adhesion

An infection process begins with exposure of the host to infectious propagules or agents. This is followed by microbial colonisation, growth and host exploitation.

Adhesion, in this case the ability of a microorganism to bind host surfaces, has been implicated in the virulence of viruses, bacteria and fungi. Notable examples include attachment of *Neisseria gonorrhoea* to urogenital epithelia (Madigan *et al.*, 2003), and *Candida albicans* to various surfaces, both of host cells and medical implants (Klotz *et al.*, 1985; Klotz, 1989; Korting & Ollert, 1994; Kanbe & Cutler, 1998). Amongst the pathogenic fungi, *C. albicans* has probably received greatest attention with regards to adherence; however, *Blastomyces dermatitidis* (Klein & Newman, 1996; Brandhorst *et al.*, 1999; Brandhorst & Klein, 2000), *Coccidioides immitis* (Hung *et al.*, 2002), *Histoplasma capsulatum* and *Sporothrix schenckii* (previous name *Sporotrichum schenckii*) (Jimenez-Lucho *et al.*, 1990), have each also been shown to produce putative cell surface adhesins.

With respect to *C. neoformans*, research has identified both adhesive potential as well as the presence of potential adhesins (Jimenez-Lucho *et al.*, 1990; Merkel & Scofield, 1994; Ibrahim *et al.*, 1995; Rodrigues *et al.*, 1997). Merkel & Scofield (1994) produced evidence to suggest that *C. neoformans* cells were capable of adhering to glial cells; vast numbers of which are found in the CNS. Interestingly Merkel & Scofield (1994) also found that acapsulate cryptococcal cells used the same method of adherence as encapsulated cells, yet were three times more adherent. Jimenez-Lucho *et al.* (1990) found that like other yeast, *C. neoformans* cells were capable of binding lactosylceramide, a glycosphingolipid found on the membranes of brain glioma cells. Ibrahim *et al.* (1995) investigated adhesion of *C. neoformans* yeast to endothelial cells; they found that cells did indeed adhere, and that acapsular cells adhered in significantly greater numbers. In addition, they also noted that acapsular cells were more likely to be internalised by such cells, thus indicating a possible route of movement from blood vessels to target organs. Regardless of this work, little has been discovered about the nature of the mechanisms which drive adhesion; as a result, analysis of cell surface structures in order to discover the exposure and coverage of potential adhesins is certainly an attractive area of research.

1.5.2 Resistance

In order to survive, colonise and proliferate within the mammalian host, microorganisms must resist challenges presented by the mammalian immune system;

additionally microbes must resist challenges presented by therapeutic regimes such as anti-microbial treatment. Naturally, the first line of defence for any microorganism, including *C. neoformans*, is the cell surface and associated structures.

The mammalian immune response initially relies upon an innate set of components consisting of macrophage type cells, and molecules such as the proteins which make up complement. Macrophages continually express receptors for various basic antigens, these receptors are used to locate microorganisms. Once located, a microorganism is bound, and then wherever possible, engulfed and destroyed. Complement and collectins enhance the action of macrophages by attaching to basic antigens on the surface of pathogens and encouraging the binding of phagocytic cells. In turn, many pathogens are capable of compromising such vertebrate immunity, they do so using a variety of differing approaches, all of which are in some way associated with cell surface phenomena (Janeway & Medzhitov, 2002). Vertebrate immunity in contrast to that produced by the likes of invertebrates, also demonstrates a highly developed adaptive response, based around antigen recognition. Antibody for instance, is produced in response to antigen presented by macrophages and other immune cells. Once available, antibody binds to a specific target antigen ultimately supporting destruction of it and, if relevant, the invasive microbes associated with it (Janeway & Travers, 1997).

C. neoformans is unique amongst pathogenic fungi as virulent strains produce a thick polysaccharide capsule *in vivo*. As discussed earlier, the capsule is known to retard phagocytosis (Kozel & Gotschlich, 1982; Kozel *et al.*, 1988) and deplete complement (Macher *et al.*, 1978), thus ameliorating innate immunity. Capsule polysaccharide is released in large quantities and is thought initially to neutralise antibody, and then induce immunological tolerance (Murphy & Cozad, 1972; Kozel *et al.*, 1977); thus capsule polysaccharide acts to reduce the efficacy of adaptive immunity. As a result of these findings the *C. neoformans* capsule has received much attention. Contrastingly, a lack of pertinent literature suggests that, to date, the cell wall surface is not well studied. However, the widely held view, put forward by Farhi *et al.* (1970), that the *C. neoformans* infectious propagule may lack capsular polysaccharide, suggests that more extensive analysis of the cell wall surface may be revealing and of importance.

Since their discovery, anti-microbials have drastically reduced the ability of microorganisms to survive within a mammalian host, and as such have been of

enormous value in patient care. The list of viable agents to which fungi involved in systemic infection are susceptible is limited (Joint Formulary Committee, 2003). Those useful against systemic infection include the polyene amphotericin B and the triazoles fluconazole and itraconazole; all of which result in the disruption of fungal membranes either *in situ*, or in production. Another compound useful against systemic infection is flucytosine, which acts by inhibiting new protein production. Finally the relatively new echinocandins disrupt cell walls by inhibiting β(1,3)glucan synthesis. Resistance to these antifungals can occur in various forms, one general form of resistance is through restriction of access of drug to target; several cell wall and cell membrane based mechanisms could contribute to this restriction, including efflux pumps and other structural changes (Ghannoum & Rice, 1999).

C. neoformans infection, in line with all infection by specific fungi, has an interesting therapeutic history as well as some unusual characteristics, all of which may indicate a need for cell wall and cell surface investigation. Until the 1950s, treatment regimes for systemic cryptococcal infection, such as meningitis, included the use of compounds ranging from actidione and penicillin, to colloidal silver and gentian violet; the failure rate of these treatments was universal (Carton, 1952). As previously stated the predominant antifungals currently used are: amphotericin B, flucytosine and relevant triazoles. Interestingly the echinocandins are of no clinical use against *C. neoformans* cell walls (Arathoon, 2001), however, the reasons behind this natural resistance to a cell wall disrupting drug have yet to be fully investigated. Research suggests that resistance is developing for those drugs which are currently effective (Rodero *et al.*, 2000; Cuenca-Estrella *et al.*, 2001). Rodero *et al.* (2000) investigated the susceptibility of *C. neoformans* isolates from patients that failed to respond whilst on amphotericin B therapy. Of five isolates examined, all were resistant to fungicidal disruption by amphotericin B; of further concern was the discovery that flucytosine and fluconazole in combination did not inhibit growth of any of the isolates. These discoveries may indicate that cross resistance between amphotericin B and fluconazole, a mechanism for which has been identified previously in the laboratory (Joseph-Horne *et al.*, 1995), could be occurring in the greater environment. Cuenca-Estrella *et al.* (2001) investigated flucytosine resistance in various *Candida* species as well as *C. neoformans*; they found that flucytosine resistance was present in 6.9 % of *C. neoformans* strains examined, and that

intermediate susceptibility was observed amongst 39.5 % of isolates. As drug resistance increases, researchers may respond with attempts to understand the various resistance mechanisms and how they affect the interaction of cells with their environment. In addition, investigation of changes occurring amongst cell wall, membrane and capsular entities of various *C. neoformans* states may identify both novel antifungal approaches, and possibly even resistance mechanisms.

1.5.3 Nutrient uptake

Microorganisms must facilitate the absorption of nutrients in order to survive and multiply. As a result, an ability to incorporate and use raw materials from the environment is extremely important. It is clear that all materials entering a yeast cell must first pass through the cell wall. In turn, active systems of transport may therefore rely on exposed cell surface moieties. With regards to pathogenic fungi an area which has received much attention is acquisition of iron.

A mammalian host environment is particularly low in free iron, especially during infection. Iron is essential for the production of working oxidoreductase enzymes, a group of enzymes used by almost all living organisms. In order to obtain sufficient amounts of ferric iron (Fe^{3+}), pathogenic microbes generally resort to one of three strategies: secreting iron-chelating chemicals (siderophores), reducing insoluble Fe^{3+} to soluble, and therefore easier to acquire, ferrous iron (Fe^{2+}), or by capturing and breaking down iron containing proteins (Jacobson *et al.*, 1998; Howard, 1999). In his extensive review on iron acquisition amongst pathogenic fungi, Howard (1999) detailed the evidence indicating which fungi utilised each of the three differing acquisition methods. Several pathogenic fungi are known to produce siderophores, including: *H. capsulatum*, *B. dermatitidis*, *Aspergillus spp.* and *S. schenckii*. The use, or more accurately, the uptake of siderophores is of interest to those studying cell surfaces of microbes; indeed a considerable number of medically important fungi do not actually produce siderophores, but instead simply 'poach' them, possibly via cell surface receptors. Fungi which do not create siderophores but do utilise those available, include *Saccharomyces cerevisiae* and *P. brasiliensis*. Pathogenic fungi also use the other common method of iron acquisition, reduction, a system where insoluble

environmental Fe^{3+} is reduced to the more soluble Fe^{2+}, which is then actively taken up. Reduction is brought about through reductant compounds such as melanin, as well as by ferric reductase enzymes. These various reducing agents are either secreted into the immediate vicinity, or held within, or on, the cell wall or plasma membrane. Pathogenic fungi known to use reductants, or ferric reductases, include *S. cerevisiae*, *C. albicans* (Howard, 1999) and *H. capsulatum* (Timmerman & Woods, 1999). Active uptake of iron, once scavenged, occurs after iron is bound by receptors and then transported across the cell surface by molecules similar to those proteinaceous iron permeases found amongst virulent *C. albicans* strains (Ramanan & Wang, 2000).

Iron acquisition by *C. neoformans* has been extensively studied. There is some indication that cells may utilise but not produce, siderophores (Howard, 1999); the exact mechanism for this action is not fully elucidated, particularly as cell surface receptors have yet to be isolated, extracted and identified. Regardless, there is much evidence suggesting that *C. neoformans* cells use, above all else, a reduction method of iron capture (Jacobson & Vartivarian, 1992; Nyhus *et al.*, 1997; Jacobson *et al.*, 1998). In detail, *C. neoformans* cells produce and secrete two reductants, the first of these is melanin, a known virulence factor, the second is 3-hydroxyanthranillic acid. As well as producing free reductants, ferric reductase enzymes are also utilised, these are held in a cell bound state (Nyhus *et al.*, 1997). Once reduced to Fe^{2+}, iron is then actively taken up using both high, and then low affinity systems depending on prevailing iron concentrations (Jacobson *et al.*, 1998).

In common with iron uptake processes, other nutrients may cross cryptococcal cell surfaces by additional active mechanisms, all of which potentially require some form of exposed uptake related structure on *C. neoformans* cell surfaces. In turn, the search for, and visualisation of, these structures is a valid goal for cell surface investigation and for *C. neoformans* research in particular.

1.6 Aims and objectives

The above material indicates that the capsule of *C. neoformans* has been extensively researched with regards to a fuller understanding of its nature, function and any role such an entity has in cryptococcal infection of human hosts. In contrast, it

is apparent that cell wall surfaces of *C. neoformans* have received much less focus and attention, despite the current belief that infection with *C. neoformans* is mediated by reduced capsule, or even acapsulate, entities (Farhi *et al.*, 1970).

The following work attempts to, at least in part, redress this imbalance, and characterise the cell surface of various *C. neoformans* strains. In the following chapters various non-specific properties of *C. neoformans* yeast cells are examined. Subsequently, carbohydrate exposure and distribution across cell wall surfaces and capsule is determined; an examination which included identification, quantification and characterisation of relevant exposed carbohydrates. To conclude this work, proteins expressed on the cell wall surface and within the capsule, are observed and quantified, then extracted, purified and examined. Experimentation draws upon a variety of techniques including: aseptic manipulation and culture, flow cytometry, confocal microscopy, gel electrophoresis and microparticulate analysis.

CHAPTER 2 – Identification, characterisation and evaluation of culture conditions

2.1 Introduction

C. neoformans can be identified from other unicellular budding fungi using a variety of methods (Casadevall & Perfect, 1998). The most convenient approaches employ commercial kits that perform a battery of tests all contained within one simple platform. The clinical yeast identification system used to identify the strains of *C. neoformans* described herein, was the 20C AUX API (Bio Merieux UK, Basingstoke, UK), which contains a battery of assimilation tests and is designed to identify clinical yeast isolates over a 24 – 48 hour period. Strains, once confirmed as *C. neoformans*, must then be identified further to variant level and then, where possible, to serotype level. Variant identification is achieved using diagnostic medium (Kwon-Chung *et al.*, 1982b; Min & Kwon-Chung, 1986). Serotyping is performed using commercial typing kits (Kabasawa *et al.*, 1991) which are capable of discerning between the five known capsule serotypes *C. neoformans*: A, B, C, D and AD (Wilson *et al.*, 1968; Kwon-Chung *et al.*, 1982a; Ikeda *et al.*, 1985).

In vitro characterisation further individualises strains allowing for more complex comparison. In the case of *C. neoformans*, characterisation includes experimentation to analyse morphology, productivity and microscopic appearance.

2.2 Materials and methods

Details of principle equipment, chemicals and materials, are included within the text; information pertaining to more common items can be found in Appendix G.

2.2.1 Isolate identification

In total, four isolates of *C. neoformans* were used in this work. Three part-characterised isolates came from the Research Centre for Pathogenic Fungi and Microbial Toxicoses, Chiba, Japan (designated CN IRM 5815, CN IRM 5854 and CN IRM 45922). The fourth isolate, a mutant anamorph of *C. neoformans* (Sanfelice) Vuillemin, designated Cap 67 (Jacobson *et al.*, 1982), was received from the American Type Culture Collection (ATCC), Manassas, Virginia, USA. Cap 67 is referred to herein with its ATCC number 52817. Strain ATCC 52817 is confirmed as being capsule free due to an inability to produce GXM.

Each of the three unidentified strains was confirmed as *C. neoformans* using 20C AUX API Strips (Bio Merieux UK, Basingstoke, UK). The method used to perform the identification was that outlined in the literature received with the strips. Canavanine-glycine-bromthymol blue (CGB) agar (Kwon-Chung *et al.*, 1982b; Min & Kwon-Chung, 1986) (Appendix A) was used for variant identification. Serotyping of the unidentified strains was performed by the Mycology Reference Laboratory, Bristol Public Health Laboratory, Bristol, UK; using M. K. Iatron serotyping kits (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan).

2.2.2 Storage and culture

Strains were stored at -70 °C in Sabouraud liquid medium (Lab M, Bury, UK) supplemented with 10 % glycerol. Prior to use, isolates were sub cultured monthly onto Sabouraud dextrose agar (Oxoid, Basingstoke, UK) and incubated at 30 °C for 72 hours before being stored at 4 °C. If necessary, up to two further sub cultures were created to keep isolates pure and 'vital'.

2.2.3 Characterisation

2.2.3.1 Microscopic appearance

Cells were examined for morphology, size and capsule presence. Each of these characteristics was determined after India ink negative staining and light microscopy observation according to Casadevall and Perfect (1998). Aliquots of 10 µl of logarithmic and stationary growth phase cultures (Sabouraud liquid medium, 37 °C with shaking at 150 rpm) were placed on microscope slides and covered with coverslips. An aliquot of 5 µl of India ink was then introduced under each coverslip. The prepared slides were then examined at x 400 and where necessary x 1000 magnification, using a calibrated eyepiece graticule. The general formation of cells was noted before the mean diameter of six random cells, before capsule, was measured. The width of capsule from cell surface outwards was also measured. Where appropriate, data were analysed using two factor analysis of variance (ANOVA) (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). The following comparisons were analysed, (i) differences in yeast cell diameter between strains and growth phase and (ii) differences in capsule width between strains and growth phase. Where necessary to illustrate significance, the least significant difference (LSD) was calculated as part of ANOVA analysis.

2.2.3.2 Growth at 37 °C

In order to identify the various stages of growth and contrast the relative productivity of each of the strains, their respective growth curves were determined. The density of cultures growing in broth was measured at fixed time points over a 56 hour period. An aliquot of 200 µl of a 48 hour growth culture (in 25 ml Sabouraud liquid medium (Lab M, Bury, UK)) was inoculated into two Erlenmeyer flasks containing 25 ml of Sabouraud liquid medium. The flasks were incubated 12 hours apart at 37 °C with shaking (150 rpm). A Novospec II spectrophotometer (Pharmacia

BioTech, Cambridge, UK) set at the optimum wavelength of 660 nm (determined by spectrophotometric analysis of the medium) was used, at two hour intervals for 8 hours in each 12 (relative), to determine the optical density (OD) of the medium in each flask, for 56 hours (relative). In order to minimise inaccurate readings, samples that gave an OD reading above 0.7 were judged as too dense and so were mixed thoroughly with Sabouraud liquid medium to a dilution of either 1 in 10 or 1 in 20 as appropriate, before a repeat reading.

2.3 Results

2.3.1 Isolate identification

The following tables confirm identification and define the four strains of *C. neoformans* referred to in all following chapters. Strain ATCC 52817 was received pre-identified and therefore was not re-identified in this work, however identifying information has been included here for a complete record (Jacobson *et al.*, 1982; Jacobson & Tingler, 1994).

Table 2.3.1a. Profile data from API 20C AUX species identification.

Strain	Profile Number	Description
CN IRM 5815	2747133	95.8 % probability *C. neoformans*
CN IRM 5854	2156131	85.8 % probability *C. neoformans*
CN IRM 45922	2557373	99.9 % probability *C. neoformans*
ATCC 52817	N/A	Pre-identified as *C. neoformans*

Table 2.3.1b. CGB variant analysis agar.

Strain	Growth + Colour Change	Variant
CN IRM 5815	Yes	*gattii*
CN IRM 5854	No	*neoformans*
CN IRM 45922	No	*neoformans*
ATCC 52817	N/A	*neoformans*

Table 2.3.1c. Serotyping analysis.

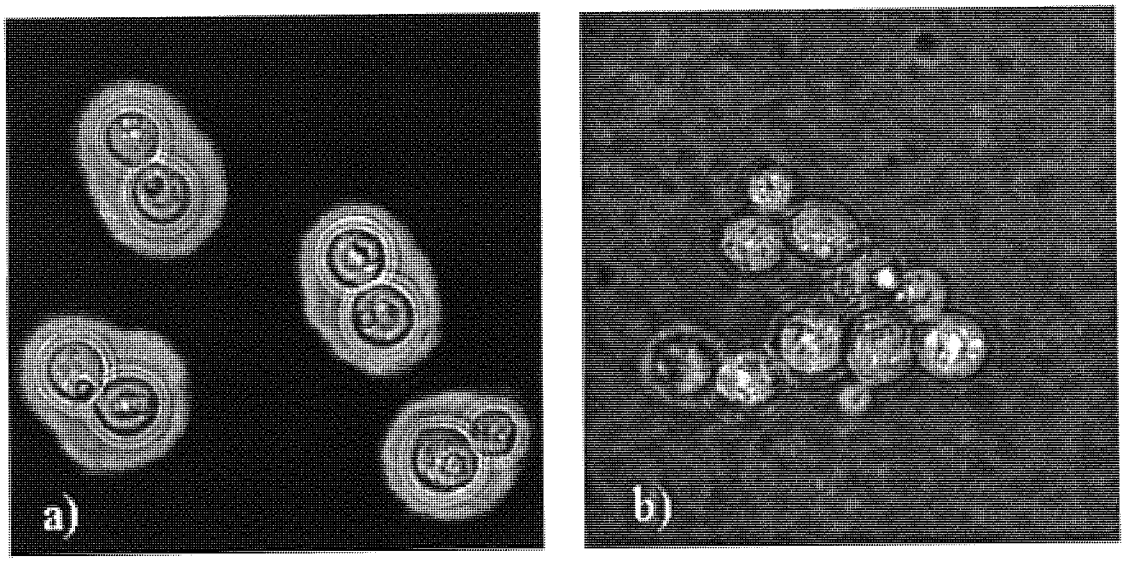
Strain	Serotype reported
CN IRM 5815	*B or C
CN IRM 5854	A
CN IRM 45922	A
ATCC 52817	†None

* Failed to agglutinate due to prozone effect (capsule overproduction).
† Serotyping is dependent on capsule production, ATCC 52817 is genetically incapable of creating a capsule, however, it is a variant of strain B-3501 which is serotype D.

2.3.2 Characterisation

2.3.2.1 Microscopic appearance

Strains CN IRM 5815, CN IRM 5854 and CN IRM 45922 appeared as singular free living cells except where cells remained attached while in the process of budding. ATCC 52817 presented as large clumps of cells which did not separate upon vortexing. Figures 2.3.2.1a&b illustrate the contrast between cell association of CN IRM 5815 and ATCC 52817.



Figures 2.3.2.1a&b. India ink negative staining images of two *C. neoformans* strains:
a) CN IRM 5815
b) ATCC 52817

Figures 2.3.2.1c&d illustrate cell and capsule size of *C. neoformans* strains respectively. Statistical analysis demonstrated a significant difference in cell size between strains ($P < 0.001$); in particular, strain 45922 was significantly smaller than all other strains ($P < 0.05$). There was also a significant difference ($P < 0.05$) between the logarithmic and stationary growth phase cell diameters of strain CN IRM 5815.

Capsular material was visible in strains CN IRM 5815 and CN IRM 45922. The capsule of CN IRM 5815 was significantly larger ($P < 0.001$) than that of CN IRM 45922.

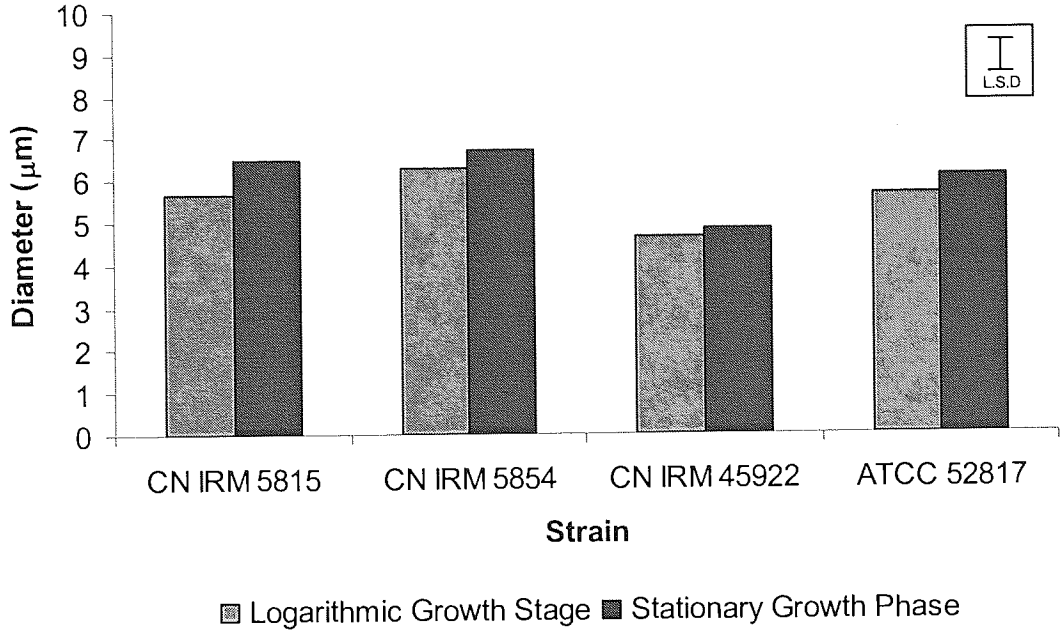


Figure 2.3.2.1c. Mean cell diameter of logarithmic and stationary growth phase *C. neoformans* strains grown in Sabouraud liquid medium at 37 °C. Results are means of 6 readings, LSD is calculated from ANOVA analysis.

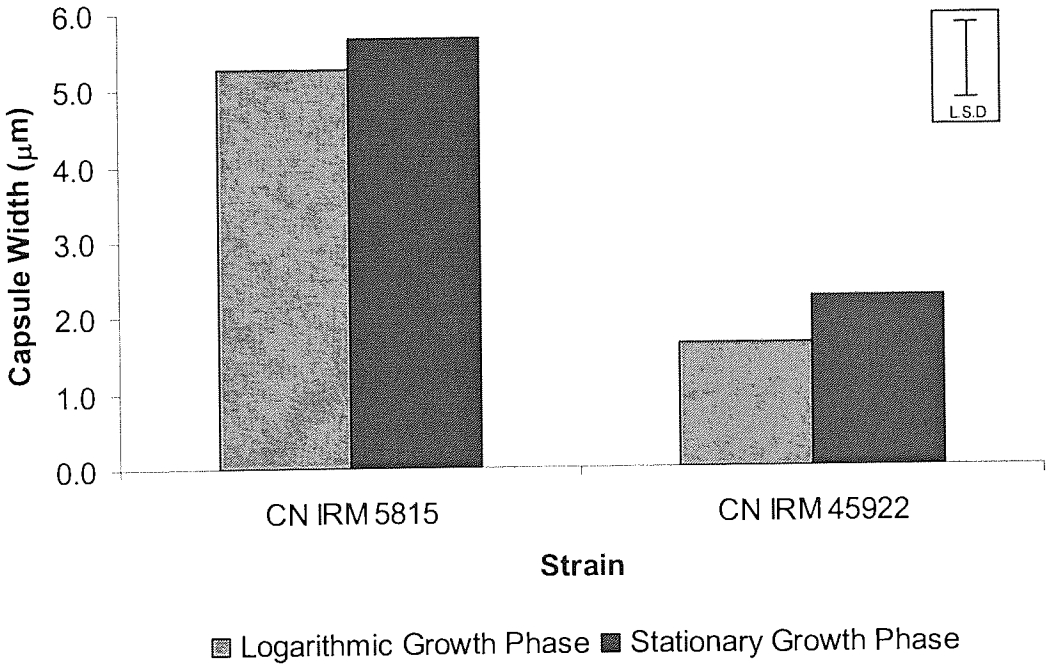


Figure 2.3.2.1d. Mean capsule width of *C. neoformans* strains expressing a capsule in Sabouraud liquid medium at 37 °C. Capsule width is defined as the distance between the cell surface and outer edge of the capsule as observed by India ink negative staining. Logarithmic and stationary growth phase results are illustrated. Results are means of 6 readings, LSD is calculated from ANOVA analysis.

2.3.2.2 Growth at 37 °C

Figure 2.3.2.2 demonstrates that, although the strains vary in fecundity, the growth profiles are in the main equivalent, giving a mid logarithmic phase at around 22 hours. The exception is acapsulate strain ATCC 52817 which is slower growing and reaches mid logarithmic phase at around 24 to 26 hours growth. All the strains reach stationary phase from around 42 hours onwards. Heavily encapsulated CN IRM 5815 is the most productive of the strains; the least productive is acapsulate strain ATCC 52817.

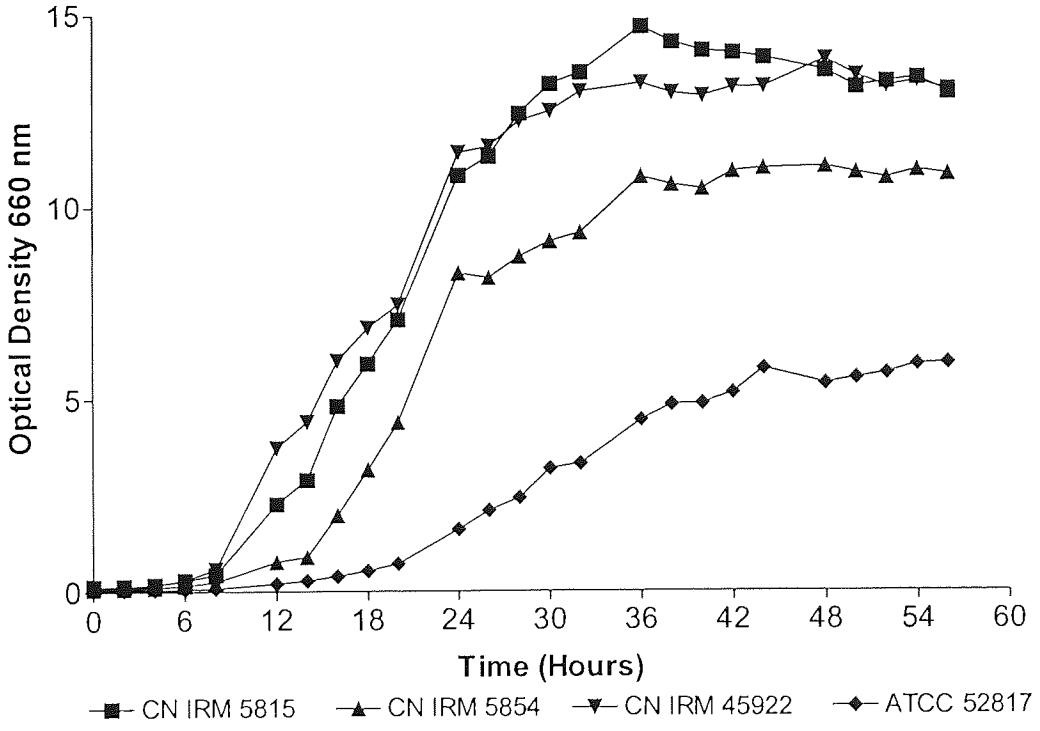


Figure 2.3.2.2. Growth profiles of four strains of *C. neoformans*. Results are means of 3 experimental readings. For clarity standard error bars are not included in the figure, however, maximum standard error measurements were as follows: ± 0.55 for CN IRM 5815; ± 0.73 for CN IRM 5854; ± 0.59 for CN IRM 45922 and ± 0.43 for ATCC 52817.

2.4 Discussion

2.4.1 Identification

Accurate and assured identification of *C. neoformans* strains held, is essential to ensure that subsequent work was executed with defined and characterised organisms. One strain was only partially identified at serotype level (CN IRM 5815), this was due to the commonly observed 'prozone effect', a lack of agglutination generally associated with heavily encapsulated cells (M. K. Iatron Inc., 2001). In line with such it was decided that this strain would be identified as variant *gattii* serotype B or C. Serotyping is dependant upon capsular polysaccharide (Casadevall & Perfect, 1998), as a result, the acapsular mutant ATCC 52817 has no serotype; however ATCC 52817 is a mutated form of ATCC B-3501 which is reported as serotype D (Jacobson *et al.*, 1982).

2.4.2 Characterisation

2.4.2.1 Cell size and capsule analysis

Cell size and encapsulation were analysed in order to define contrasting characteristics of the strains held. Although India ink light microscopy is well known and often used (Bergman, 1965; Jacobson & Tingler, 1994; Casadevall & Perfect, 1998), it is important to note that further examination of CN IRM 5854 would be necessary in order to demonstrate a complete lack of capsular material (Jacobson *et al.*, 1982; Jacobson & Tingler, 1994). CN IRM 5815 was found to have a significantly larger capsule than CN IRM 45922, the only other strain with a visible capsule. This observation gives a clear distinction between the two variant forms employed in subsequent experimentation. Furthermore, these differences are useful, as by

comparison of results obtained from investigations employing such variant forms, the influence of capsule presence and size may be ascertained.

2.4.2.2 Growth at 37 °C

C. neoformans is noted as an extremely resilient organism capable of survival and growth in most fungal media up to a temperature of 37 °C (Howard, 1961; Casadevall & Perfect, 1998). The work performed in the following chapters was exclusively concerned with cells grown at 37 °C as it is homologous with the temperature of the human body. Sabouraud liquid media, a well-known commonly used medium that allows for growth of cells in yeast phase, was used throughout this work.

Analysis of growth was performed in order to estimate the time points at which each of the strains entered the different phases of growth. This analysis is useful for two reasons; firstly, it allows for the calculation of cellular mass at specific time points, ensuring that sufficient cells are harvested for an experiment. Secondly, knowing when cells are in a specific growth phase allows for valid comparison between strains.

CHAPTER 3 – Non-specific adhesion phenomena

3.1 Introduction

Unicellular organisms interact with their environment through their cell surface; knowledge of interface characteristics is therefore fundamental. One general characteristic of clinically important organisms is the ability to attract and adhere to the surfaces of a host, thus supporting more effective host colonisation (Klotz *et al.*, 1985; Hazen & Hazen, 1987; Klotz, 1989; Lachica, 1990; Korting & Ollert, 1994; Kanbe & Cutler, 1998). Paradoxically it has been suggested that the attractive nature of an organism can increase cellular contact with host defence mechanisms and entities, specifically macrophages (Nosanchuk & Casadevall, 1997).

The various forces influencing attraction and adherence of microorganisms to surfaces can be divided into specific and non-specific types. Non-specific forces are created by the placement of different cell constructs in varying concentration, on or around the cell. This varying cell surface organisation alters the general Lifshitz-Van der Waals, electrostatic and acid-base properties of the surrounding space in such a manner as to bring about attraction or repulsion of nearby surfaces. The forces involved act from the cell surface outwards and can be categorised according to the distances over which they are effective. At distances greater than 50 nm the only forces capable of influencing other bodies are Lifshitz-Van der Waals intermolecular forces. As the two bodies get closer (over distances of 2 – 20 nm) a microorganisms cell surface charge may be of consequence. When objects get as close as 2 nm to a cells outer surface, hydrophobic interactions will potentially then have an important role in contact between surfaces (James, 1991; Marshall, 1991). Of particular interest with respect to *C. neoformans* yeast, is the variation of these non-specific forces amongst varying strains, especially amongst those with differing capsule phenotypes.

3.1.1 Cell surface charge

Cell surface charge is the net value of the charges of all cell surface components; it is particularly relevant to those microorganisms found and active in an aqueous environment. The charge a cell creates is dependant upon ionisation of cell surface components and therefore varies with pH, temperature and the ionic strength of the surrounding medium (James, 1991; Bos *et al.*, 1999). Cell surface charge is usually, if not always, negative in both prokaryotic and eukaryotic microorganisms (James, 1991). Of most relevance to clinically important microorganisms is the observation that the relative strength of surface negative charge, will affect the magnitude of repulsion experienced from and by other negatively charged bodies.

Cell surface charge cannot be measured directly; instead it is defined using a relative measurement known as the ζ potential. Figure 3.1.1 shows how free ions behave in the presence of a cell. Immediately surrounding the cell is a layer 0.5 nm thick, known as the Stern layer, which is made up of attracted ions that are held in close contact to the surface by chemical adsorption or localised electrostatic interaction. The Stern layer is separated from an outer diffuse layer by the plane of shear. At the plane of shear, ions from the diffuse layer meet the ions in close contact with the cell surface. These two phases will move independently from each other if an electric charge is applied, the charge at this point can in turn be determined from this movement and is known as the ζ potential. The ζ potential is reflective of the charge at the cell surface and is calculated using the Smoluchowski equation:

$$\mu_e = \frac{\varepsilon\zeta}{\eta}$$

where μ_e is the electrophoretic mobility measured, ε is the permittivity of the liquid medium and η is its viscosity. Factors which may influence the permittivity and viscosity of the liquid medium, particularly temperature, must be kept constant at all times in order to allow for accurate comparison of data (James, 1991).

Figure 3.1.1. An illustration of the charged areas surrounding a cell's surface, including the relative positioning of the cell surface charge and ζ potential (taken from James, 1991).

3.1.2 Cell surface hydrophobicity (CSH)

Cell surface hydrophobicity (CSH) is a measure of cellular interaction and formation of weak electrostatic hydrogen bonds with water. A strongly hydrophobic object brings about a passive removal of water layers that exist between it and other hydrophobic surfaces, drawing them closer and creating a more energetically stable state (Rosenberg & Doyle, 1990; Van Der Mei *et al.*, 1991). The overall hydrophobic property of a cell is in fact a conglomerate of the various hydrophobic (hydrophobins) and hydrophilic (hydrophilins) cell surface components. These cell surface components are present in varying concentrations (Rosenberg & Doyle, 1990; Doyle, 2000).

There is much evidence to suggest that cells with marked CSH achieve greater attachment to host tissues and prosthetic devices than less hydrophobic counterparts (Van Der Mei *et al.*, 1991; Doyle, 2000). Paradoxically highly hydrophobic cells are more vulnerable to phagocytosis by neutrophils as a result of this marked hydrophobicity (Absolom, 1986).

CSH is relatively simple to measure, yet data acquired is often difficult to compare. The reasons for this are twofold, firstly each of the various techniques has potential for marked inter-experimental variation; secondly the form of hydrophobicity measured varies from technique to technique. Three methods for assessing CSH were employed as outlined below; microbial adherence to hydrocarbons (MATH) (Rosenberg & Doyle, 1990), hydrophobicity interaction chromatography (HIC) (Smyth *et al.*, 1978) and hydrophobic microsphere attachment (HMA) assay (Hazen & Hazen, 1987). Each of these assays has various strengths and weaknesses and as such when used in conjunction support robust comparison and extrapolation.

The MATH assay indicates CSH by monitoring the change in population size of an organism, suspended in an aqueous buffer, when it is exposed to a hydrocarbon. This method of partitioning is especially suited towards organisms in unicellular form and provides a quantitative view of CSH. The main disadvantages of the MATH assay are that cells coming in contact with hydrocarbons are almost certainly adversely affected (Pembrey *et al.*, 1999), and that cell surface charge can affect results (Geertsema-Doornbusch *et al.*, 1993).

HIC protocols pass a quantified population of cells, in aqueous buffer, over octyl- or phenyl-sepharose gel, an electrically neutral hydrophobic solid state material. The population is reassessed and hydrophobicity determined as a comparative measure of the drop in cell numbers as a result of cell attachment to respective sepharose gels. HIC protocols are useful in measuring charge-independent attachment to solid surfaces; conversely however, entrapment of cells in the column has been known to give errant readings (Hazen, 1990; Rosenberg & Doyle, 1990; Van Der Mei *et al.*, 1991).

HMA is a semi-quantitative method in which a population of cells in aqueous buffer is introduced to hydrophobic polystyrene microspheres, and then incubated for varying periods of time before attachment is observed. The main advantage of HMA is that the distribution of hydrophobic sites on filamentous and unicellular surfaces can be assessed. The main disadvantage is its semi-quantitative status.

3.2 Materials and methods

Details of principle equipment, chemicals and materials, are included within the text; information pertaining to more common items can be found in Appendix G.

3.2.1 Cell surface charge

Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm), logarithmic or stationary phase cells were removed and washed twice with 1 mM KCl. Immediately before use 1 mM KCl was carefully adjusted to pH values in the range pH 3 to pH 9 using concentrated hydrochloric acid and 5 M KOH, cells were then suspended in this pH adjusted medium to a concentration of 1 x 10^7 cells ml^{-1}. The ζ potential of the samples was then measured with a ZetaPlus Zeta Potential Analyzer (Brookhaven Instruments Corporation, New York, USA) using Laser Doppler Velocimetry (LDV).

Approximately 1.2 ml of sample was homogenised thoroughly and placed in a clear sided plastic cuvette containing two electrodes. The cuvette was inserted into the ZetaPlus machine and a charge was applied across the electrodes. A laser (659 nm wavelength, 27 mW output power) was then projected through the sample. Doppler shifted light, scattered due to movement of the cells, was then detected at 15 degrees from the laser beam, and transformed to a measure of mobility. The electrophoretic mobility of the sample was then converted using the Smoluchowski equation (see section 3.1.1) to a ζ potential. In order to ensure the constant viscosity and permittivity of the sample medium, temperature was maintained at 25 °C. Five population readings were gained for each of three separate samples. Population distributions for each set of readings were compared by examination of half width data.

Data were analysed using two factor ANOVA (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). The following comparisons were analysed, (i) differences in ζ potential between strains and growth phase at pH 7 and (ii) differences in ζ potential between strains and pH. Where necessary to illustrate significance, the LSD was calculated as part of the ANOVA analysis.

3.2.2 Cell surface hydrophobicity (CSH)

3.2.2.1 Microbial adhesion to hydrocarbons (MATH)

The method used for MATH analysis was that outlined by Smith *et al.* (1998). Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm), logarithmic or stationary phase cells were removed, washed, suspended in phosphate urea magnesium (PUM) buffer (Appendix B) and adjusted to an OD of 0.5 at 470 nm (A470) using a Novospec II spectrophotometer (Pharmacia BioTech, Cambridge, UK). Replicate 1.5 ml samples of the suspension were placed in identical small acid-washed glass tubes before addition of 300 µl of n-hexadecane. Samples were left to stand for 10 min before vortexing for 30 seconds and then left for a further 10 min. The layer of n-hexadecane

was then removed by pipette. After 15 min at 4 °C the remainder of the n-hexadecane, now solid, was removed using plastic inoculation loops. The OD of the remaining aqueous sample was then ascertained (A470) and the relative CSH calculated using the equation depicted in Figure 3.2.2.1; results that gave negative values were reported as zero.

$$\text{Relative cell surface hydrophobicity (\%)} = \frac{\text{Initial A470 - Final A470}}{\textit{Initial}\ \text{A470}} \times 100$$

Figure 3.2.2.1. Calculation of relative cell surface hydrophobicity for MATH analysis.

Data were analysed using two factor ANOVA (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). Differences in relative CSH between strains and growth phase were analysed. Where necessary to illustrate significance, the LSD was calculated as part of the ANOVA analysis.

3.2.2.2 Hydrophobic interaction chromatography (HIC)

Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm), logarithmic or stationary phase cells were removed, washed, suspended in PUM buffer and adjusted to an OD of 0.5 at 470 nm (A470). Two 1 ml samples were dispensed onto the top of two glass Pasteur pipettes loosely plugged with 0.1 g of glass wool. One of each pair of pipettes contained 1 ml of control sepharose CL-4B, the other contained 1 ml of hydrophobic phenyl-sepharose CL-4B. After passage of cells, each column was washed with 4 ml of PUM buffer and the resultant fractions were pooled, centrifuged and reconstituted to 1 ml before the OD was read at 470 nm (A470). The relative CSH of each sample was calculated using the equation given in Figure 3.2.2.2. As with the MATH assay, results that gave negative values were reported as zero.

$$\text{Relative cell surface hydrophobicity (\%)} = \frac{\text{Control A470 - Experimental A470}}{\textit{Control}\ \text{A470}} \times 100$$

Figure 3.2.2.2. Calculation of relative cell surface hydrophobicity for HIC analysis.

Data were analysed using two factor ANOVA (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). Differences in relative CSH between strains and growth phase were analysed. Where necessary to illustrate significance, the LSD was calculated as part of the ANOVA analysis.

3.2.2.3 Hydrophobic microsphere attachment (HMA)

The HMA assay performed here was modified from that used by Hazen & Hazen (1987). Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm), logarithmic or stationary phase cells were removed, washed, suspended in PUM buffer and adjusted to approximately 4 x 10^6 cell ml^{-1}. Fluorescein isothiocyanate (FITC) labelled plain latex microspheres of 1 μm diameter (Polysciences Europe GmbH, Eppelheim, Germany) were mixed with 500 μl PUM buffer to a concentration of 2 x 10^9 ml^{-1} and washed twice before being combined in triplicate with appropriate cell suspensions. After 1 and 24 hours incubation at 23 °C samples were examined using fluorescence microscopy. Two hundred cells from each sample were examined and the number from this population with three or more microspheres attached was recorded. Representative images were taken using an AxioCam digital microscope camera (Imaging Associates, Bicester, UK). In order to obtain satisfactory prints, contrast and brightness of images were altered using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, USA), no raw data was removed and no synthetic data was added in this process.

3.3 Results

3.3.1 Cell surface charge

Figure 3.3.1a is an example of the typical population profile achieved through use of the Brookhaven ZetaPlus equipment. For each of three replicate samples the mean of five population readings is produced. The mean half width for all the populations analysed was 5.5 mV, with a standard error of 0.054 mV, the population profiles were therefore relatively uniform irrespective of strain, growth phase and pH.

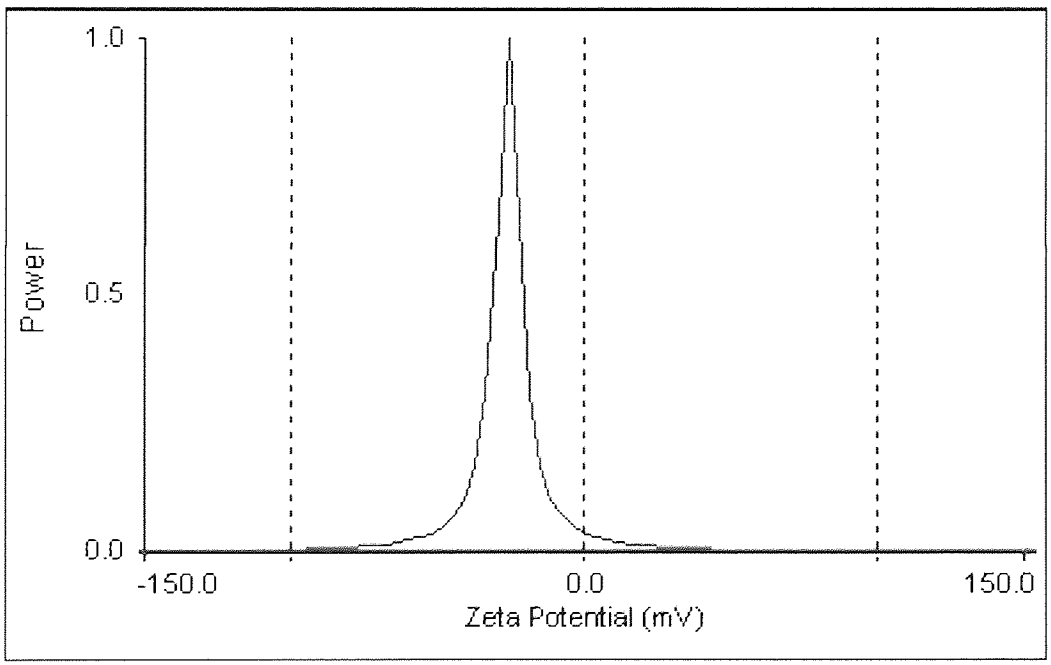


Figure 3.3.1a. A typical profile received from the ZetaPlus Zeta Potential Analyser for a strain of *C. neoformans* (CN IRM 5815 in logarithmic growth phase at pH 7).

Figure 3.3.1b compares the ζ potential readings of four strains of *C. neoformans* in both logarithmic and stationary growth phase at pH 7. Note, although there is no significant difference between growth phases, there is a significant difference between strains ($P < 0.001$) and that this is mainly due to the results obtained from ATCC 52817, which gave ζ potential readings markedly closer to neutral than all other strains.

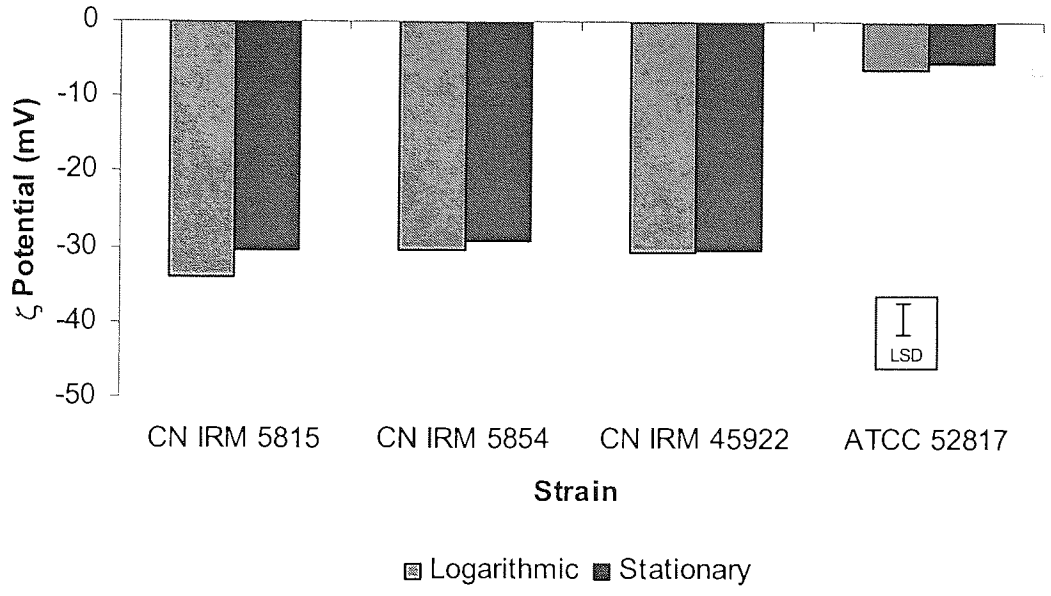


Figure 3.3.1b. ζ potentials of *C. neoformans* in logarithmic and stationary phase growth suspended in 1 mM KCl adjusted to pH 7. Results are means of three separate populations; LSD is calculated from ANOVA analysis.

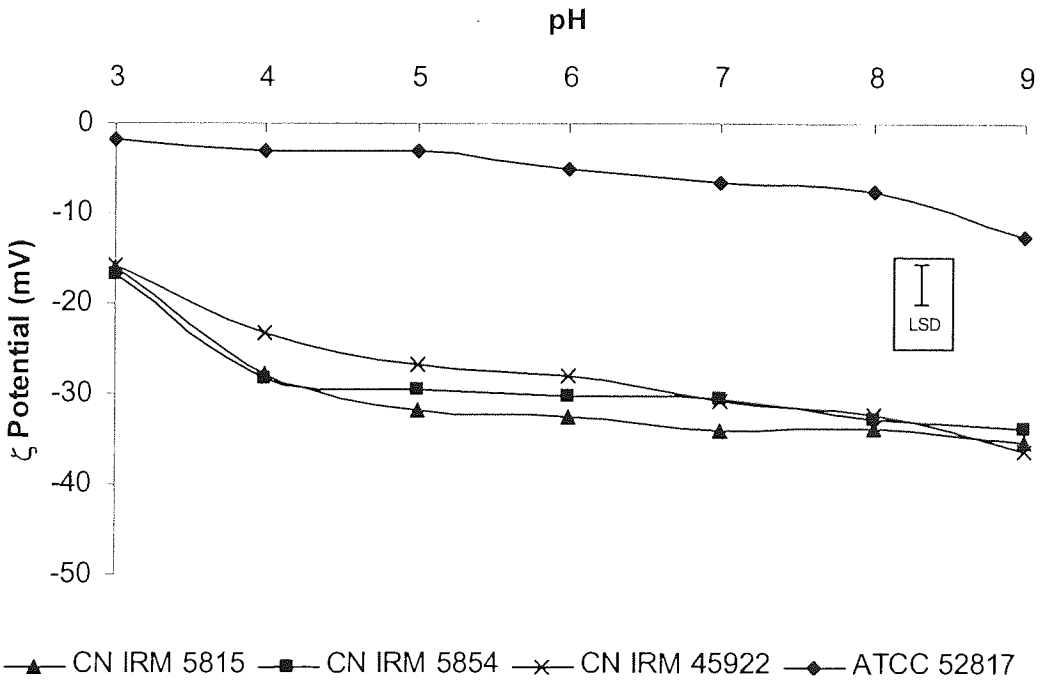


Figure 3.3.1c. ζ potentials of *C. neoformans* in logarithmic phase growth suspended in 1 mM KCl adjusted to a broad pH spectrum. Results are means of three separate populations; LSD is calculated from ANOVA analysis.

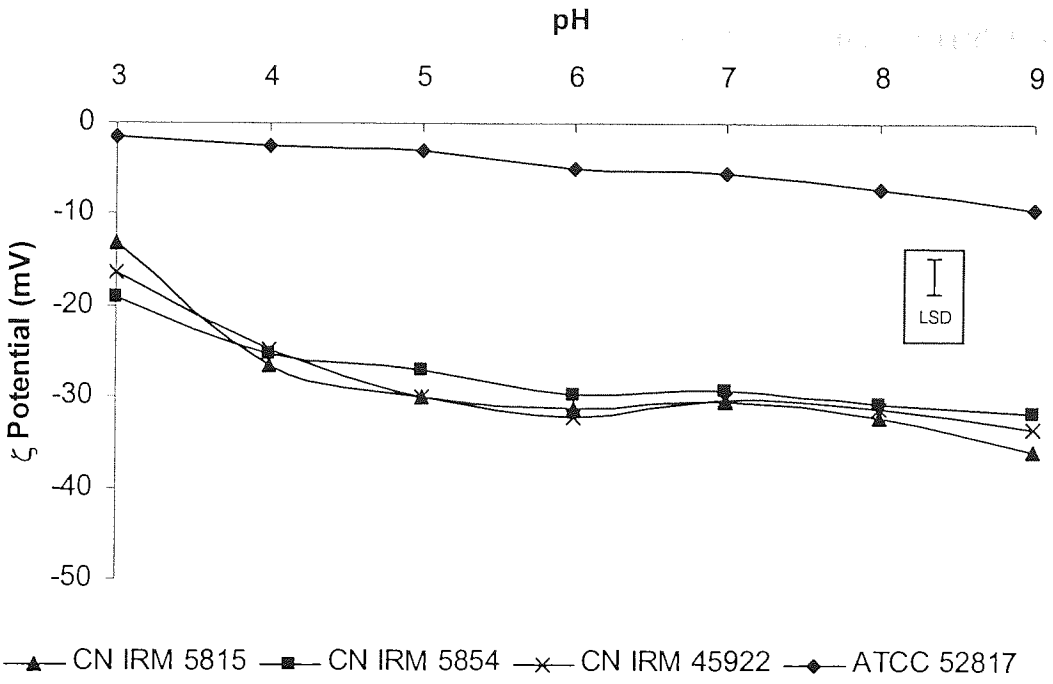


Figure 3.3.1d. ζ potentials of *C. neoformans* in stationary phase growth suspended in 1 mM KCl adjusted to a broad pH spectrum. Results are means of three separate populations; LSD is calculated from ANOVA analysis.

Figures 3.3.1c&d (above) illustrate the variation of ζ potential across the pH spectrum for logarithmic and stationary growth phases respectively. In both log and stationary phase populations, ζ potential gradually becomes more negative with increasing pH. A significant difference was observed between strains ($P < 0.001$), which, as LSD figures suggest, is largely due to strain ATCC 52817 which is markedly more neutral in charge when compared to all other strains.

3.3.2 Cell surface hydrophobicity (CSH)

3.3.2.1 Microbial adhesion to hydrocarbons (MATH)

Table 3.3.2.1 shows that without exception all four strains in both growth stages were extremely hydrophilic when assayed using the MATH procedure. ANOVA analysis of the results indicated no significant difference between growth

stages, the same statistical analysis also showed no significant difference between the heavily encapsulated strain CN IRM 5815 and the capsule free strain ATCC 52817 (LSD = 1.34 %).

Table 3.3.2.1. % relative cell surface hydrophobicity of strains of *C. neoformans* assessed using the MATH assay. Results are means of three experimental readings (± standard error).

Strain	Growth Phase	
	Logarithmic	Stationary
CN IRM 5815	0.00 (± 0.00)	0.00 (± 0.00)
CN IRM 5854	2.39 (± 1.39)	1.36 (± 0.92)
CN IRM 45922	2.51 (± 1.85)	0.77 (± 0.77)
ATCC 52817	0.70 (± 0.42)	0.00 (± 0.00)

3.3.2.2 Hydrophobic interaction chromatography (HIC)

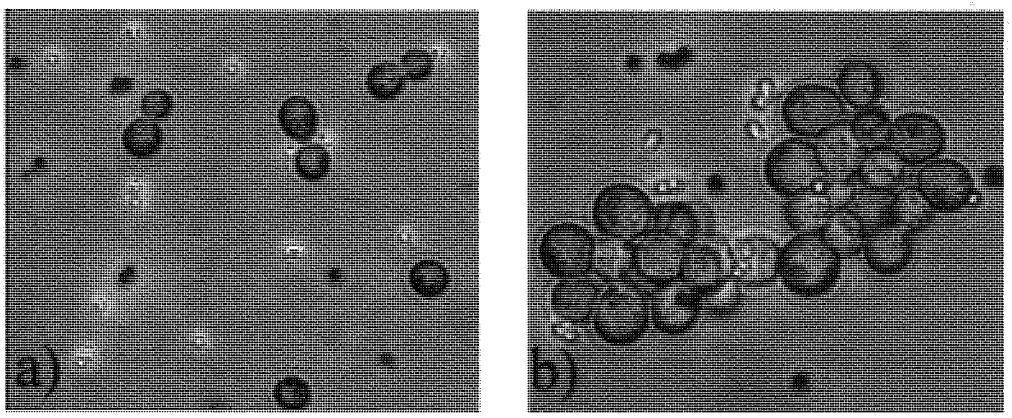
It can be seen from Table 3.3.2.2 that with HIC analysis, as with MATH analysis, *C. neoformans* strains analysed are extremely hydrophilic. There was no significant difference between strains or growth phases. Strain ATCC 52817 gave no useable data with this technique due to entrapment of clumped cells in sepharose gel matrices and glass wool plugs.

Table 3.3.2.2. % relative cell surface hydrophobicity of strains of *C. neoformans* assessed using the HIC assay. Results are means of three experimental readings (± standard error).

Strain	Growth Phase	
	Logarithmic	Stationary
CN IRM 5815	0.32 (± 0.32)	0.31 (± 0.31)
CN IRM 5854	0.69 (± 0.69)	0.00 (± 0.00)
CN IRM 45922	2.22 (± 2.22)	1.06 (± 1.06)
ATCC 52817	No Data	No Data

3.3.2.3 Hydrophobic microsphere attachment (HMA)

Figures 3.3.2.3a&b are images of microsphere-cell association. As can be seen the microsphere to cell ratio was optimised to ensure clear definition between those microspheres that were bound to cells and those in close proximity. Figure 3.3.2.3a has no cells with three microspheres bound and as such all cells were scored as lacking bound microspheres hence hydrophilic. In contrast, Figure 3.3.2.3b shows a number of cells with at least three visibly bound microspheres, in turn indicative of hydrophobic interaction between cells and microspheres. It is important to note that changes in focus plane are needed in order to assess each cell accurately and that images are for qualitative analysis only.



Figures 3.3.2.3a&b. Images of hydrophobic microsphere attachment.
a) CN IRM 5815 in logarithmic growth phase after 1 hour incubation with microspheres.
b) ATCC 52817 in logarithmic growth phase after 1 hour incubation.

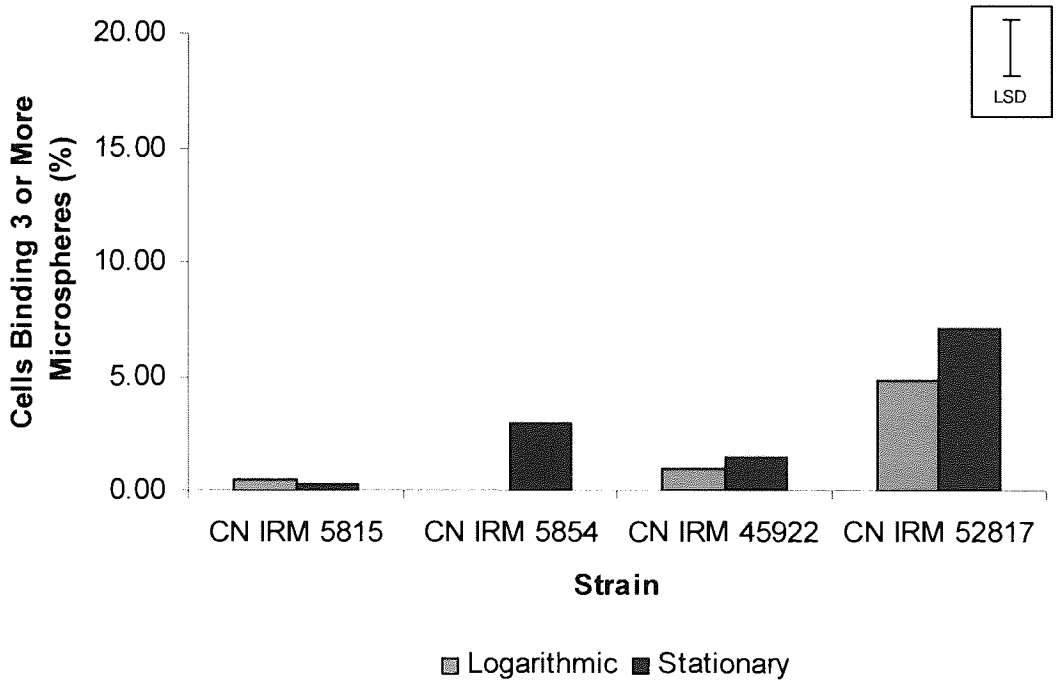


Figure 3.3.2.3c. Percentage of *C. neoformans* cells binding three or more hydrophobic microspheres after one hour of incubation at 23 °C. Results are means of 3 experimental readings of a population of 200 cells. LSD is calculated from ANOVA analysis.

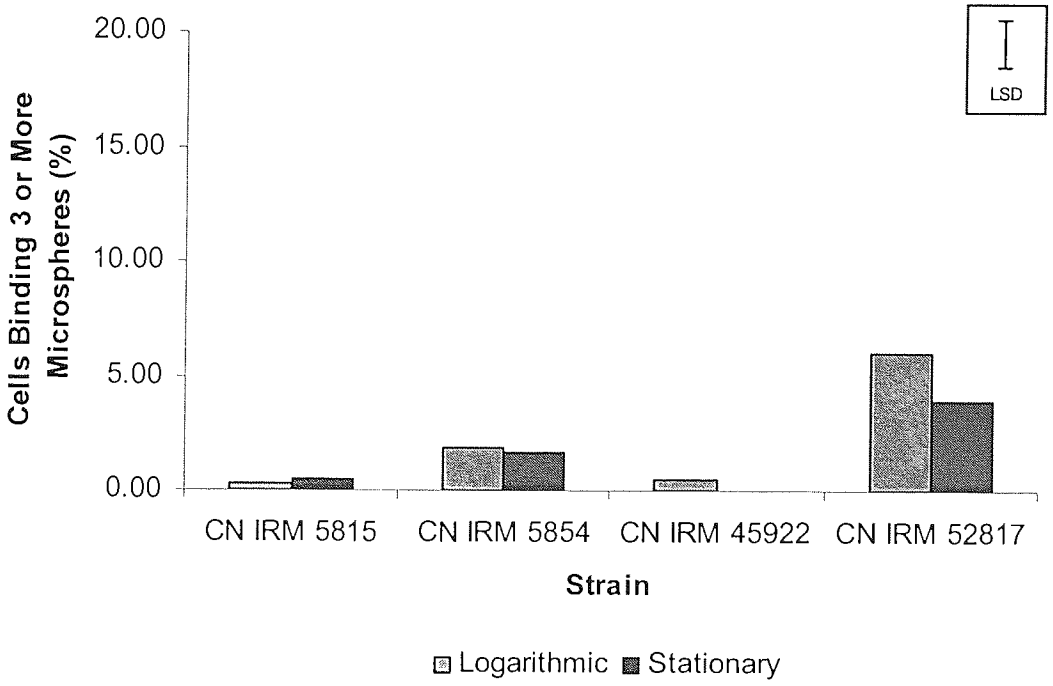


Figure 3.3.2.3d. Percentage of *C. neoformans* cells binding three or more hydrophobic microspheres after 24 hours incubation at 23 °C. Results are means of 3 experimental readings of a population of 200 cells. LSD is calculated from ANOVA analysis.

Figures 2.3.2.3c&d (above) indicate that the HMA assay, in line with those of MATH and HIC, found *C. neoformans* strains to be hydrophilic regardless of incubation period with microspheres, or growth phase. The average number of positively bound cells, regardless of strain, incubation time and growth phase, was 4.15 (± 0.73) per 200. Despite a general demonstration of hydrophilicity, strains did vary and a significant difference in microspheres bound was found between strains in the same growth phase ($P < 0.001$). LSD analysis indicates that, in common with ζ-potential analysis, ATCC 52817 is largely responsible as it was significantly different to all other strains of similar maturity. The length of incubation did not significantly increase the amount of positively bound cells for any strain in any growth phase, other than ATCC 52817 in stationary phase ($P = 0.048$). Due to the general paucity of microsphere attachment an accurate study as to the nature and orientation of microsphere binding sites was precluded.

3.4 Discussion

3.4.1 Cell surface charge

Klotz *et al.* and Lock *et al.* (1985; 1987) suggest that microorganism ζ potential determinations are indicative of microorganism behaviour in the presence of proximal charged surfaces. Furthermore, measuring the interaction of pH and ζ potential indicates which ionogenic groups, amino, carboxyl or phosphate, are amongst a cell's outermost surfaces (Gittens & James, 1963; Bayer & Sloyer, 1990; James, 1991); additionally, ζ potential can indicate how surface ionogenic moieties alter the physico-chemistry of organisms like *C. neoformans* when naturally exposed to environments of variable pH (Casadevall & Perfect, 1998).

Studies of the cell surface charge of *C. neoformans* (Kozel *et al.*, 1980; Kozel & Gotschlich, 1982; Nosanchuk & Casadevall, 1997) concluded that encapsulated *C. neoformans* strains are strongly negative in charge and that acapsulate mutant strains showed a weakly negative status. Here, a modern method of charge assessment has been used to analyse thoroughly the ζ potential of known encapsulated wild type and acapsulate *C. neoformans* strains, both at differing growth phases and with pH variation. In line with previous studies, wild type *C. neoformans* strains were found to have a marked negative charge. However, the acapsulate genetic mutant ATCC 52817 exhibited considerably less of a negative charge, confirming the work of Nosanchuk & Casadevall (1997). The contrast between the acapsulate strain and wild type strains indicates that all three wild type strains under investigation produce some capsular material. In addition, the weakly negative charge of the unshielded cell wall of the acapsulate strain, suggests that the cell wall of *C. neoformans* contributes little, if anything, to the markedly negative charge of wild type strains.

Cell culture maturity, regardless of the encapsulation status of differing strains employed, had no significant affect upon *C. neoformans* strains ζ potential. For the encapsulated wild type strains of *C. neoformans* this suggests that they remain fully encapsulated throughout their lifecycle. Interestingly, the acapsulate strain ATCC 52817 also manifests the same cell surface charge regardless of culture age, suggesting that the cell wall of ATCC 52817, and potentially of encapsulated strains,

either changes very little with cellular maturity, or that any changes in cell surface nature which may occur with ageing do not influence charge significantly.

The affect of pH upon ζ potential gives some insight as to the nature of the cell surface producing it. Figures 3.3.1c&d indicate that overall the charge of the three wild type strains of *C. neoformans* decreased with increasing pH, however there is a noticeable plateau from around pH 5 to pH 8. Unlike a noticeably stepped change in charge, a plateau indicates that only one ionogenic group is largely responsible for producing the charged attribute of a cell population (James, 1991). This finding, and reports that capsule polysaccharide is known to contain approximately 90 % GXM (Cherniak & Sundstrom, 1994; Casadevall & Perfect, 1998), support the widely held assumption that glucuronic acid and therefore carboxyl groups are major contributors to cell surface charge of *C. neoformans* (Kozel *et al.*, 1980; Kozel & Gotschlich, 1982; Nosanchuk & Casadevall, 1997).

The weakly negative charge of ATCC 52817, previously observed by Nosanchuk & Casadevall (1997), was more thoroughly investigated here by the manipulation of pH. The affect of pH on ATCC 52817 ζ potential is dissimilar to the wild type strains as it describes a consistently linear, though weak, decrease in charge with increasing pH. These observations lead to the conclusion that the cell wall surface of ATCC 52817 is made up almost entirely of neutrally charged groups with low levels of variably charged groups, the majority of which appear to be negatively charged. Such an interpretation is supported by the conclusions of James *et al.* (1990), who examined the constituents of the cell wall of ATCC 52817 (Cap 67), and found that it was made up largely of neutral glucose based molecules; they also noted that glucosamine, hexosamine and phosphate based material was present in smaller amounts. These latter components would, if exposed, have a variable effect on the charge of the cell.

The consequences of a marked negative charge, both for microorganisms in general and specifically *C. neoformans*, remain obscure. Lock *et al.* (1987) found that hydrophilic *Escherichia coli* of considerable negative ζ potential, were more resistant to phagocytosis by polymorphonuclear leukocytes than weakly negative hydrophobic counterparts. Kozel *et al.* (1980) went further by chemically removing the negative charge associated with *C. neoformans* and then assessing its vulnerability to mice peritoneal macrophages, in turn, concluding that charge plays a concomitant but minor role in evasion of phagocytosis. Although it may compromise phagocytosis, and hence

provide enhanced survival, a number of studies suggest that microorganisms that elicit a low negative charge, more readily adhere to surfaces in the immediate cellular environment. In particular, Klotz *et al.* (1985) examined the adherence of *Candida* species to plastic surfaces; they concluded that yeast manifesting a weak charge adhered in greater numbers to negative plastic surfaces. Indeed, the more neutral charge of ATCC 52817 may be a contributing factor to the clumping or flocculation observed in this strain by microscopy (Figure 3.3.2.3b).

3.4.2 Cell surface hydrophobicity (CSH)

Hydrophobicity assessment, in common with cell surface charge determination, may be indicative of a particular pathogens interaction with host surfaces, and with other microorganisms. The contrasting nature of *C. neoformans* strains, and of the various methods employed by this study, allows for effective characterisation of cellular hydrophobicity and, in addition, any contribution to it that the capsule of *C. neoformans* may provide.

MATH analysis, of four strains of *C. neoformans*, indicated that all four were extremely hydrophilic regardless of culture age, they each gave a relative CSH reading markedly lower than the 30 % level used by other investigators as an indicator of considerable hydrophilicity (Jones *et al.*, 1996). Furthermore, results indicate that both the capsule and the cell wall surface are largely hydrophilic. An observation supportive of speculation that capsule and cell wall surfaces have very little, if any, dense areas of hydrophobins, thus indicating a lack of even relatively small areas of hydrophobicity. Using the MATH assay various authors have linked encapsulation to hydrophilicity in medically important bacteria (Ofek *et al.*, 1983; Rosenberg *et al.*, 1983; Williams *et al.*, 1986; Reifsteck *et al.*, 1987; Benedi *et al.*, 1989). In turn, the only medically important encapsulated fungus, *C. neoformans*, is, like these encapsulated bacteria, also hydrophilic. Interestingly, when lacking a capsule *C. neoformans* retains this hydrophilic property.

HIC protocols differ from MATH assessment by employing a solid neutrally charged surface for test cells to adhere to (Smyth *et al.*, 1978). This lack of charge excludes any interference by electrostatic interaction therefore giving a less complex

analysis of hydrophobicity. Cryptococcal HIC analysis of natural strains supported similar conclusions to that derived from MATH work. Cell culture age had no affect on the extreme hydrophilicity recorded. Acapsulate mutant ATCC 52817 was found unsuitable for use with this technique, a consequence of the tendency for cells of this strain to aggregate giving rise to large bundles of cells which failed to readily pass through column matrices. This difficulty has often been associated with HIC analysis (Hazen, 1990; Rosenberg & Doyle, 1990; Van Der Mei *et al.*, 1991).

The results of the HMA assay confirm the opinion that *C. neoformans* is an extremely hydrophilic organism. Cell culture age significantly affected the adherence of microspheres to strain CN IRM 5854 after an hour of incubation, however no other strain was affected and the scale of change from 0 % to 3 % adherence may not be considered biologically relevant as it does not signify a change from a hydrophilic to a hydrophobic cellular state. Overall binding by microspheres to strain ATCC 52817 was more marked in comparison to wild type strains employed. However the level of binding experienced was sparse and as such cannot be considered sufficient to query the hydrophilic status of this strain. In addition, although microscopic observation clearly indicated microspheres associated with ATCC 52817, there may be some doubt as to whether the microspheres were hydrophobically attracted. A possible alternative explanation for the observed association is that the microspheres became entangled within the aggregated groups of cells when the two were combined.

The contribution of hydrophobicity to flocculation of microorganisms and colonisation of alien surfaces is well documented (Amory *et al.*, 1988; Rosenberg & Doyle, 1990; Van Der Mei *et al.*, 1991). CSH of medically important fungi, particularly *Candida* species (the focus of many studies), has been looked at in depth by Hazen (1990). Hazen concluded that of the various *Candida* species, *Candida tropicalis* has most consistently been attributed with the greatest hydrophobicity, whereas *C. albicans* has variously been labelled as both weakly and strongly hydrophobic in its unicellular state. In their work Klotz *et al.* (1985) found that *C. albicans* hydrophobicity levels varied markedly between strains and also between methods of assessment. In his review of fungal hydrophobicity Hazen (1990) stated that culture age had a variable affect upon CSH of *C. albicans*. Here *C. neoformans* shows no real variability in CSH between the strains studied. Direct comparison between data derived from different studies is generally seen as limited in value, a consequence of the broad array of inter-experimental anomalies associated with both

the MATH and HIC assays. However, it is clear from the HIC analysis results presented by Kozel (1983), and from the work described herein, that *C. neoformans* strains are uniformly hydrophilic regardless of strain, capsule or culture age. This conclusion contrasts with the reported hydrophobicity of *C. albicans*, which is known to vary markedly with strain and culture age (Hazen, 1990).

3.4.3 Conclusion

In-depth analysis of the ζ potential of several *C. neoformans* strains suggests that regardless of culture maturity, wild type *C. neoformans* strains manifest marked negative charge. This charge is, in the main, created by glucuronic acid contained in a polysaccharide capsule. When stripped of its capsule, *C. neoformans* yeast appear to exhibit a weak negative charge, probably caused by a lack of charged structures associated with the cell wall.

Thorough investigation of the hydrophobicity of *C. neoformans* cells demonstrated a hydrophilic nature amongst strains examined. This hydrophilicity is manifest probably for the concomitant reason that encapsulated *C. neoformans* cells exhibit a strongly negative cell surface charge. A weak cell surface charge and strong hydrophobicity are widely thought to promote adherence to host surfaces and promote infection (Hazen, 1990; Rosenberg & Doyle, 1990; Van Der Mei *et al.*, 1991). In contrast, *C. neoformans* cells do not manifest such non-specific force characteristics, thereby interacting more weakly with host surfaces. *C. neoformans* outer surface constitution may therefore result in reduced predation. In the clinical context this may be predation by phagocytic cells of the vertebrate immune system, and in the greater environmental context from voracious amoeboid phagocytes.

CHAPTER 4 – Biotin based carbohydrate analysis

4.1 Introduction

C. neoformans manifests non-specific cell surface attributes which may reduce the likelihood of contact with host tissues and predator entities (see Chapter 3 along with Kozel *et al.*, 1980; Kozel, 1983; Nosanchuk & Casadevall, 1997). Regardless of this state of affairs, research has indicated that *C. neoformans* adheres to mammalian cells (Jimenez-Lucho *et al.*, 1990; Ibrahim *et al.*, 1995), and has even been shown to exhibit facultative parasitism (Merkel & Scofield, 1994; Feldmesser *et al.*, 2000; Feldmesser *et al.*, 2001). These seemingly contrasting observations may be resolved by further investigation into interaction processes associated with the *C. neoformans* cell surface and capsule. A well documented host-microbial interaction related phenomenon is the expression of cell surface carbohydrate in the form of glycosylated structures (Doyle, 1994). Carbohydrates serve many functions in the yeast cell wall including structural and receptor based roles (Gooday, 1995). It is this former set of functions, often carried out by glycoproteins, which is of interest when studying the interaction of *C. neoformans* cells with their proximal and greater environment. Glycoproteins are glycosylated structures in which carbohydrates are attached covalently to amino acid residues of peptides. They are found universally in eukaryotic cell components including mammalian cell membranes and yeast cell walls (Odds, 1979; Gooday, 1995; Stryer, 1995). In particular, glycoproteins have been shown to modulate various functions including adherence and germination in *C. albicans* (Tronchin *et al.*, 1989; Korting & Ollert, 1994; Kanbe & Cutler, 1998). In turn, this study selectively examined carbohydrate moieties on the *C. neoformans* cell surface with the aim of identifying possible expression of glycosylated structures, particularly glycoproteins.

Carbohydrate – potentially associated with glycoprotein – on the cell surface of *C. neoformans* yeast was assessed quantitatively and qualitatively using biotin hydrazide and fluorescein-streptavidin. Biotin is a 244 Dalton vitamin with a marked affinity for avidin and its prokaryotically associated sister molecule streptavidin (Wilchek & Bayer, 1988). Biotin hydrazide can be used to attach biotin to oxidised

carbohydrates. In order to selectively bind glycosylated structures, particularly glycoproteins, controlled oxidisation of cell surface molecules is required. As a result, the extent and type of carbohydrate biotinylation achieved using biotin hydrazide, is dependent upon the strength of the oxidising agent used and length of exposure prior to addition of biotin hydrazide (O'Shannessy & Quarles, 1987). The method employed here was adapted from those used by Hughes *et al.* (1999) and Kähne & Ansorge (1994), and was designed to facilitate identification of cell surface glycoproteins. Once bound, biotin was then labelled with fluorescein-streptavidin before quantitative analysis using a fluorescence activated cell sorter (FACS). The phenomenon of ligand-receptor binding, seen here as biotin-carbohydrate binding, can only be studied in depth if examined using saturation binding analysis (Rang *et al.*, 1999). As a result, binding of various concentrations of biotin hydrazide was analysed in order to gain further information on the ligand receptor relationship. Qualitative analysis was then performed using confocal microscopy.

4.2 Materials and methods

Details of principle equipment, chemicals and materials, are included within the text; information pertaining to more common items can be found in Appendix G.

4.2.1 Biotinylation of *C. neoformans* cell surface carbohydrates

Two isolates of *C. neoformans*, CN IRM 5815 and ATCC 52817, were selected for use in this work. Selection was based upon contrasting capsule production; CN IRM 5815 produces an extensive capsule, ATCC 52817 is acapsulate.

Mild and selective oxidation of surface carbohydrates to produce aldehyde groups was performed using the following technique. Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm) in triplicate. Logarithmic or stationary phase cells were removed, washed twice, and suspended in phosphate buffered saline (PBS). Cell concentration

was adjusted to 2 x 10^7 cell ml^{-1}. Cell suspensions of 1 ml were transferred to sterile Eppendorf tubes in triplicate, and by the addition of 50 mM sodium periodate solution, were adjusted to a 0.5 mM solution of sodium periodate-PBS. Following a 15 minute incubation in the dark at room temperature, cells were pelleted by centrifugation, washed five times with, and resuspended in, 0.9 ml of ice cold PBS.

Biotinylation of oxidised carbohydrates was performed using varying concentrations of biotin hydrazide. Samples in triplicate were adjusted to the required concentration by the addition of biotin hydrazide dissolved in 100 µl of dimethyl sulphoxide (DMSO). After mixing, cells were incubated at room temperature for 30 minutes with regular agitation, pelleted by centrifugation and washed three times with PBS.

The final stage of biotinylation is the binding of fluorescein-streptavidin (Molecular Probes, Leiden, The Netherlands) to the carbohydrate bound biotin, this procedure was adapted from that used by Bhalgat *et al.* (1998). Cells were re-suspended in 1 ml PBS containing 5 µg ml^{-1} fluorescein-streptavidin and incubated on ice in the dark for 30 minutes with regular agitation before being pelleted by centrifugation, and washed three times with PBS in order to remove excess fluorescein-streptavidin. Before further analysis cells were pelleted by centrifugation and fixed in 1 % w/v paraformaldehyde / PBS solution.

4.2.2 Flow cytometry saturation binding analysis

Saturation binding analysis was performed on cells treated with five concentrations of biotin hydrazide; 0.01, 0.05, 0.1 and 0.5 mg ml^{-1}. Flow cytometry analysis was adapted from that used by Smith *et al.* (1999; 2001). Fluorescence of 10,000 yeast cells was determined with a Becton Dickinson FACS 440 using an argon laser (200 mW), with an excitation wavelength of 488 nm and emitted light detector at 530 nm (± 15 nm), adjusted to a fixed channel using standard Brite Beads (Beckman Coulter, High Wycombe, UK), prior to determining fluorescence. Yeast samples were vortexed before introduction to sheath fluid and subsequent determination of fluorescence.

Binding concentration data were analysed using non-linear regression in the following manner: autofluorescence values were subtracted from each data point and resulting data fitted to a logistic function of the form:

$$\text{Bound(B)} = \frac{\text{Fmax} \bullet [\text{BH}]^{\text{Hn}}}{[\text{BH}]^{\text{Hn}} + \text{EC50}}$$

where Fmax is the fluorescence value when all binding sites are saturated, BH is the concentration of biotin hydrazide and EC50 is the concentration of biotin hydrazide that produces 50 % of the maximum saturation. Hn is the Hill coefficient, a measure of the cooperativity in the system (Rang *et al.*, 1999). Data fitting was by means of non-linear regression analysis performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, USA). The binding curve from each individual experiment was analysed to obtain estimates of Fmax, EC50 and Hn.

Data were analysed using two factor ANOVA and where necessary the Student's t-test (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). Differences in carbohydrate induced yeast cell fluorescence between strains and growth phase were analysed. Where necessary to illustrate significance, the LSD values were calculated as part of the ANOVA analysis.

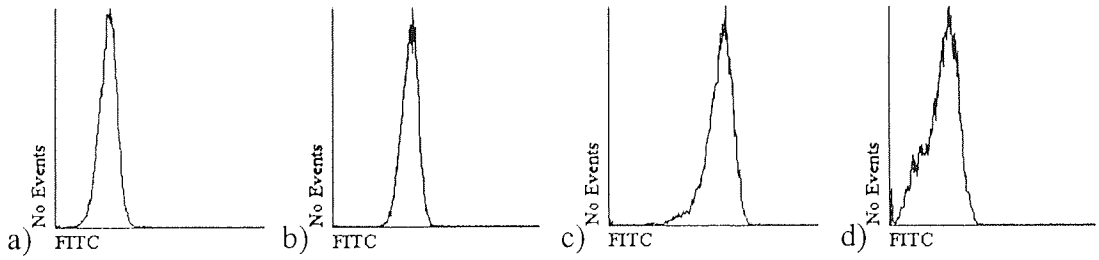
4.2.3 Confocal Microscopy

Aliquots of 7.5 µl logarithmic and stationary phase cells, biotinylated to a final concentration of 0.2 mg ml^{-1} biotin hydrazide using the procedure described above (section 4.2.1), were dispensed into wells of separate multispot microscope slides (Hendley-Essex, London, UK). Cell suspensions were then air dried and fixed by the addition of 7.5 µl cold acetone, which was subsequently allowed to evaporate under warm air. Wells were then sealed with 5 µl "Vectorshield" (Vector Laboratories, Burlingame, USA), a coverslip firmly affixed with "Tippex" correction fluid and stored at 5 °C in the dark until fluorescence characterisation by confocal microscopy. Images were acquired with a Zeiss Axiovert / Biorad MRC 1024 OS laser scanning

confocal microscope facility, utilising Laser Sharp 2000 software (Bio-Rad Laboratories, Hemel Hempstead, UK). An argon laser (100 mW), excitation wavelength 488 nm moderated by a series of neutral density filters, gain, offset and zoom functions was utilised to induce and optimise image fluorescence intensity, contrast and composition. Slides were viewed with an oil immersion Zeiss Neuflor 1.3 NA objective (Carl Zeiss, Welwyn Garden City, UK) and subjective material captured as 512 x 512 pixel images in turn converted from Bio-Rad PICT to tagged image file format (TIFF) format. In order to obtain satisfactory prints, contrast and brightness of images were altered using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, USA), no raw data was removed and no synthetic data was added in this process.

4.3 Results

4.3.1 Flow cytometry saturation binding analysis



Figures 4.3.1a-d. Flow cytometry population fluorescence profiles of biotin hydrazide binding to *C. neoformans* strains (at 0.01 mg ml^{-1}): (a) CN IRM 5815 (logarithmic growth phase), (b) CN IRM 5815 (stationary growth phase), (c) ATCC 52817 (logarithmic growth phase), (d) ATCC 52817 (stationary growth phase).

Fluorescein-streptavidin-biotin binding data were ascertained from profiles similar to those represented in Figure 4.3.1a-d (above), the profiles indicate the nature of fluorescence derived from a population of cells and therefore demonstrate the spectrum of carbohydrate coverage between population members and, potentially, between populations. The leptokurtic or sharply peaked appearances of profiles 4.3.1a-d, regardless of strain and culture age, are suggestive of great homogeneity in

carbohydrate exposure. Of notable contrast is the asymmetric appearance of logarithmic and stationary phase profiles of ATCC 52817 (Figures 4.3.1c&d), indicative of greater levels of variation in fluorescence hence some heterogeneity of carbohydrate exposure amongst population members within this particular strain, when compared to CN IRM 5815.

FACS equipment also reports mean average fluorescence values for each sampled population, it is these values that have been used to produce the comparisons in the following set of figures. Figure 4.3.1e compares biotin hydrazide binding to CN IRM 5815 and ATCC 52817 in both growth phases at the critical biotin hydrazide concentration of 0.1 mg ml^{-1}. This concentration is critical as it represents the beginning of the binding plateau and onset of binding site saturation (see Figures 4.3.1f&g). Data indicate that marked levels of carbohydrate were recorded on the cell surface of both strains examined. Two factor ANOVA analysis of these data confirms that the variation between strains is significant ($P < 0.001$). However, due to reciprocity amongst culture age data, no significant difference was noted between growth phases when both strains were analysed together ($P = 0.08$). Taken separately, each strain varies significantly between logarithmic and stationary phase (t-test, $P < 0.05$).

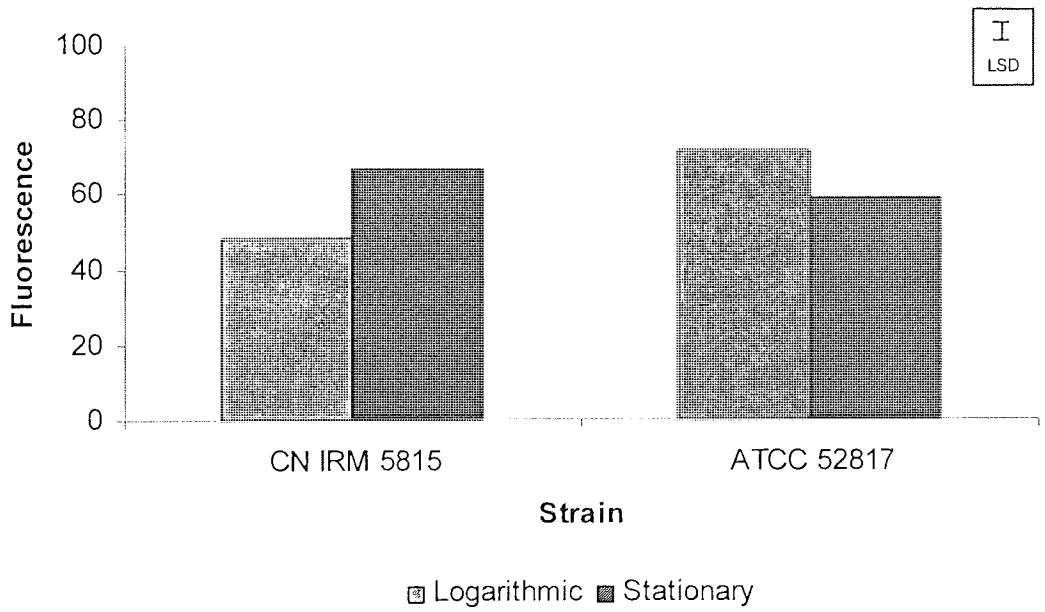


Figure 4.3.1e. Biotin hydrazide binding to *C. neoformans* surface expressed carbohydrates. Strains were grown to logarithmic and stationary growth phase. Biotin hydrazide concentration was 0.1 mg ml^{-1}. Results are means of three experimental readings; LSD is calculated from ANOVA analysis.

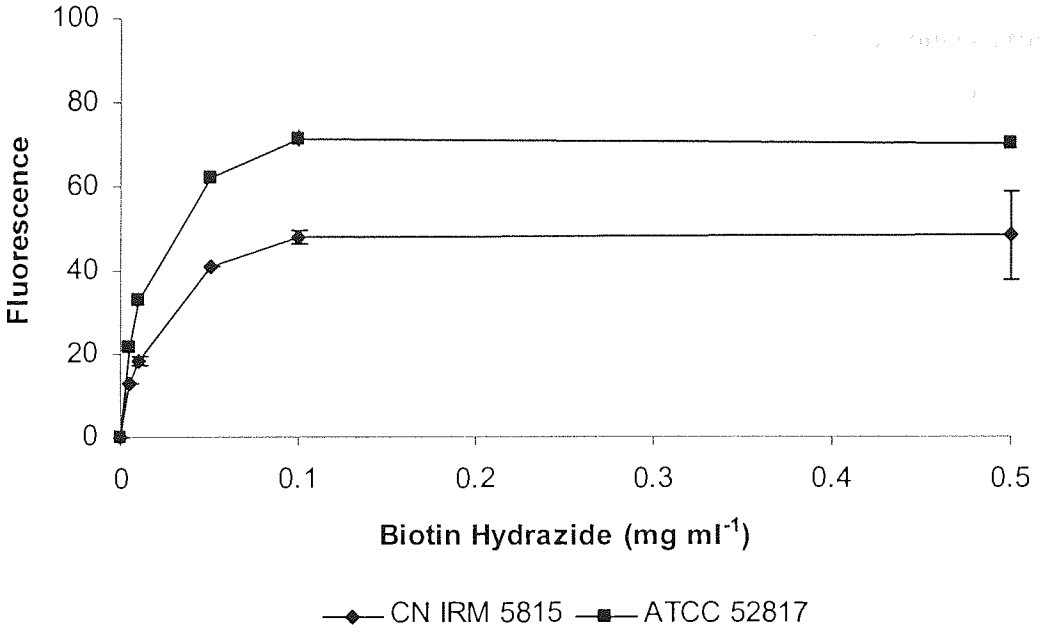


Figure 4.3.1f. The affect of biotin hydrazide concentration upon biotin hydrazide / fluorescein-streptavidin binding to carbohydrates on the cell surface of *C. neoformans* strains grown to logarithmic growth phase. Results are means of three experimental readings ± standard error.

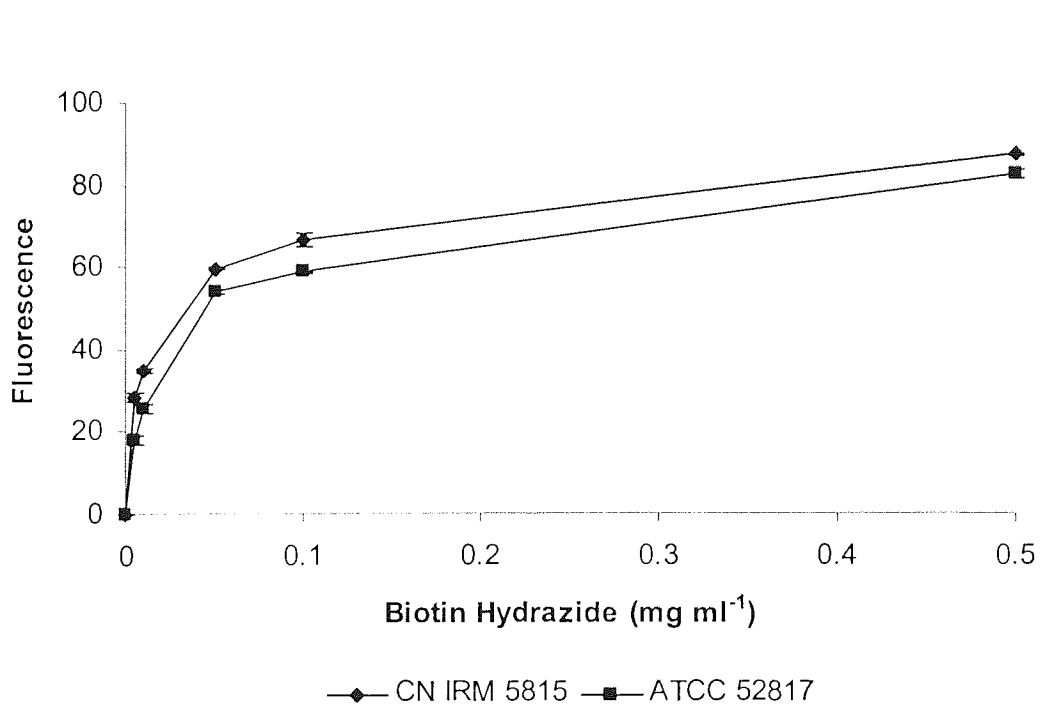


Figure 4.3.1g. The affect of biotin hydrazide concentration upon biotin hydrazide / fluorescein-streptavidin binding to carbohydrates on the cell surface of *C. neoformans* strains grown to stationary growth phase. Results are means of three experimental readings ± standard error.

Figures 4.3.1f&g (above) are concentration response plots comparing biotin hydrazide binding of carbohydrate to both strains in logarithmic and stationary growth phases respectively. ANOVA analysis found a significant difference between strains and concentrations in both growth phases ($P < 0.001$ for all four analyses). This was due to low variability amongst the majority of replicates, as demonstrated by standard error bars on each graph. The one exception was heterogeneity of replicate data at the highest concentration used (0.5 mg ml^{-1}) for CN IRM 5815 in logarithmic phase. This heterogeneity is in turn the possible consequence of 0.5 mg ml^{-1} biotin hydrazide being close to its solubility extreme.

Non-linear regression analysis yielded the results presented in Table 4.3.1. Of note is the relatively weak Fmax value of CN IRM 5815 in logarithmic growth phase. Also of interest are the Hill coefficient values (Hn) which fall into two groups based upon culture age.

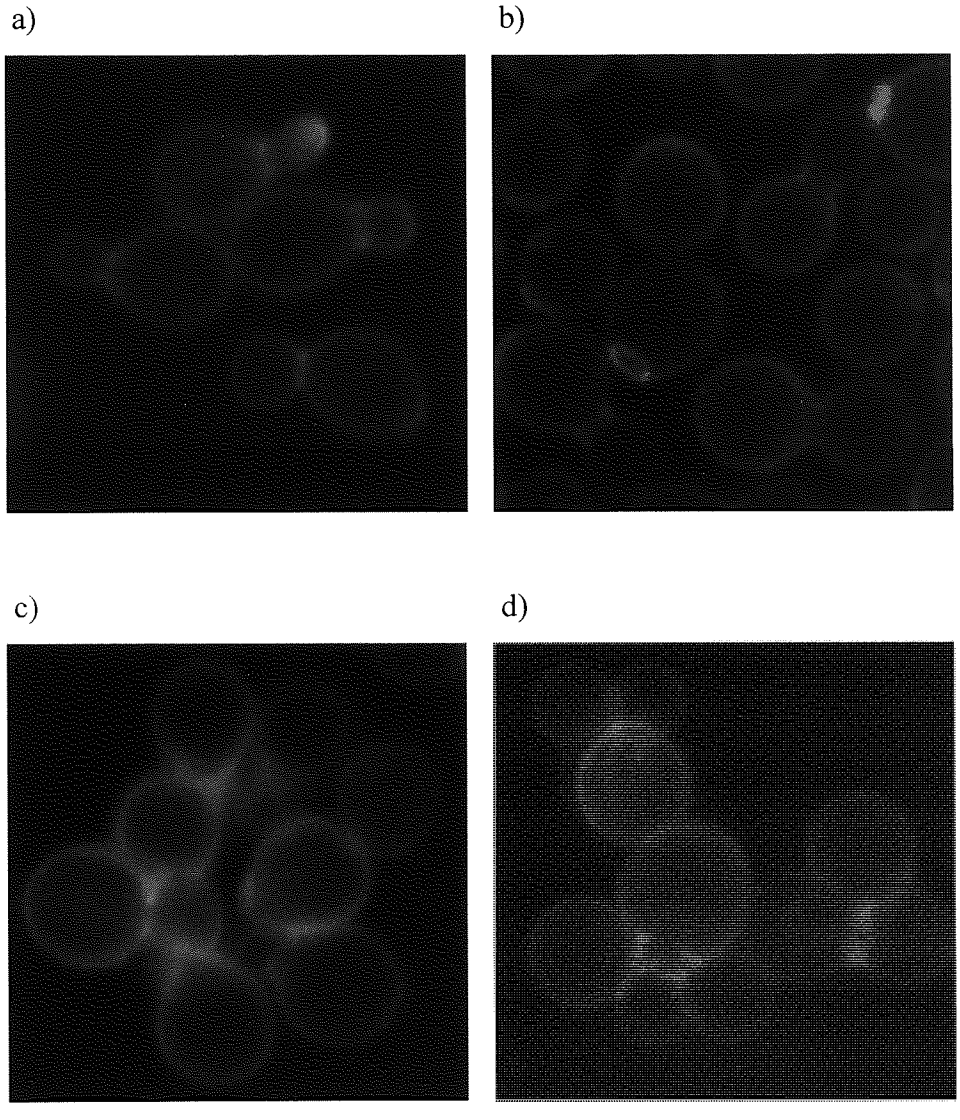
Table 4.3.1. Non-linear regression analysis of biotin hydrazide / fluorescein-streptavidin binding to carbohydrates on the cell surface of *C. neoformans* (± standard error).

	CN IRM 5815		ATCC 52817	
	Logarithmic	Stationary	Logarithmic	Stationary
Fmax	52.6 (± 3.5)	82.1 (± 2.6)	75.8 (± 1.3)	81.5 (± 2.5)
EC50 (mg ml^{-1})	0.016 (± 0.004)	0.013 (± 0.002)	0.012 (± 0.001)	0.024 (± 0.003)
Hn	1.21 (± 0.42)	0.52 (± 0.04)	1.21 (± 0.11)	0.67 (± 0.06)

4.3.2 Confocal microscopy

Figures 4.3.2a-d are representative images obtained by confocal microscopy. Although the images presented are merely reflective of the level of fluorescence a

population exhibits, all images are representative of the cell surface nature of biotin / fluorescein-streptavidin binding within the population. First and foremost the images selected show widespread though weak overall fluorescence coverage of cells, thus indicating limited albeit general carbohydrate coverage. The images also depict stronger fluorescence at mother and daughter cell interfaces; also apparent is that, regardless of culture age, images of CN IRM 5815 manifest little capsule fluorescence indicative of limited biotin binding to elements of the capsular matrix.



Figures 4.3.2a-d. Confocal images of biotin / fluorescein-streptavidin mediated *C. neoformans* yeast cell fluorescence.
a) CN IRM 5815 (logarithmic). b) Strain CN IRM 5815 (stationary).
c) ATCC 52817 (logarithmic). d) ATCC 52817 (stationary).

4.4 Discussion

Although biotinylation using biotin hydrazide and related molecules is an established technique (Skutelsky & Bayer, 1979; O'Shannessy & Quarles, 1987; Bayer *et al.*, 1988; Kähne & Ansorge, 1994; Hughes *et al.*, 1999), the coupling with fluorescein-streptavidin leading to both a quantified and qualified determination of carbohydrate on a microbial surface, appears unique. It is accepted that to reinforce the reliability of this analysis and to identify further the structures detected, experimentation into the nature of individual structures would be of particular use. Regardless, the general view of coverage and density this technique allows is particularly informative and is likely to be of value with respect to cell surface evaluation of cells from other organisms, including multicellular types. With respect to *C. neoformans* this procedure is useful as it gives an insight into the effect of capsular interference upon perceived coverage of carbohydrate and potentially glycoproteins. However, regardless of both strain and cell maturity, extensive biotinylation occurred across the entire cell surface of *C. neoformans*, with a noticeable increase in density observed around actively budding sites. Biotinylation of the capsule of CN IRM 5815 did not occur, indicating a lack of exposed carbohydrate residues susceptible to oxidation. Despite a lack of marked capsule fluorescence, contrasting results between the greatly encapsulated strain CN IRM 5815, and the acapsulate strain ATCC 52817 indicate that capsule presence may have had a variable effect upon biotinylation of the remainder of the cell. However the mechanism responsible appears too complex to speculate upon using the evidence presented here.

4.4.1 Flow cytometry analysis of biotin hydrazide binding

Flow cytometry work provided both a quantitative measure of carbohydrate exposure by a cell population, and also some insight into exposure of carbohydrate by population members. Post flow cytometry analysis gives an insight into relative numbers of susceptible carbohydrates as well as the avidity and complexity of biotin hydrazide-carbohydrate binding.

The population profiles of both strains, regardless of culture age, were leptokurtic in appearance at the critical biotin hydrazide concentration of 0.1 mg ml^{-1}. Such an occurrence suggests that individual population members present similar amounts of susceptible carbohydrates. Comparison between the strains indicates that ATCC 52817 is leptokurtic, but also differs somewhat by exhibiting an asymmetric distribution, indicating that cells exhibit greater variation below the modal fluorescence than above. This observation may be explained by the fact that ATCC 52817 is prone to aggregation (section 2.3.2.1.) as cells contained within aggregated groups would likely be less exposed to oxidation as well as both the biotin hydrazide and fluorescein-streptavidin used in the procedure. Should such reduced exposure occur, the result would be a weaker binding response in these cells and the observed increase in heterogeneity below the modal fluorescence.

Although saturation binding analysis, performed on mean fluorescence values achieved from flow cytometry of fungal material, has been used before (Smith *et al.*, 1999; Smith *et al.*, 2001), it appears unique with respect to biotin hydrazide / fluorescein-streptavidin carbohydrate detection. Maximum predicted binding, represented as Fmax values, was greater for stationary than for logarithmic phase cells, indicating that actively growing cells appear to express fewer biotin hydrazide susceptible carbohydrates than mature ones. Strain CN IRM 5815 logarithmic phase cells gave a markedly weaker Fmax than all others, indicating that actively growing, greatly encapsulated cells have notably fewer available biotin hydrazide binding sites not only than acapsulate cells, but also heavily encapsulated mature stationary phase cells.

Cell surface exposure of glycosylated structures, in particular glycoprotein, is of interest as it has been implicated as a contributing factor to several virulence mechanisms, including adherence of pathogenic fungi to host cells and surfaces (Bircher & Hohl, 1997; Kanbe & Cutler, 1998), nutrient uptake (Braun, 1999) and hydrophobicity (Masuoka & Hazen, 1999). In this work, it seems that *C. neoformans* cells, like other fungi, may produce marked amounts of glycosylated structures on cell surfaces. However, *C. neoformans* is unlike the *C. albicans* and *Coniothyrium minitans* studied by Smith *et al.* (1999; 2001), as it has a capsule. The moderating effect of the capsule is, according to the analysis performed here, probably due to shielding of binding sites by capsule production-related activities, and capsule components themselves, or indeed may simply be due to the capsule matrix reducing

the exposure time of each binding step. Also of interest are the differences and similarities observed between strains and cell maturity. Fmax data indicates that strains show greater fluorescence in stationary phase than logarithmic. Hn values markedly greater or less than one indicate that neither stationary nor logarithmic growth phase cells had identical, independently achieved, binding sites. In fact, logarithmic cells of both strains indicated positive cooperativity, a state in which binding of one molecule of biotin hydrazide encourages further binding. Stationary cells on the other hand showed negative cooperativity, indicating that the moieties binding ligands in this phase have either altered markedly with age, or alternatively, have replaced cooperative structures present on the surface of logarithmic cells.

4.4.2 Confocal microscopy

Use of confocal microscopy is valuable as it allows qualitative evaluation of the cell surface distribution of receptor ligand binding. Confocal microscopy is especially valuable with regards to *C. neoformans* as it allows an assessment of the role the capsule plays in biotin hydrazide binding.

As well as confirming flow cytometry findings through direct observation of binding, confocal work revealed four important features of biotin hydrazide binding to *C. neoformans* cell surfaces. The first observation of some consequence was the lack of interaction of biotin hydrazide with the cryptococcal capsule. Considering the known carbohydrate content of the capsule (Casadevall & Perfect, 1998), the absence of biotinylation both on and in this structure, is encouraging as the primary components of the capsule, making up over 90 % of its total mass, are GXM and GalXM, neither of which are glycoproteins. Notably however, one other minor component of the capsule, mannoprotein, is a glycoprotein, and yet was not detected. The reason for the lack of detection of mannoprotein is not clear; it may be due to shielding by the other capsule components, or more likely, that it was not present at levels allowing ready detection. The finding that the capsule did not fluoresce leads to the further realisation that biotin hydrazide and fluorescein-streptavidin freely penetrated the capsule matrix to reach the cell surface. The third and fourth matters of consequence were that binding had occurred evenly across observed cell surfaces

except at potential interfaces between mother and daughter cells; here a noticeable area of marked fluorescence was present, highlighting a collar or scar of carbohydrate ligands. This final observation leads to the assumption that localities of cell surface dynamism and flux, such as mother daughter interfaces, expose carbohydrate moieties attractive to biotin hydrazide in greater quantities than surrounding surfaces. This could explain the greater fluorescence observed in stationary cells in comparison to logarithmic growth phase cells. Cells in a stationary phase population have a greater number of bud scars than those drawn from a logarithmic phase population; should such scars or surface perturbations remain exposed, increased fluorescence could be expected. The discovery of spatially localised carbohydrate exposure is not unique amongst fungi, in fact work by Buck & Andrews (1999) examining glycoprotein exposure on the cell surface of the encapsulated yeast *Rhodosporidium toruloides* found that the fluorescent lectin Con A, which binds to mannose based glycoproteins, bound to localised polar regions of the yeast cells. Further analysis by Buck & Andrews (1999) found that Con A eliminated attachment of *R. toruloides* yeast to both barley leaf segments and polystyrene, indicating that the glycoprotein structures Con A bound were critical to adhesion.

4.4.3 Conclusion

In conclusion, biotin hydrazide susceptible carbohydrate moieties are present on the surface of *C. neoformans* cells and, due to the procedure used, are potentially components of glycoproteins. Confocal microscopy revealed that bound moieties are more densely congregated around the mother daughter cell interface. Binding sites were not found amongst capsular material, indicating that the protocol used is, as expected, specific to a narrow group of carbohydrate based structures. Susceptible carbohydrate moieties were found in greater amounts on cells from mature cultures. Variation in binding cooperativity suggests a significant alteration of exposed carbohydrates with age, either through a change in orientation or possibly even replacement with alternative components. The two strains examined were found to have significantly different binding profiles, probably as a result of contrasting capsule status. In order to assess fully the significance of the carbohydrates examined,

alternative analysis would be necessary to identify the specific carbohydrates involved.

CHAPTER 5 – Lectin based carbohydrate analysis

5.1 Introduction

Biotin hydrazide based biotinylation studies have clearly shown the presence of carbohydrates on the cell surface of *C. neoformans* which are potentially component parts of glycoproteins. Further examination of these carbohydrates and the structures associated with them, may define their function. A range of lectins were therefore used to quantitatively and qualitatively identify specific carbohydrate residues on *C. neoformans* cell surfaces.

Lectins are carbohydrate-binding proteins that are neither enzymes nor antibodies; they are ubiquitous and are thought to be found in all life forms. Lectins have specific affinities for a limited number of carbohydrates and are generally found on cell surfaces (Doyle, 1994). They have been used to characterise cell surface glycoconjugates of various microorganisms, including amoeba (Ribeiro *et al.*, 1997), bacteria (Doyle, 1994) and various fungal forms such as conidia and spores from *C. minitans* (Smith *et al.*, 1999), and yeast and hyphae from *C. albicans* (Smith *et al.*, 2001).

Similarly to Chapter 4, saturation binding analysis can be used when examining the binding of lectins to cell surface carbohydrates; these analyses give information on the nature of the ligand-receptor relationship, as well as an indication as to the quantity of target structures involved. In addition, a complete study should examine the magnitude of non-specific binding via the use of ligand blocking. Also of potential use with respect to glycoprotein analysis is tunicamycin, an antimicrobial agent which blocks the dolichol pathway leading to the production of N-glycosylated proteins in eukaryotes including yeasts (Chaffin, 1985). Tunicamycin can be used to block glycoprotein production, and therefore expression on the cell surface of yeasts, and as a result can give information on the nature of the lectin target receptor.

This study employs a panel of fluorescent lectins, with varying specific binding attributes, to further assess the carbohydrates discovered through biotin hydrazide biotinylation. *C. neoformans* fluorescence after lectin exposure was analysed both quantitatively and qualitatively using flow cytometry and confocal

microscopy respectively. Where appropriate, further experimentation of lectin tagged carbohydrate expression took the form of saturation binding analysis, inhibitory hapten blocking – through the use of high concentrations of free ligand-analogous molecules (Vector Laboratories inc., 2001) – and tunicamycin based blocking of protein glycosylation.

5.2 Materials and methods

Details of principle equipment, chemicals and materials, are included within the text; information pertaining to more common items can be found in Appendix G.

5.2.1 Binding a panel of FITC-lectins to *C. neoformans*

Lectin binding using a panel of eight fluorescent lectins, each varying markedly in specificity, was performed on four strains of *C. neoformans*; CN IRM 5815, CN IRM 5854, CN IRM 45922 and ATCC 52817. The concentration of each lectin was set at 30 μg ml^{-1}, a concentration considerably greater than the working range given in literature supplied by the vendor (Vector Laboratories, Burlingame, USA). As actively growing cells may show a greater variation in cell surface components than stationary cells, strains were analysed in logarithmic growth phase. The eight lectins used are described in detail in Table 5.2.1.

Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm) in triplicate. Logarithmic phase cells were removed, washed three times and suspended in 10 mM supplemented HEPES / 0.15 M NaCl buffer (Appendix C) at a concentration of 1.5 x 10^7 cell ml^{-1}. Aliquots of 200 μl were removed and placed in Eppendorfs before addition of either 200 μl of lectin at 60 μg ml^{-1} or 200 μl 10 mM supplemented HEPES / 0.15 M NaCl buffer as a negative control. Samples were incubated for 30 minutes in the dark at 4°C before being harvested, washed three times with 10 mM HEPES / 0.15 M NaCl buffer and finally fixed in 10 mM HEPES / 0.15 M NaCl buffer with 1% w/v paraformaldehyde. Samples were stored in the dark at 4 °C before determination of

fluorescence by flow cytometry analysis using a method adapted from that used by Smith *et al.* (1999; 2001). Fluorescence of 10,000 yeast cells was determined with a Becton Dickinson FACS 440 using an argon laser (200 mW), with an excitation wavelength of 488 nm and emitted light detector at 530 nm (± 15 nm), adjusted to a fixed channel using standard Brite Beads (Beckman Coulter, High Wycombe, UK), prior to determining fluorescence. Yeast samples were vortexed before introduction to sheath fluid and subsequent determination of fluorescence.

Data acquired was analysed as follows: relative abundance of fluorescence for each lectin was determined from population mean fluorescence values, which were divided by the specific activity of each lectin (fluorescein / lectin ratio), to produce a relative value of lectin binding.

Table 5.2.1. Lectins used, along with carbohydrate binding details. Lectins may be specific for the same carbohydrate, but each has markedly different binding affinities (Vector Laboratories inc., 2001).

* Fluorescein : lectin ratio.

5.2.2 Flow cytometry analysis

Flow cytometry was carried out on *C. neoformans* yeast cells exposed to select FITC-lectins (Con A & WGA) at several concentrations in order to assess receptor site saturation. This experimentation was carried out in the same manner as above (5.2.1) with the following alterations:

- Both logarithmic and stationary phase cells were analysed.
- When used Con A concentrations varied in line with the following: 1, 2.5, 5, 7.5, 10, 20, 30, and 50 $\mu g\ ml^{-1}$.
- When used WGA concentrations varied in line with the following: 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 20, 30, and 50 $\mu g\ ml^{-1}$.

Flow cytometry of each replicate sample produced a population profile and a mean average fluorescence value. Data, in the form of mean fluorescence values, were analysed using two factor ANOVA. The following comparisons were analysed: (i) differences in yeast cell fluorescence between strain and growth phase for the test lectins and (ii) differences in yeast cell fluorescence between strain and lectin concentration. Where necessary to illustrate significance, the LSD was calculated as part of the ANOVA analysis.

5.2.3 Saturation binding analysis

Binding concentration data, in the form of mean fluorescence values for each concentration point, were analysed using non-linear regression in the following manner: autofluorescence values were subtracted from each data point and the resulting data fitted to a logistic function of the form

$$\text{Bound}(B) = \frac{\text{Fmax} \bullet [L]^{Hn}}{[L]^{Hn} + EC50}$$

where Fmax is the fluorescence value when all binding sites are saturated, L is the concentration of FITC-lectin and EC50 is the concentration of FITC-lectin that produces 50 % of the maximum saturation. Hn is the Hill coefficient, a measure of the cooperativity in the system (Rang *et al.*, 1999). Data fitting was by means of non-linear regression analysis performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, USA). The binding curve from each individual experiment was analysed to obtain estimates of Fmax, EC50 and Hn. Relative Fmax data was produced by normalising Fmax values for each lectin and growth phase in order that the weakest value achieved equalled one.

5.2.4 Hapten interference analysis

Hapten interference analysis was performed in order to determine the level of non-specific FITC-lectin binding. This experiment was identical to that described previously (5.2.1), except that both logarithmic and stationary phase cells were used, and that FITC-lectins were preincubated with appropriate haptens before introduction to cells; controls were pre-incubated with supplemented HEPES / 0.15 M NaCl buffer only. Hapten concentrations used are indicated in Table 5.2.4. FITC-lectins and haptens were incubated together at double strength concentration for 30 minutes at 4°C in the dark, before addition of an equal volume of cell sample (400 µl final volume). Sample fluorescence was assessed by flow cytometry analysis.

The influence of respective inhibitory haptens on FITC lectin binding to yeast cells of each strain was analysed using two factor ANOVA. At each growth phase and for each FITC-lectin a comparison was made between strain and hapten presence / absence. Where necessary to illustrate significance, the LSD was calculated as part of ANOVA analysis.

Table 5.2.4. FITC-lectins and the haptens used (Vector Laboratories inc., 2001). Concentrations stated are correct for the final cell / FITC-lectin / hapten mixtures.

5.2.5 Tunicamycin interference analysis

Glycoprotein production and expression was blocked with tunicamycin using the following procedure adapted from those of various researchers (Chaffin, 1985; Vai *et al.*, 1987; Morosoli *et al.*, 1988; Buck & Andrews, 1999). Con A and WGA binding was assessed on strains CN IRM 5815 and ATCC 52817.

The selected strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm) in triplicate until mid-logarithmic growth phase, at which point tunicamycin was introduced to test cultures to a concentration of 9 $\mu g\ ml^{-1}$, control cultures were left unaltered. Separate cultures, both with and without tunicamycin, were then subjected to the lectin binding experiment outlined in section 5.2.1 at 4 and 24 hours post tunicamycin inoculation. Sample fluorescence was assessed by flow cytometry analysis.

Data were analysed using the Student's t-test (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). Differences in FITC-lectin binding, measured as mean population fluorescence, were compared between treated and untreated cells. In order to compare the affect of tunicamycin treatment on both strains, two factor ANOVA was applied (Microsoft Excel 2000, Microsoft Corp., Seattle, USA).

5.2.6 Confocal microscopy

Confocal microscopy was performed on selected samples from the FITC-lectin binding experiments described in section 5.2.2. Cells from strains CN IRM 5815, CN IRM 5854, CN IRM 45922 and ATCC 52817 were examined in both logarithmic and stationary growth phase and after incubation with FITC-lectins WGA and Con A.

Aliquots of 7.5 µl logarithmic and stationary phase cells, exposed to a final concentration of 30 µg ml^{-1} FITC-lectin, and fixed in HEPES / 0.15 M NaCl with 1% w/v paraformaldehyde, were in turn dispensed into wells of separate multispot microscope slides (Hendley-Essex, London, UK). Cell suspensions were then air dried and fixed by the addition of 7.5 µl cold acetone, which was subsequently allowed to evaporate under warm air. Wells were then sealed with 5 µl "Vectorshield" (Vector Laboratories, Burlingame, USA), a coverslip firmly affixed with "Tippex" correction fluid and stored at 5 °C in the dark until fluorescence characterisation by confocal microscopy. Images were acquired with a Zeiss Axiovert / Biorad MRC 1024 OS laser scanning confocal microscope facility, utilising Laser Sharp 2000 software (Bio-Rad Laboratories, Hemel Hempstead, UK). An argon laser (100 mW), excitation wavelength 488 nm moderated by a series of neutral density filters, gain, offset and zoom functions was utilised to induce and optimise image fluorescence intensity, contrast and composition. Slides were viewed with an oil immersion Zeiss Neuflor 1.3 NA objective (Carl Zeiss, Welwyn Garden City, UK) and subjective material captured as 512 x 512 pixel images in turn converted from Bio-Rad PICT to TIFF format. In order to obtain satisfactory prints, contrast and brightness of images were altered using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, USA), no raw data was removed and no synthetic data was added in this process.

5.3 Results

5.3.1 Binding a panel of FITC-lectins to *C. neoformans*

Figure 5.3.1 compares the mean relative fluorescence, detected by flow cytometry, of four test *C. neoformans* strains exposed to each FITC-lectin panel member. Con A gave the greatest mean fluorescence, followed by WGA and then GNL. These results suggested that more intensive work should focus upon binding of the two FITC-lectins giving the greatest and most consistent fluorescence regardless of strain; therefore FITC-lectins Con A and WGA only, are investigated further in the following sections of work.

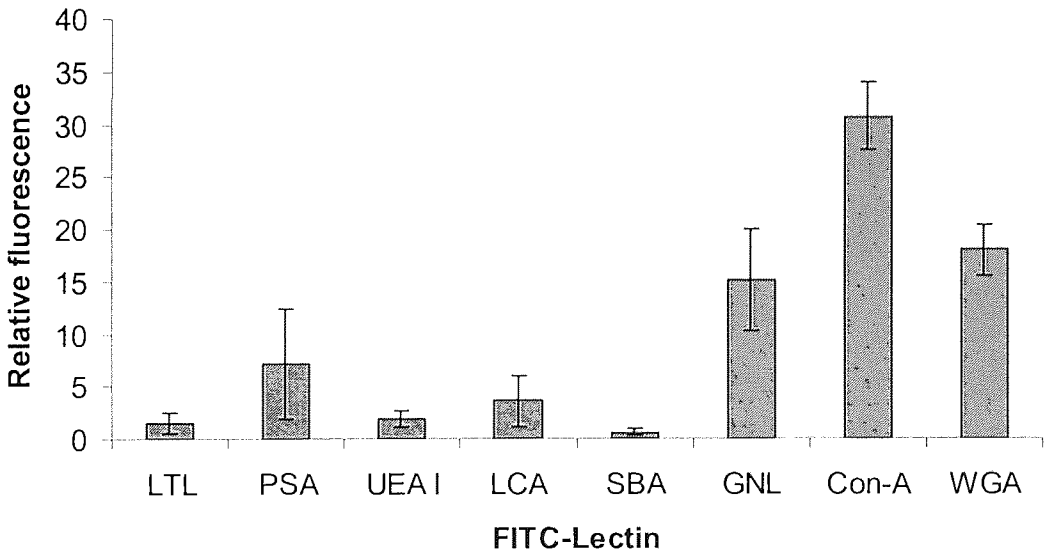


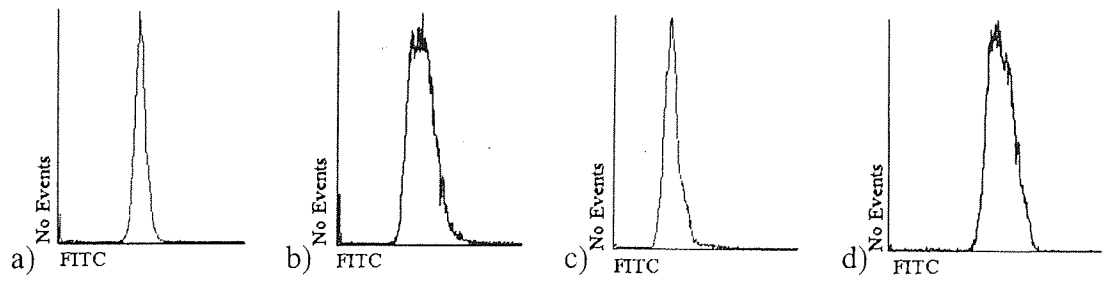
Figure 5.3.1. Binding, indicated by relative fluorescence, of eight FITC-lectins to four *C. neoformans* strains. Results are means of 12 experimental readings, 3 each from four strains of *C. neoformans*, and are ± standard error.

The lectin profile given in Figure 5.3.1 indicates that in general *C. neoformans* strains express very little, if any, SBA-specific N-acetyl-galactosamine or galactose residues. In addition, UEA I and LTL based data indicate relatively little exposure of

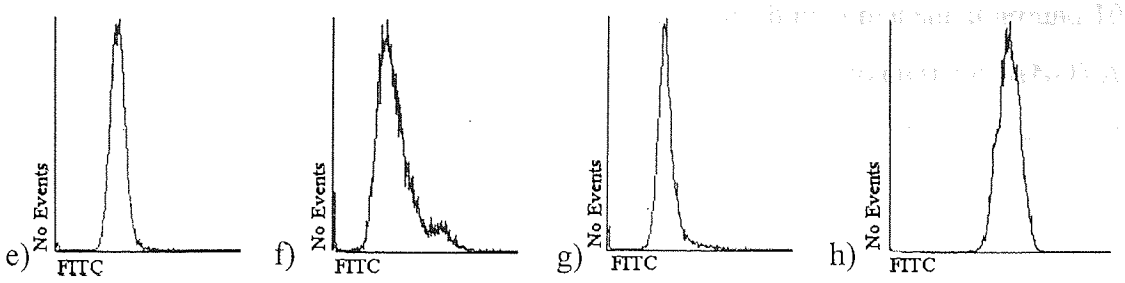
α-fucose. Mannose and / or glucose are detected in marked amounts by Con A and GNL, however, LCA and PSA specific mannose and glucose are not as abundant. WGA based data indicates that considerable amounts of N-acetyl-glucosamine are also present on cell surfaces.

5.3.2 Flow cytometry analysis

Flow cytometry analysis generates the mean fluorescence of a cell population as well as cell fluorescence population profiles. Mean fluorescence data is used in all further analysis; however before it can be used, population profiles must be evaluated in order to assess the homogeneity of each sample population. Figures 5.3.2a-l are examples of the population profiles achieved through flow cytometry work. Con A related profiles (Figures 5.3.2a-h) are markedly leptokurtic, those gained from strains with a capsule visible under India ink (Figures a, c, e & g) show a greater degree of homogeneity, and thus sharper peaks than strains appearing capsule free (Figures b, d, f & h). When compared on the basis of growth phase, Con A profiles in general do not differ markedly in shape, an exception is the shoulder of cells exhibiting fluorescence greater than the mean in stationary phase profiles of strain CN IRM 5854 (visible in Figure 5.3.2f).

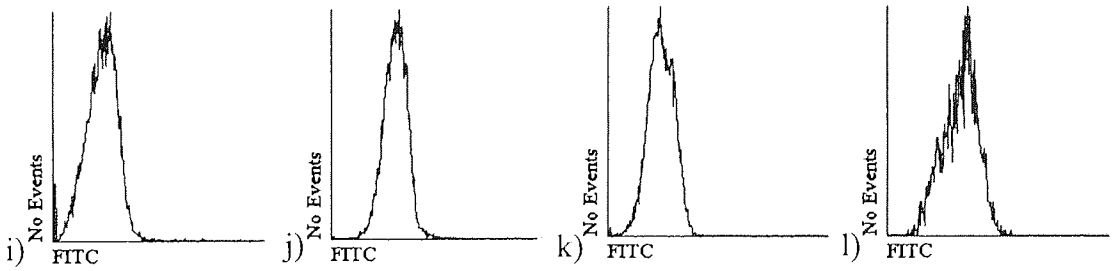


Figures 5.3.2a-d. Flow cytometry population fluorescence profiles of *C. neoformans* strains (a) CN IRM 5815, (b) CN IRM 5854, (c) CN IRM 45922 and (d) ATCC 52817 subjected to Con A binding at a concentration of 30 μg ml^{-1} in logarithmic growth phase.



Figures 5.3.2e-h. Flow cytometry population fluorescence profiles of *C. neoformans* strains (e) CN IRM 5815, (f) CN IRM 5854, (g) CN IRM 45922 and (h) ATCC 52817 subjected to Con A binding at a concentration of 30 μg ml^{-1} in stationary growth phase.

The profiles in Figures 5.3.2i-l are representative population profiles from cells incubated with WGA. Stationary phase profiles are not included here as their profile shape did not differ markedly from logarithmic phase profiles. WGA binding profiles describe a less homogenous population than related Con A based profiles. The one exception is strain CN IRM 5854, which shows little difference in profile between lectins. Unlike Con A, WGA profiles do not indicate a marked difference in binding based upon capsule status.



Figures 5.3.2i-l. Flow cytometry population fluorescence profiles of *C. neoformans* strains (i) CN IRM 5815, (j) CN IRM 5854, (k) CN IRM 45922 and (l) ATCC 52817 subjected to WGA binding at a concentration of 30 μg ml^{-1} in logarithmic growth phase.

Figures 5.3.2m&n describe the variable binding of FITC-lectin Con A, to *C. neoformans* strains CN IRM 5815, CN IRM 5854, CN IRM 45922 and ATCC 52817, using various different concentrations of lectin, and under differing growth phases. Regardless of strain or growth phase, cells showed a significant increase in

fluorescence with relatively small increases in Con A, reaching a plateau at around 10 μg ml^{-1} (ANOVA, $P < 0.001$). Despite this shared general characteristic, ANOVA analysis indicates that there was a significant difference in fluorescence emitted from strains at the various concentration points ($P < 0.001$), this was manifest by cell populations in either growth phase. ANOVA analysis of the affect of cell culture maturity, performed on data received from 30 μg ml^{-1} treatments, indicated a significant difference in emitted fluorescence between growth phases, regardless of strain ($P < 0.001$). Acapsulate strain ATCC 52817 appears to manifest greater lectin binding than all other strains regardless of culture maturity. Fluorescence data from strains CN IRM 5815 and CN IRM 5854 indicates that these strains bind more Con A in logarithmic growth phase than stationary phase, a marked disparity not present in the binding profiles of strains CN IRM 45922 and ATCC 52817.

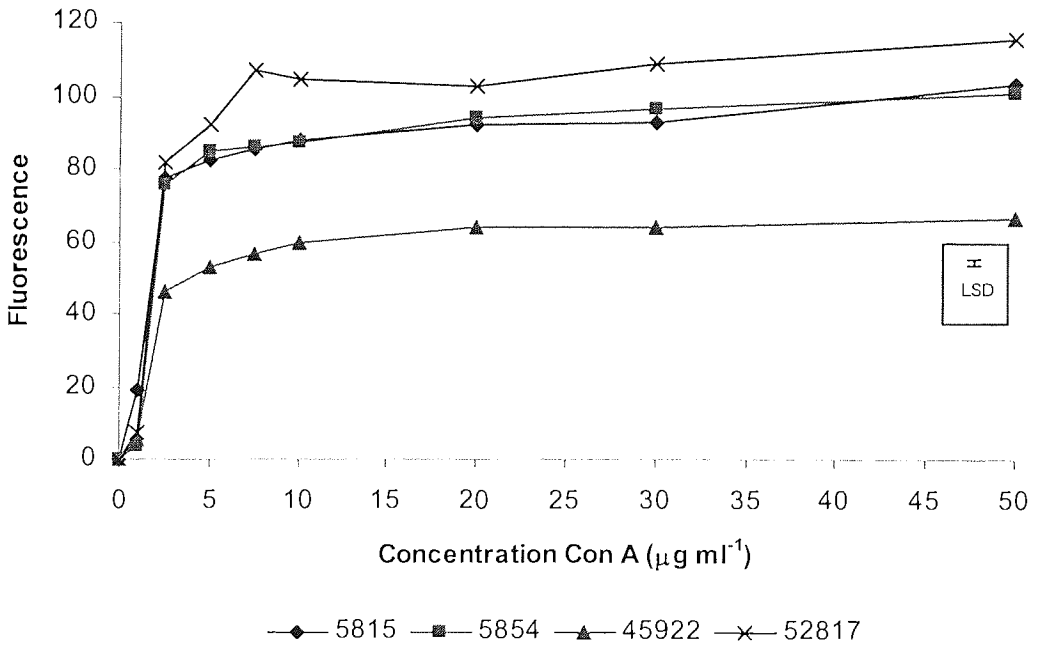


Figure 5.3.2m. Con A binding to *C. neoformans* strains in logarithmic growth phase. Results are means of three experimental readings; LSD is calculated from ANOVA analysis.

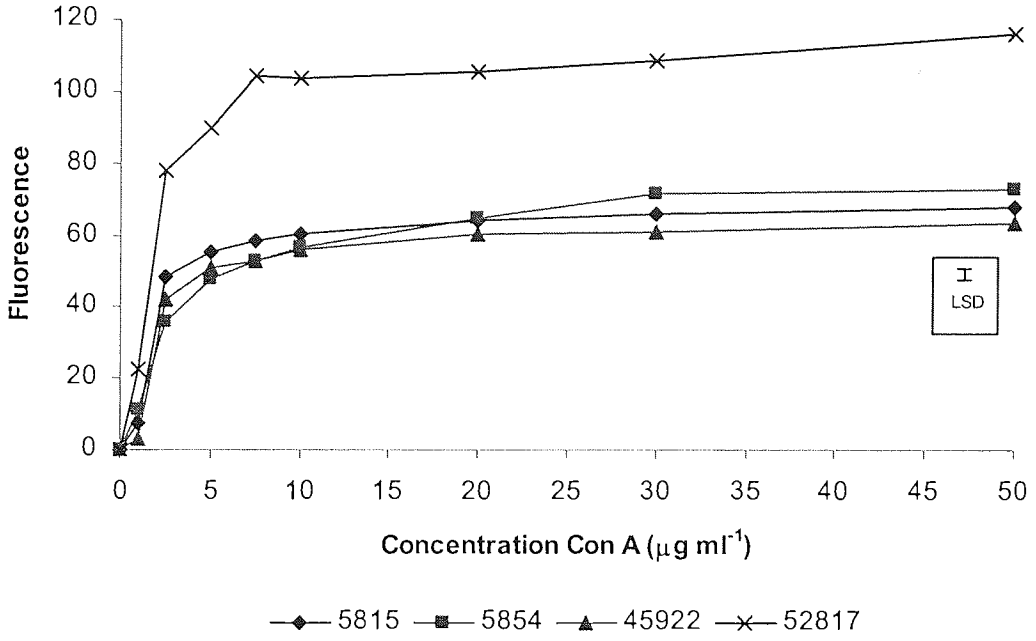


Figure 5.3.2n. Con A binding to *C. neoformans* strains in stationary growth phase. Results are means of three experimental readings; LSD is calculated from ANOVA analysis.

Figures 5.3.2o&p demonstrate the variable binding of FITC-lectin WGA to *C. neoformans* strains CN IRM 5815, CN IRM 5854, CN IRM 45922, and ATCC 52817, using steadily increasing concentrations of lectin and under differing growth phases. As with Con A based work, cell populations of both strains and growth phases manifest significant increases in fluorescence with relatively small increases in concentration (ANOVA, $P < 0.001$). Unlike Con A however, a plateau of maximum fluorescence occurred at around 2.5 μg ml^{-1} WGA. Again similar to Con A based fluorescence, WGA fluorescence varied significantly between strains (ANOVA, $P < 0.001$), this was a finding maintained regardless of growth phase. Comparison by culture maturity, using 30 μg ml^{-1} concentration point data, described a significant difference between growth phases (ANOVA, $P < 0.001$). Regardless of cell age, and in line with Con A, WGA appears to bind in greatest amounts to acapsulate strain ATCC 52817. However, unlike Con A, WGA appears to bind least to CN IRM 5815, again regardless of culture maturity.

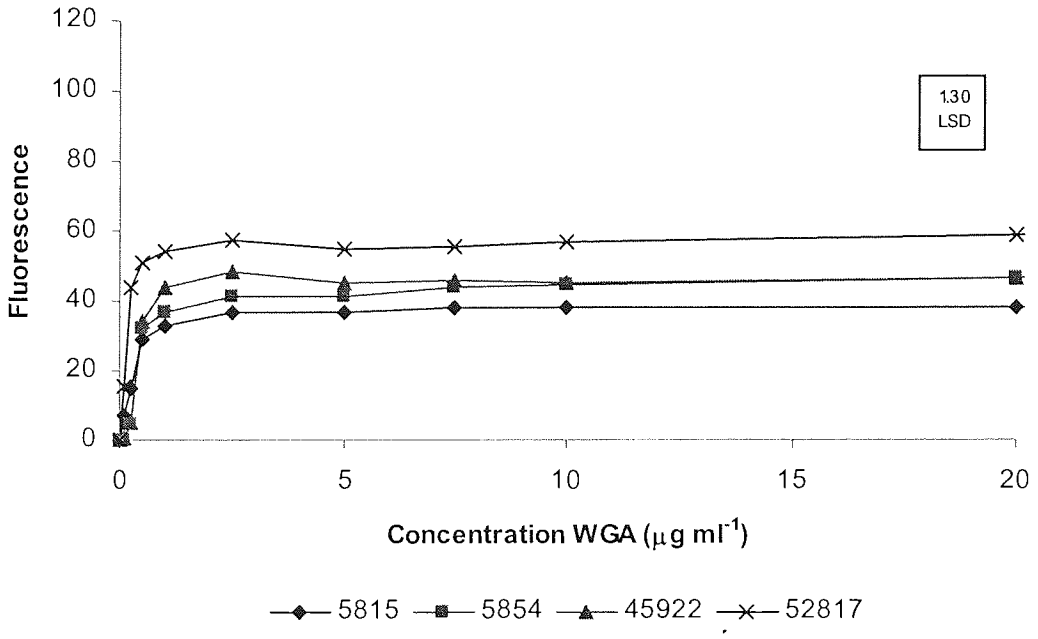


Figure 5.3.2o. WGA binding to *C. neoformans* strains in logarithmic growth phase. Results are means of three experimental readings; LSD is calculated from ANOVA analysis.

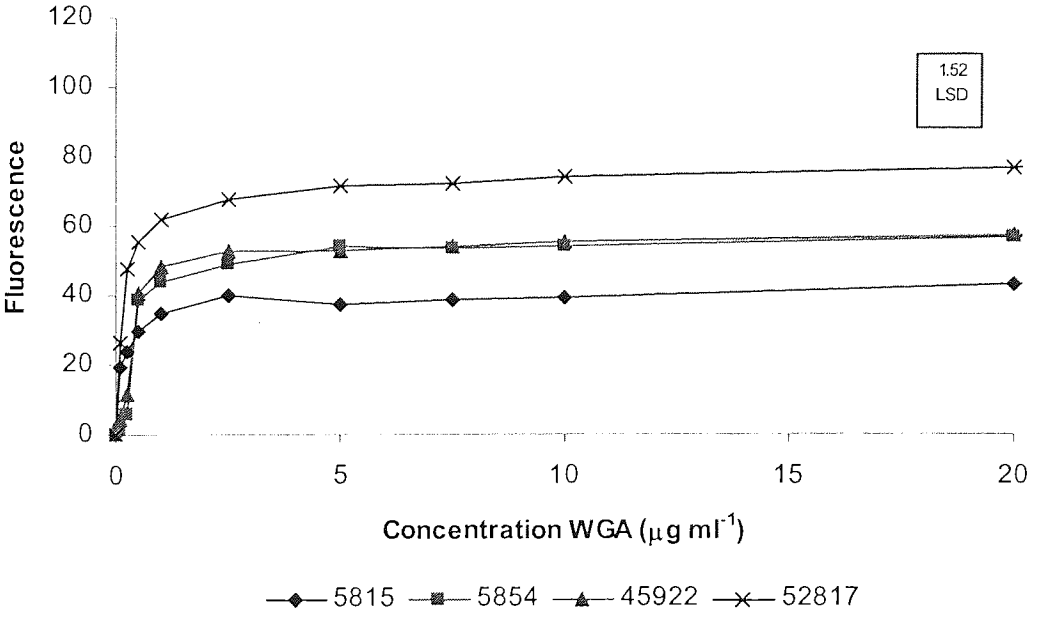


Figure 5.3.2p. WGA binding to *C. neoformans* strains in stationary growth phase. Results are means of three experimental readings; LSD is calculated from ANOVA analysis.

5.3.3 Saturation binding analysis

Analysis of flow cytometry data has furnished the material outlined in Tables 5.3.3a-c. Table 5.3.3a characterises Con A binding to *C. neoformans* strains. EC50 values are at or below 3.56 μg ml^{-1} for all strains, indicating marked avidity of *C. neoformans* yeast cells for Con A. Also with respect to EC50 data, acapsulate strain ATCC 52817 is the only strain manifesting a greater avidity for Con A in stationary phase than in logarithmic growth phase. Hn values, with the exception of CN IRM 5854 in stationary phase, are markedly greater than one, indicating a situation in which the binding of one receptor to a ligand encourages the binding of others. Fmax values produced are greater for logarithmic phase cells than for stationary, however, standard error values indicate that this is not marked for strains CN IRM 45922 and ATCC 52817.

Table 5.3.3a. Binding characteristics of Con A to strains of *C. neoformans* in logarithmic (Log) and stationary (Stat) growth phase (± standard error).

Strain : Growth Phase		EC50 (μg ml^{-1})	Hn	Fmax
CN IRM 5815 :	Log	1.74 (±0.30)	2.93 (±0.35)	104.3 (±3.5)
	Stat	2.16 (±0.38)	3.02 (±0.33)	72.8 (±2.7)
CN IRM 5854 :	Log	2.20 (±0.51)	4.79 (±0.81)	108.0 (±5.3)
	Stat	3.56 (±0.27)	1.15 (±0.10)	78.3 (±1.5)
CN IRM 45922 :	Log	2.34 (±0.42)	2.99 (±0.38)	72.1 (±2.9)
	Stat	2.55 (±0.48)	3.16 (±0.48)	68.9 (±2.9)
ATCC 52817 :	Log	2.19 (±0.47)	3.67 (±0.47)	123.3 (±5.7)
	Stat	1.99 (±0.27)	1.98 (±0.18)	121.6 (±3.4)

Table 5.3.3b gives the binding characteristics of WGA to *C. neoformans* strains. EC50 values are extremely low the greatest being 0.55 μg ml^{-1}, which, in comparison to Con A, indicates that *C. neoformans* strains have markedly greater avidity for WGA. In general cell maturity does not seem to affect EC50 values, with the exception of strain CN IRM 5815 which exhibits a markedly lower EC50 in stationary phase. Hn values sharply depart from one for all strains and growth phases, with the exception of ATCC 52817 in stationary phase. Hn values of mature CN IRM 5815 cells were markedly lower than one, unlike all other strains and growth phases. In contrast to Con A binding, all WGA based Fmax values are greater amongst stationary phase cells than those of logarithmic phase, suggesting greater exposure of carbohydrate residues in mature cells. It is important to note however that standard errors reported for CN IRM 5815 indicate only a slight difference between growth phases.

Table 5.3.3b. Binding characteristics of WGA to strains of *C. neoformans* in logarithmic (Log) and stationary (Stat) growth phase (± standard error).

Strain : Growth Phase		EC50 (μg ml^{-1})	Hn	Fmax
CN IRM 5815 :	Log	0.31 (±0.02)	1.55 (±<0.01)	40.3 (±0.5)
	Stat	0.15 (±0.01)	0.69 (±<0.01)	40.9 (±0.5)
CN IRM 5854 :	Log	0.54 (±0.10)	3.91 (±0.02)	48.2 (±1.4)
	Stat	0.55 (±0.08)	3.02 (±0.01)	59.3 (±1.6)
CN IRM 45922 :	Log	0.44 (±0.08)	4.36 (±0.01)	50.2 (±1.6)
	Stat	0.43 (±0.06)	2.70 (±0.02)	59.6 (±1.3)
ATCC 52817 :	Log	0.14 (±0.02)	1.84 (±0.02)	59.6 (±0.9)
	Stat	0.18 (±0.01)	0.81 (±0.01)	75.8 (±0.5)

In order to allow for comparison between lectins, Table 5.3.3c compares normalised Fmax data from WGA and Con A based work. Values are normalised relative to strain CN IRM 5815 in logarithmic phase subjected to WGA lectin. Strain ATCC 52817 emits greatest fluorescence, regardless of other factors, indicating that this acapsulate strain expresses the greatest concentration of carbohydrate residues. Strains have markedly greater relative Fmax values when treated with Con A than WGA.

Table 5.3.3c. Relative Fmax data of *C. neoformans* strains subjected to FITC-lectin binding both at logarithmic (Log) and stationary (Stat) phase. Data are relative to CN IRM 5815 in logarithmic phase using WGA.

	Con A		WGA	
	Log	Stat	Log	Stat
CN IRM 5815	2.59	1.81	1.00	1.02
CN IRM 5854	2.68	1.95	1.20	1.47
CN IRM 45922	1.79	1.71	1.25	1.48
ATCC 52817	3.06	3.02	1.48	1.88

5.3.4 Hapten interference analysis

Figure 5.3.4a compares Con A experimental fluorescence with that from samples incorporating inhibitory hapten. A marked reduction in fluorescence occurred as a result of hapten utilisation, a difference confirmed as significant upon ANOVA analysis ($P < 0.001$).

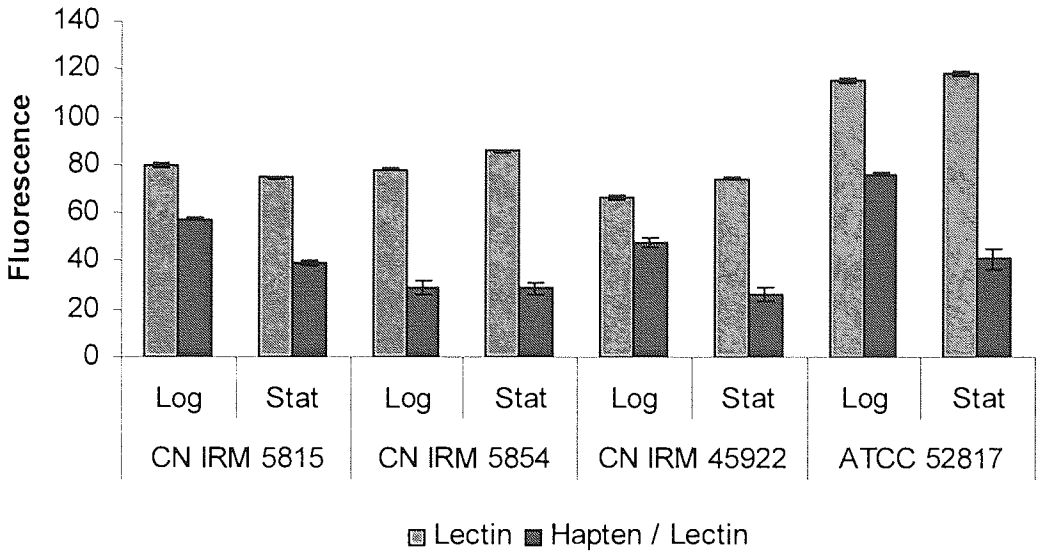


Figure 5.3.4a. The affect of hapten presence on FITC-lectin Con A binding to *C. neoformans* strains in logarithmic (Log) and stationary (Stat) growth phase. Results are means of three experimental readings ± standard error.

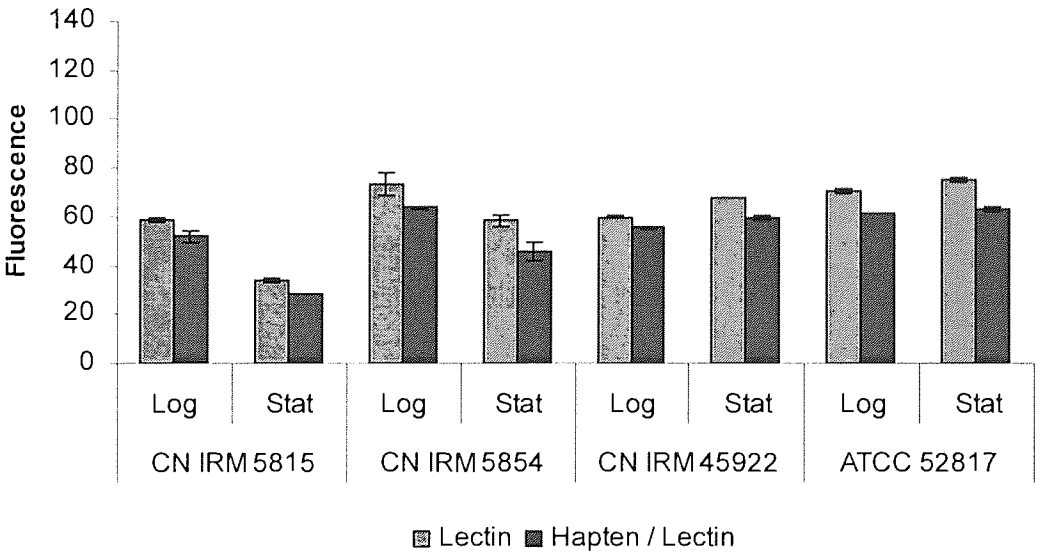


Figure 5.3.4b. The affect of hapten presence on FITC-lectin WGA binding to *C. neoformans* strains in logarithmic (Log) and stationary (Stat) growth phase. Results are means of three experimental readings; LSD is calculated from ANOVA analysis ± standard error.

Figure 5.3.4b (above) shows the affect of respective hapten utilisation upon FITC-lectin WGA binding to *C. neoformans* strains. Similarly to Con A, WGA

binding is significantly reduced by hapten presence (ANOVA, $P < 0.001$), however the reduction experienced is not as marked.

5.3.5 Tunicamycin interference analysis

Figure 5.3.5a compares the affect tunicamycin has upon expression of Con A susceptible carbohydrates on the cell surface of two strains of *C. neoformans*. Cells exposed to tunicamycin in mid-logarithmic phase were then analysed at 4 and 24 hours post exposure. At 4 hours post exposure there is no significant difference in fluorescence between treated and untreated CN IRM 5815 (t-test, $P = 0.34$), whereas tunicamycin significantly decreased the fluorescence associated with ATCC 52817 ($P < 0.05$). At 24 hours post treatment strain CN IRM 5815 again shows no significant difference (t-test, $P = 0.36$), strain ATCC 52817 however shows a significant increase in fluorescence detected from cells treated with tunicamycin (t-test, $P < 0.001$).

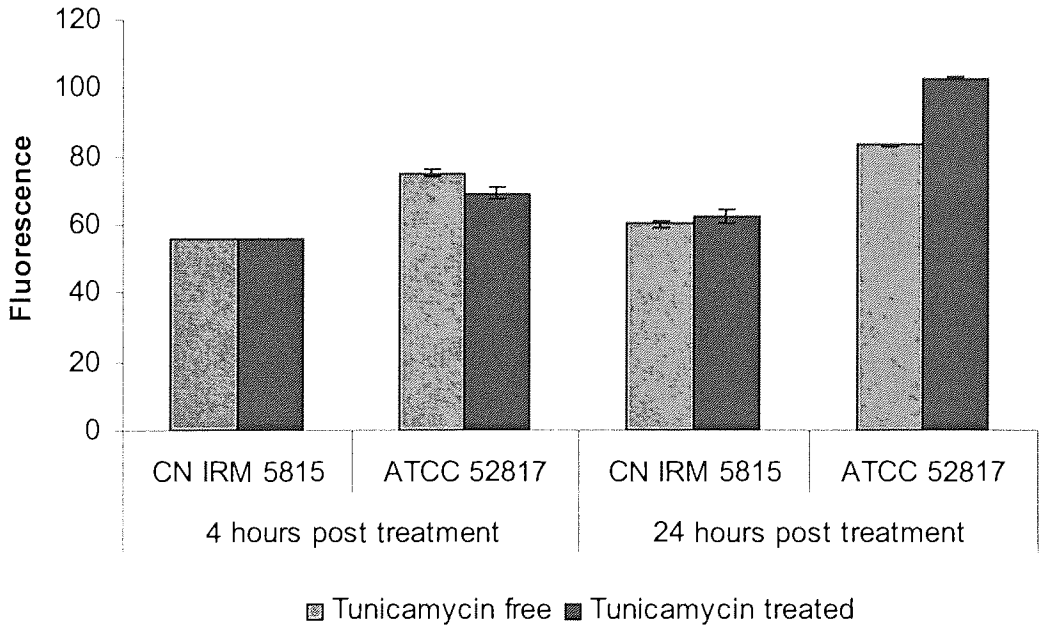


Figure 5.3.5a. The affect of tunicamycin on FITC-lectin Con A binding to *C. neoformans* strains CN IRM 5815 and ATCC 52817. Results are means of three experimental readings ± standard error.

Figure 5.3.5b outlines the affect of tunicamycin treatment on FITC-lectin WGA binding to *C. neoformans* strains CN IRM 5815 and ATCC 52817. After 4 hours treatment with tunicamycin cells exhibit significantly weaker fluorescence regardless of strain (ANOVA, $P < 0.001$). Contrastingly after 24 hours of exposure to tunicamycin, both strains exhibited significantly greater fluorescence (ANOVA, $P < 0.001$).

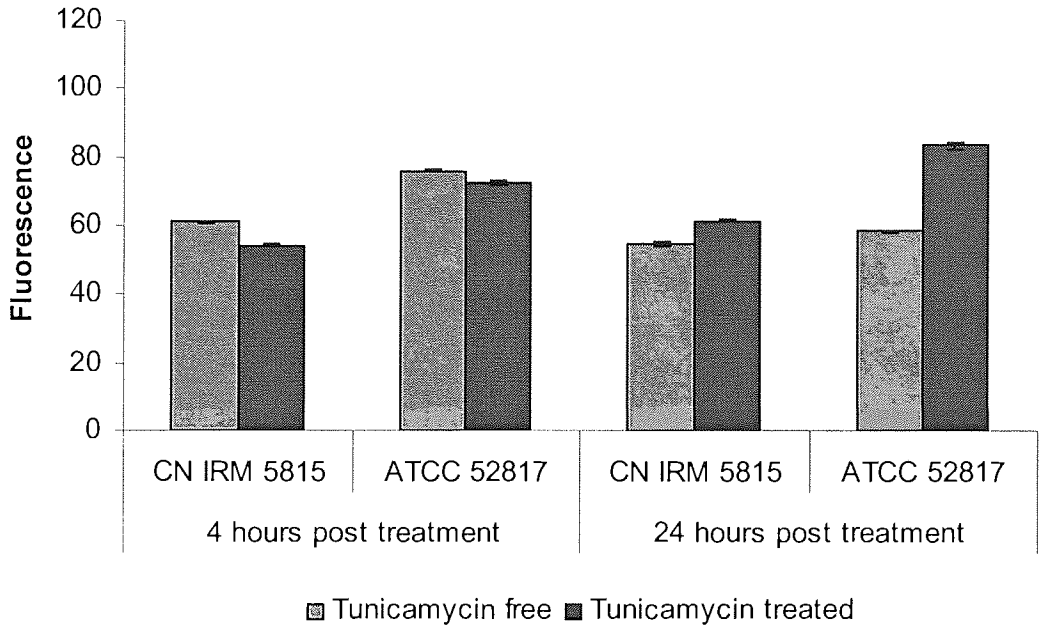


Figure 5.3.5b. The affect of tunicamycin on FITC-lectin WGA binding to *C. neoformans* strains CN IRM 5815 and ATCC 52817. Results are means of three experimental readings ± standard error.

5.3.6 Confocal microscopy

Figures 5.3.6a-f are representative confocal microscopy images of FITC-lectin Con A mediated cell fluorescence. Figures 5.3.6a-d illustrate the localised nature of Con A based binding amongst two wild type strains. Figure 5.3.6b, representing fluorescence alone, shows that although Con A effectively penetrated the capsule, some fluorescence can be seen associated with the capsular matrix. Figures 5.3.6e&f

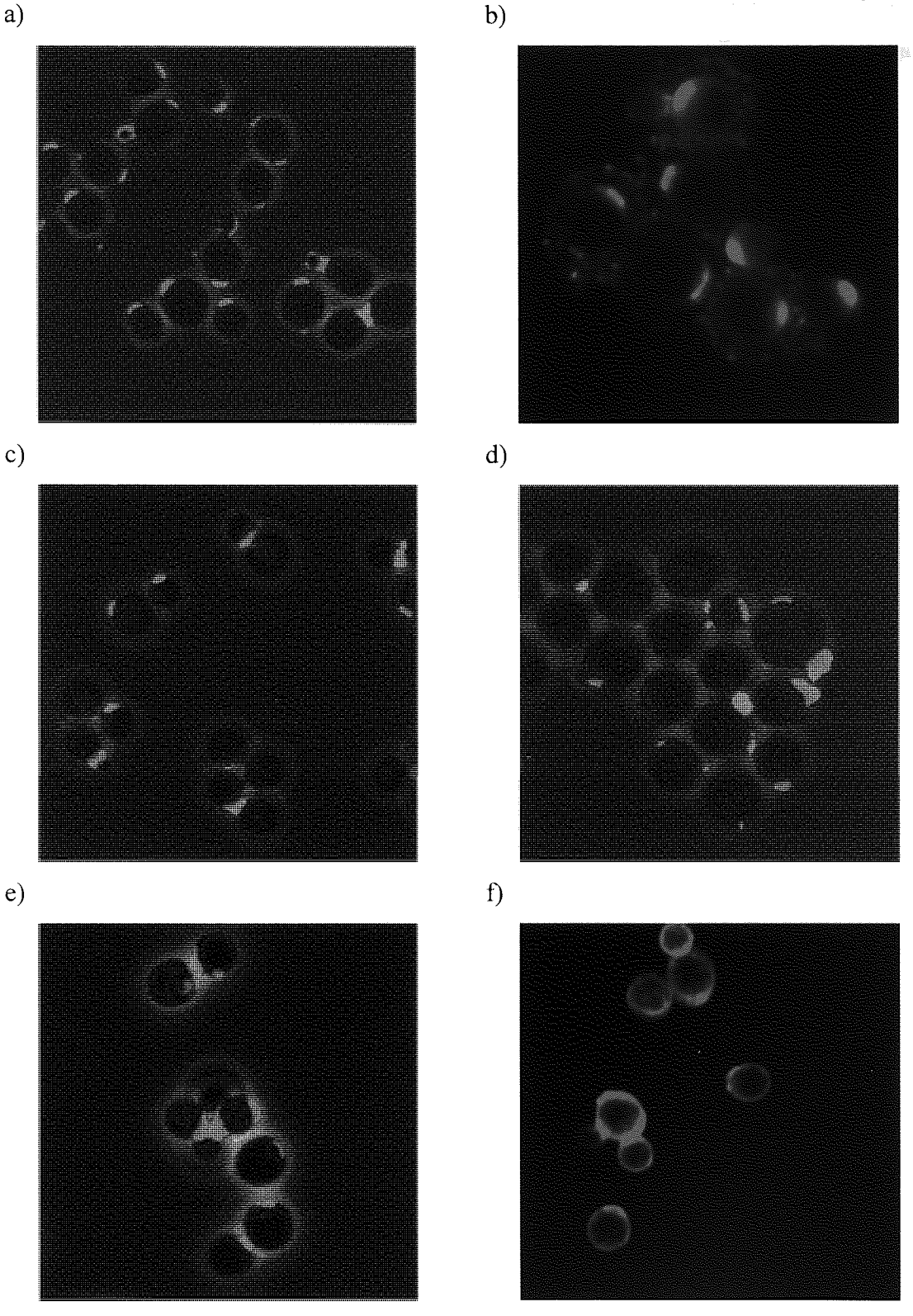


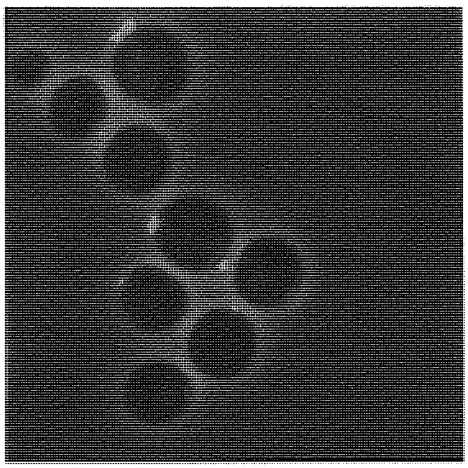
Figure 5.3.6a-f. Confocal images of FITC-lectin Con A mediated *C. neoformans* yeast cell fluorescence.

a) CN IRM 5815 (logarithmic).
b) CN IRM 5815 (stationary) fluorescence only.
c) CN IRM 5854 (logarithmic).
d) CN IRM 5854 (stationary).
e) ATCC 52817 (logarithmic).
f) ATCC 52817 (stationary) fluorescence only.

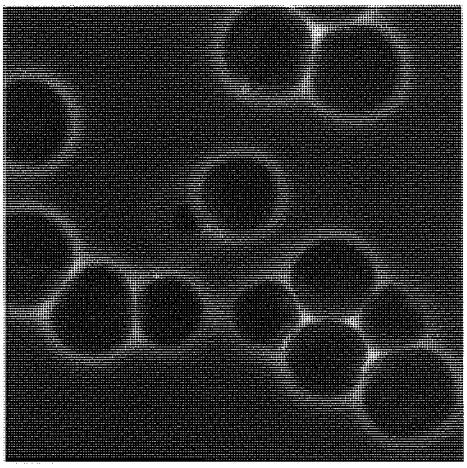
illustrate Con A mediated fluorescence with respect to acapsulate strain ATCC 52817; in particular Figure 5.3.6f, a fluorescence only image, shows fluorescence is not localised but emanates from most of the cell surface.

WGA mediated fluorescence is represented by Figures 5.3.6g-i. WGA, similarly to Con A, bound to cell surface areas associated with cell proliferation, however this binding is less apparent than that for Con A. Figure 5.3.6i indicates that, as with Con A mediated fluorescence, strain ATCC 52817 bound WGA over a greater area than other strains examined.

g)



h)



i)

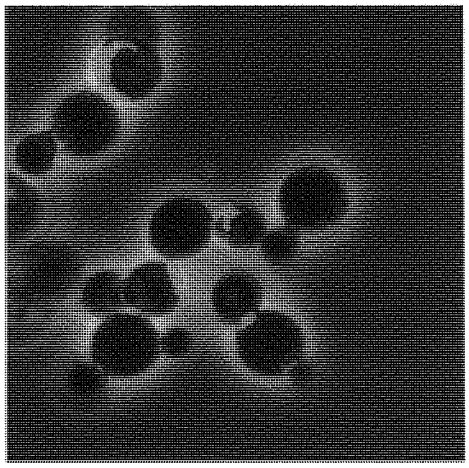


Figure 5.3.6g-i. Confocal images of FITC-lectin WGA mediated *C. neoformans* yeast cell fluorescence.
g) CN IRM 5815 (stationary). h) CN IRM 5854 (logarithmic).
i) ATCC 52817 (stationary).

5.4 Discussion

Fluorescent lectins have been used extensively to assess carbohydrate residue exposure on cell surfaces (Doyle, 1994). In particular, fluorescent lectins have been used to visualise and identify carbohydrate exposure on various fungal surfaces including, *C. minitans*, *C. albicans*, *R. toruloides*, *Phytophthora palmivora* and *Uromyces* species (Freytag & Mendgen, 1991; Bircher & Hohl, 1997; Buck & Andrews, 1999; Smith *et al.*, 1999; 2001). These studies found varying levels of avidity amongst the species employed, for the lectins involved, thus giving an insight into variation of carbohydrate exposure between differing species and forms of growth. In turn, this technique has proven equally useful in assessing carbohydrate residue exposure on the surface of varying strains of *C. neoformans*, giving insight not only into the composition of the cell wall surface, but also uniquely, the capsule matrix.

In a study aimed at identifying *C. neoformans* cell wall glucan content, James *et al.* (1990) concluded that the *C. neoformans* cell wall contains 86 % glucose complexed in various molecules. Casadevall and Perfect, as part of an extensive review (1998), also note that the *C. neoformans* cell wall has been found to contain immunogenic mannoprotein and probably the N-acetyl-glucosamine based component chitin. The capsule of *C. neoformans* has also been extensively studied and is known to contain three major components; GXM, GalXM and mannoprotein, with GXM making up roughly 90 % of the total capsule content (Casadevall & Perfect, 1998). Neither GXM nor GalXM will complex with lectin Con A, whereas mannose residues of mannoprotein may (Cherniak & Sundstrom, 1994).

This study reveals that FITC-lectins Con A and WGA bound readily to cryptococcal cell surfaces, thereby demonstrating the presence of mannose / glucose and N-acetyl-glucosamine respectively. The avidity of ligand for receptor(s) was marked for both lectins, especially WGA. Amongst the various other FITC-lectins, mannose / glucose binding lectins bound in varying, albeit low, levels. However, of particular interest is the data obtained from GNL binding assays, as GNL unlike Con A, PSA and LCA, shows little avidity for glucose residues (Vector Laboratories inc., 2001). Data obtained from GNL based studies, therefore indicates whether binding of Con A, PSA and LCA is localised to mannose or glucose. Relatively high binding of

GNL in comparison to PSA and LCA confirms that mannose is potentially responsible for a majority of Con A binding to *C. neoformans* strains, this despite the fact that a previous investigation of the contents of the *C. neoformans* cell wall found very little evidence to suggest mannose presence (James *et al.*, 1990). In addition, the data accumulated from GNL, LCA and PSA binding experiments signifies that several different forms or orientation of mannose / glucose residue are present on the *C. neoformans* cell surface. Lectins which bind fucose and galactose / N-acetyl-galactosamine residues, failed to bind in sufficient quantities, indicating a lack of exposure of these carbohydrate residues on *C. neoformans* cell surfaces. Confocal microscopy clearly shows that amongst wild type strains, the majority of the *C. neoformans* cell surface does not expose mannose / glucose or N-acetyl-glucosamine residues; exposure of such moieties was localised, and hence potentially highly concentrated, in areas associated with dynamic cellular change and trauma such as bud scars and budding daughter cells. Lectin avidity of Con A and WGA were studied in greater detail.

5.4.1 Avidity of lectin Con A

Con A is commonly used to identify glycoproteins as it is known to bind α-linked mannose a carbohydrate potentially found in many glycoprotein core oligosaccharides (Vector Laboratories inc., 2001). Studies of fungal yeast and conidial surfaces using fluorescein Con A (Smith *et al.*, 1999; Smith *et al.*, 2001), have indicated marked affinity for, and a uniform distribution of, Con A binding sites. However, results achieved here, when compared to *C. albicans* binding data reported by Smith *et al.* (2001), indicate that in contrast to *C. albicans*, *C. neoformans* has relatively fewer binding sites for Con A. This relative paucity of mannose / glucose exposure is not necessarily due to a lack of relevant carbohydrates in the cell wall; instead it may be due to preclusive masking and / or orientation of residues. Furthermore, Freytag & Mendgen (1991), as well as Sachdev (1979), reported that Con A may bind preferentially to hydrophobic areas as has been shown for other fluorescent probes; the overall hydrophilicity of the cryptococcal surface (as detailed in Chapter 3) may therefore have reduced binding by this lectin.

A comparison of Con A saturation binding data of various *C. neoformans* strains, indicates that EC50 values vary little between strains. Hn values do vary but generally indicate cooperative binding. Differences such as these may simply be a result of strain variation. Fmax values vary markedly between strains; interestingly it appears that strain CN IRM 5854, which lacks a visible capsule, along with acapsulate strain ATCC 52817, both gave greater Fmax values regardless of culture age. This indicates that severely reduced or acapsulate cells either have greater exposed carbohydrate in the cell wall, or that capsular presence and associated components hinder receptor ligand interaction.

The paucity of Con A binding to encapsulated strains confirms the finding of Cherniak & Sunderstrom (1994) that the mannose residues, found in GXM and GalXM, are not bound by this lectin. Confocal images show little fluorescence emanating from the capsule area of *C. neoformans*. Fluorescence that did occur may have resulted from Con A binding to mannoprotein, known to be found within the capsule matrix (Casadevall & Perfect, 1998). The greater Con A related Fmax values achieved with the acapsulate strain ATCC 52817, and to a lesser extent with the reduced capsule strain CN IRM 5854, indicates that capsular material, which ATCC 52817 lacks and which is poorly manifested in CN IRM 5854, may reduce surface exposure and / or accessibility of carbohydrate Con A receptors; this is likely to be either through concealment, attachment in such a manner as to preclude further moiety access to it on the yeast cell surface, or indeed both.

Hapten treatment indicated that mannose / glucose carbohydrate residues were largely responsible for fluorescence. Interestingly, tunicamycin interference analysis did not give conclusive data as to the nature of the residues observed; however it did indicate that, if the residues bound were components of glycoproteins, they do not appear to turnover at a high rate. Such an apparent lack of glycoprotein turnover might be a consequence of experimental protocol, and due to the age of the cells at the point of tunicamycin treatment. At the point of treatment, tunicamycin may not have affected glycoprotein presence in the cell wall of logarithmic growth phase cells because they may have achieved a level of maturity, which resulted in sufficient cell surface mannose / glucose related glycoproteins present in the cell wall to allow for completion of the cell lifecycle.

5.4.2 Avidity of lectin WGA

Detection of N-acetyl-glucosamine by FITC-lectin WGA binding, has considerable value in determining the presence of chitin, a common component of fungal cell walls (Doyle, 1994; Vector Laboratories inc., 2001) which speculation suggests is present in the cell wall of *C. neoformans* yeast (Casadevall & Perfect, 1998). Cryptococcal cell surface N-acetyl-glucosamine, as detected by FITC lectin WGA and associated ligand receptor characterisation, allows the comparison of exposure patterns between strains and species handled in the same manner. Here saturation binding analysis suggests that WGA binds to *C. neoformans* strains with marked avidity for both log and stationary populations. Relative Fmax data and confocal microscopy work implies that WGA ligands are present in lower numbers on the cell surface than Con A ligands, indicating either that N-acetyl-glucosamine residues are expressed in fewer numbers, or alternatively that N-acetyl-glucosamine orientation compromises WGA access and binding.

Saturation binding analysis indicated few readily distinguishable trends. However, the markedly low EC50 data, in comparison to that achieved by Con A, indicates that WGA bound to ligands on the cryptococcal cell surface with great avidity. In addition, similarly to Con A, data achieved from WGA binding indicated that acapsulate strain ATCC 52817 gave the greatest fluorescence of any strain, regardless of culture age. However, in contrast to Con A lectin binding, Fmax data suggests that WGA binds stationary phase cells in greater amounts than logarithmic phase cells; this may indicate that N-acetyl-glucosamine is exposed less on immature daughter cells as mature stationary cells are in greater numbers in stationary phase populations. Should such speculation be valid, a deficiency of N-acetyl-glucosamine on daughter cell surfaces could be a consequence of incomplete cell wall synthesis and architecture.

Confocal microscopy indicated WGA, similarly to Con A, penetrated capsule material. Lack of fluorescence within capsular material, as shown both by confocal microscopy imaging and the markedly low EC50 values exhibited by WGA binding to encapsulated cells, indicates that WGA appears to pass through the capsule with relatively little hindrance, and without any visible binding to capsular components. This lack of interaction with capsular components confirms the currently held view of

capsule composition, i.e. that it does not contain N-acetyl-glucosamine associated structures (Casadevall & Perfect, 1998). Furthermore, such a finding also suggests that little, if any, N-acetyl-glucosamine containing material is shed from the *C. neoformans* cell wall.

Hapten interference data suggests that, although incidental binding is occurring in relatively greater amounts than is apparent for Con A, there is still a significant amount of confirmed WGA–N-acetyl-glucosamine binding. Tunicamycin data describes a definite reduction in binding 4 hours post tunicamycin treatment. However as with Con A, tunicamycin caused an increase in carbohydrate bound at 24 hours post treatment. This seemingly contradictory data for WGA could be due to an initial reduction in glycoprotein production at 4 hours post treatment, followed by over compensatory production leading up to 24 hours post treatment. However, the contradictory nature of tunicamycin data demonstrates that WGA binding is not necessarily linked to glycoprotein production, instead, recorded N-acetyl-glucosamine may be a structural component of the cell wall.

5.4.3 Comparative examination of lectin based work

A comparative examination of this lectin based work is not easily undertaken as, with notable exceptions (Smith *et al.*, 1999; Smith *et al.*, 2001), fluorescent lectins have not been used to examine cell surface carbohydrate expression in similar quantitative detail. However, some useful comparison can be achieved when past experimentation and associated results data is viewed semi-quantitatively or qualitatively. Mendgen *et al.* (1985) examined the avidity of a panel of fluorescent lectins to infection structures of rust fungi, of particular interest was the pattern of binding observed; Con A was found to bind to most fungal surfaces irrespective of morphology, whereas WGA bound preferentially to germ tubes. Freytag & Mendgen (1991) continued the examination of rust fungi in an attempt to discover common structures and localisation of carbohydrate expression relevant to pathogenicity. Results from two distinct species of fungi showed a marked and significant level of diffuse binding of WGA to basidiospores and germ tubes. Also observed was a weaker, though still marked, binding of Con A to these same structures. A lectin based study of the surface of *P. palmivora*, a fungal phytopathogen, indicated that Con A,

LCA and WGA reduced adherence of infection structures, suggesting the presence of mannose / glucose and N-acetyl-glucosamine based adhesive materials (Bircher & Hohl, 1997). Smith *et al.* (1999) examined lectin binding to conidia and germlings of the mycoparasite *C. minitans* and found that conidia and germlings bound both Con A and WGA in marked amounts. Such work with diverse fungi, described above, indicates that similarly to *C. neoformans* yeast cells, ligand carbohydrates specific for both WGA and Con A have been discovered commonly on various fungal structures; however, unlike *C. neoformans*, the pattern of binding was less specific, tending instead to be more evenly spread across most fungal surfaces examined.

More relevantly, work with lectins has also been performed on yeast structures (Korting & Ollert, 1994; Smith *et al.*, 2001). This work has, until now, focussed almost entirely upon the dimorphic ascomycete *C. albicans*, mainly due to the possibility that many of the assumed virulence factors of this fungus, including adhesion processes and phenotypic switching, are thought to be related to glycoprotein expression on the cell surface (Korting & Ollert, 1994). One study in particular examined in detail attachment of a panel of fluorescent labelled lectins to *C. albicans* yeast and hyphal forms (Smith *et al.*, 2001). Results from the comprehensive series of experiments performed on yeast cells, found that *C. albicans* strains, similarly to *C. neoformans*, bound Con A in greatest amounts, followed by WGA and then GNL. However, comparison of flow cytometry data indicates that *C. albicans* cells bound Con A in greater amounts that *C. neoformans* cells. A further comparison of confocal images indicates that this is most likely due to the more localised binding of Con A to *C. neoformans* in comparison to the more diffuse binding observed amongst *C. albicans* yeast. Furthermore, as suggested by the manifest intensity of fluorescence observed by digital imaging, the relative density of carbohydrate residues at localities of budding and bud scars found amongst *C. neoformans* cells may be greater than the equivalent areas on *C. albicans* cells.

5.4.4 Clinical and environmental relevance of carbohydrate based expression

Carbohydrate residues detected on microbes have important functions and their exposure may have many consequences. Mannose residues have been

extensively linked to essential functions of fungi. In yeast cells, mannose has been associated with adhesion in *C. albicans*, *S. cerevisiae* (Korting & Ollert, 1994; Kanbe & Cutler, 1998; Ngondi-Ekome *et al.*, 2003) and *R. toruloides* (Buck & Andrews, 1999). Investigations focussed upon *C. albicans* yeast forms, have led to the conclusion that colonisation of host tissues is facilitated by adhesion, a process governed by moieties on the yeast cell surface (Korting & Ollert, 1994); in particular, glycoproteins containing mannose are involved (Kanbe & Cutler, 1998). The efficacy of such adhesive structures, if expressed by *C. neoformans* on the cell wall surface, is questionable as capsular material could obscure and isolate them. However, Merkel & Scofield (1994) produced convincing evidence that *C. neoformans* yeast express carbohydrate based adhesins on cell surfaces. These adhesins may indeed be blocked by capsular material, however, there is evidence that Con A binding adhesins may work from within the capsule of yeast. Buck & Andrews (1999) discovered that the phyto-saprophytic encapsulated yeast *R. toruloides*, exposed localised regions of Con A binding material which, unlike and despite capsular material, were responsible for adhesion. As well as adhesive functions, mannose based structures on *C. albicans* cell surfaces have also been associated with hydrophobicity (Masuoka & Hazen, 1999) and nutrient uptake (Braun, 1999), a role they may also fulfil for *C. neoformans*, albeit in a manner localised in areas of daughter cell development.

Consideration of the uses of carbohydrate based cell surface structures, suggests that exposure of mannose residues is of benefit to pathogenic organisms; however, expression of carbohydrate residues in quantity can also be detrimental to a microbe. Mannose binding has been implicated in the process of lectinophagocytosis a phenomenon in which lectins, sensitive for mannose, N-acetyl-glucosamine and other carbohydrate residues, are expressed on phagocytic cell surfaces, in turn facilitating phagocytosis (Ofek & Sharon, 1988). The phenomenon of lectinophagocytosis has been observed in the greater environment amongst protozoa such as *Acanthamoeba* (Brown *et al.*, 1975). Similarly, such a phenomenon is manifest within colonised hosts as part of the vertebrate immune system. This occurs both directly in the form of non-opsonic-phagocytosis induced by mannose type lectin found on the cell surface of macrophages, and indirectly in the form of serum based MBL (Janeway & Medzhitov, 2002) and mannan-specific immunoglobulin G antibodies (Zhang *et al.*, 1997). These indirect opsonins activate the complement cascade which, in turn, attracts phagocytes to the site of infection. Mannose type lectin, although named because it is a receptor

for mannose, will also bind glucose, N-acetyl-glucosamine and L-fucose (Ofek & Sharon, 1988). Paucity of *C. neoformans* cell surface exposure of mannose, glucose, N-acetyl-glucosamine and fucose, particularly amongst encapsulated cells, may compromise lectinophagocytosis, thereby reducing the effectiveness of the vertebrate immune system. Furthermore, paucity of carbohydrate residue exposure may also facilitate survival in a greater environment than that associated with vertebrates.

5.4.5 Conclusion

In conclusion, *C. neoformans* was found to have marked avidity for fluorescent lectins which bound mannose, glucose and N-acetyl-glucosamine. Considerably less surface binding of Con A and WGA, with respect to similar experiments involving *C. albicans* yeast forms, occurred. This was probably due to the more localised binding observed around areas of bud development; a finding in marked contrast to the extensive binding which occurs across cell surfaces of *C. albicans* (Smith *et al.*, 2001). Comparison amongst experimental *C. neoformans* strains did however demonstrate that mannose and glucose residues were found to be exposed in greater numbers, and in a less localised and more diffuse pattern, on genetically acapsulate variant *neoformans* strain ATCC 52817, indicating that the capsule may have a role in reducing or moderating carbohydrate residue exposure. It is probable that the capsule acts to reduce both host immune response to *C. neoformans*, as well as recognition by predators in the greater environment. This may occur not only through reduction of the amount of exposed carbohydrate residue, but also through denial of predator access, both to residues and residue binding elements of the immune system, such as MBL and carbohydrate specific antibodies. Finally, the role of exposed carbohydrates is not fully characterised, however, there is evidence that mannose and glucose based moieties are potential glycoproteins and may have an adhesive function. This, coupled with the apparent association of exposed mannose and glucose residues with those cell surface localities involved with daughter cell production, suggests that mother-daughter cell interaction should receive further consideration. Detected N-acetyl-glucosamine residues may be component parts of the

fibrous polymer chitin, and as a result could play a structural role as chitin is commonly found maintaining the structure of fungal cell walls (Gooday, 1995).

CHAPTER 6 – Isolation, analysis and extraction of cell surface proteins

6.1 Introduction

Thus far, a variety of cell surface entities have been thoroughly examined, including those carbohydrates which may constitute elements of glycoproteins. Glycoproteins are of particular interest as many have been identified as possible virulence factors of pathogenic microorganisms (Korting & Ollert, 1994; Mims *et al.*, 1995; Klein & Newman, 1996; Rodrigues *et al.*, 1997; Kanbe & Cutler, 1998; Madigan *et al.*, 2003). Material outlined below quantitatively and qualitatively examines proteinaceous cell surface constituents of *C. neoformans*, employing protocols and techniques which are relatively unique in their concept and approach. These analyses are founded on biotinylation studies employing the biotin derivative sulfo-N-hydroxysuccinimidobiotin (sulfo-NHS-biotin). Sulfo-NHS-biotin reacts with, and binds to, amino residues of proteins. A key component of sulfo-NHS-biotin is a sulphonate group which renders the compound incapable of penetrating cell membranes and walls (Cole *et al.*, 1987); this attribute is especially valuable whilst attempting to analyse cell surface material. Cell surface proteins, complexed with sulfo-NHS-biotin, can then be tagged with fluorescein-streptavidin in the same manner as with biotin hydrazide (Chapter 4). In turn, the fluorescent sulfo-NHS-biotin complex can be used during *in situ* quantitative processing via flow cytometry and associated saturation binding analyses. It can also be used for *in situ* qualitative examination of surface protein exposure via confocal microscopy. Furthermore, bound yet untagged sulfo-NHS-biotin may be of use in isolation and purification of cell surface proteins, as proteins complexed with sulfo-NHS-biotin may be isolated from cell extracts using streptavidin-based affinity chromatography.

Flow cytometry has been used successfully, as outlined elsewhere in this report, to produce comparative analyses of binding, both between strains and also between treatments. In this context, further analysis of data from concentration response experiments, as shown from previous work, may furnish information on the nature and quantity of exposed protein amino residues. Information of this nature is particularly useful with regards to *C. neoformans* as a comparison of protein exposure

between contrasting strains can be performed. Of special interest is the comparison of strains with and without a capsule.

In common with previous chapters, confocal microscopy has been used to characterise the distribution and exposure of relevant moieties on the *C. neoformans* cell surface. Comparison of such imagery, with that derived from biotin hydrazide and lectin based work, may also be informative. Common localisation of protein and carbohydrate residues would further indicate glycoprotein presence. Furthermore, comparison between encapsulated and acapsulate strains of *C. neoformans* is potentially of considerable value in providing a novel insight into the interaction of capsule and cell surface residue exposure.

In the final part of this experimentation, proteins tagged with sulfo-NHS-biotin were successfully isolated and purified. The procedure used is of great value as it allows the isolation of intact cell surface proteins without the inclusion of contaminating cytoplasmic material.

6.2 Materials and methods

Details of principle equipment, chemicals and materials, are included within the text; information pertaining to more common items can be found in Appendix G.

6.2.1 Flow cytometry analysis of sulfo-NHS-biotin biotinylation

Flow cytometry analysis was performed on two strains of *C. neoformans*, CN IRM 5815 and ATCC 52817. These strains were chosen because of their contrasting status. *C. neoformans* CN IRM 5815 is variant *gattii* and heavily encapsulated *in vitro*, whereas ATCC 52817 is a variant *neoformans* and a capsule free mutant. Five concentrations of sulfo-NHS-biotin were assessed: 0.01, 0.05, 0.1 and 0.5 mg ml^{-1}. The initial binding and tagging was performed in two steps, cells are biotinylated and then the biotin is tagged with fluorescein-streptavidin.

The biotinylation step was adapted from procedures used by Lisanti *et al.*, Kähne & Ansorge and Altin & Pagler (1989; 1994; 1995). Strains of *C. neoformans* were grown in 25 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm) in triplicate, logarithmic or stationary phase cells were removed, pelleted, washed three times, then suspended in ice cold PBS containing 1mM $MgCl_2$ and 0.1 mM $CaCl_2$ pH 7.4 (PBS C/M). Cell concentration was adjusted to 2 x 10^7 cells ml^{-1}. Cell suspensions of 0.9 ml were transferred to sterile Eppendorf tubes in triplicate, adjusted to the required concentrations of 0.01, 0.05, 0.1 and 0.5 mg ml^{-1}, by addition of room temperature sulfo-NHS-biotin (Pierce, Rockford, USA) dissolved in 100 µl of PBS C/M. After mixing, cells were incubated on ice for 30 minutes with regular agitation. Cells were pelleted, and then washed three times with ice cold PBS C/M.

Tagging of protein bound sulfo-NHS-biotin with fluorescein-streptavidin (Molecular Probes, Leiden, The Netherlands) was adapted from the procedure used by Bhalgat *et al.* (1998). Cells were pelleted, re-suspended in 1 ml PBS C/M containing 5 µg ml^{-1} fluorescein-streptavidin, and incubated on ice in the dark for 30 minutes with regular agitation. In order to remove excess fluorescein-streptavidin, cells were pelleted, and then washed three times with ice cold PBS C/M. Before further analysis cells were fixed in 1 % w/v paraformaldehyde / PBS solution.

Flow cytometry analysis was adapted from that used by Smith *et al.* (1999; 2001). Fluorescence of 10,000 yeast cells was determined with a Becton Dickinson FACS 440 using an argon laser (200 mW), with an excitation wavelength of 488 nm and emitted light detector at 530 nm (± 15 nm), adjusted to a fixed channel using standard Brite Beads (Beckman Coulter, High Wycombe, UK), prior to determining fluorescence. Yeast samples were vortexed before introduction to sheath fluid and subsequent determination of fluorescence.

Binding concentration data were analysed using non-linear regression in the following manner: autofluorescence values were subtracted from each data point and resulting data fitted to a logistic function of the form:

$$\text{Bound}(B) = \frac{\text{Fmax} \bullet [SB]^{Hn}}{[SB]^{Hn} + EC50}$$

where Fmax is the fluorescence value when all binding sites are saturated, SB is the concentration of sulfo-NHS-biotin, and EC50 is the concentration of sulfo-NHS-biotin that produces 50 % of the maximum saturation. Hn is the Hill coefficient, a measure of the cooperativity in the system (Rang *et al.*, 1999). Data fitting was by means of non-linear regression analysis performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, USA). The binding curve from each individual experiment was analysed to obtain estimates of Fmax, EC50 and Hn.

Data were analysed using two factor ANOVA (Microsoft Excel 2000, Microsoft Corp., Seattle, USA). Differences in protein induced yeast cell fluorescence, between strains and between growth phases, were analysed at varying concentrations of sulfo-NHS-biotin. Where necessary to illustrate significance, the LSD was calculated as part of the ANOVA analysis.

6.2.2 Confocal microscopy

Confocal microscopy was performed upon cells that had undergone biotinylation and fluorescein-streptavidin tagging in the same manner as described above (section 6.2.1). Strains CN IRM 5815 and ATCC 52817 were examined at 0.2 mg ml^{-1} sulfo-NHS-biotin. Aliquots of 7.5 µl logarithmic and stationary phase cells fixed in 1% w/v paraformaldehyde / PBS, were in turn dispensed into wells of separate multispot microscope slides (Hendley-Essex, London, UK). Cell suspensions were then air dried and fixed a second time, by the addition of 7.5 µl cold acetone, which was subsequently allowed to evaporate under warm air. Wells were then sealed with 5 µl "Vectorshield" (Vector Laboratories, Burlingame, USA), a coverslip firmly affixed with "Tippex" correction fluid and stored at 5 °C in the dark until fluorescence characterisation by confocal microscopy. Images were acquired with a Zeiss Axiovert / Biorad MRC 1024 OS laser scanning confocal microscope facility, utilising Laser Sharp 2000 software (Bio-Rad Laboratories, Hemel Hempstead, UK). An argon laser (100 mW), excitation wavelength 488 nm moderated by a series of neutral density filters, gain, offset and zoom functions was utilised to induce and optimise image fluorescence intensity, contrast and composition. Slides were viewed with an oil immersion Zeiss Neuflor 1.3 NA objective (Carl Zeiss, Welwyn Garden City, UK)

and subjective material captured as 512 x 512 pixel images in turn converted from Bio-Rad PICT to TIFF format. In order to obtain satisfactory prints, contrast and brightness of images were altered using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, USA), no raw data was removed and no synthetic data was added in this process.

6.2.3 Isolation and purification of cell surface proteins

Isolation and purification was performed on the acapsulate strain ATCC 52817. Of the two strains used in experimentation outlined in this chapter, CN IRM 5815, due to its large capsule, was considered unsuitable as capsular matrix material may have hindered and contaminated cell surface protein isolation. Furthermore, strain ATCC 52817 has been shown to express greater amounts of cell surface carbohydrates, potentially of a glycoproteinaceous nature, than other strains, and therefore was thought likely to support successful glycoprotein isolation. Stationary phase cultures were used as they appear to bind more biotin, as well as yielding greater numbers of yeast than logarithmic phase material. Isolation and purification was carried out in four separate steps: biotinylation, protein extraction, protein purification and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Biotinylation was adapted from the protocols of section 6.2.1. Strains of *C. neoformans* were grown in 100 ml of Sabouraud liquid medium (Lab M, Bury, UK) at 37 °C with shaking (150 rpm), stationary phase cells were removed, washed three times with, and suspended in, ice cold PBS C/M, before the addition of room temperature sulfo-NHS-biotin (Pierce, Rockford, USA) to a concentration of 0.5 mg ml^{-1}. Control cells were incubated with ice cold PBS C/M only. Samples were mixed, incubated on ice for 30 minutes with regular agitation, pelleted by centrifugation, and then washed three times with ice cold PBS C/M.

Protein extraction was performed using a method adapted from that used by Pitzurra *et al.* (1997) and is outlined in Figure 6.2.3a. Cell samples were suspended in 0.1 % w/v SDS – 1 % v/v 2-mercaptoethanol (SDSMerc), and heated to 90 °C for 10 minutes. Samples were pelleted by centrifugation at 15,000 *g* for 30 minutes, and after

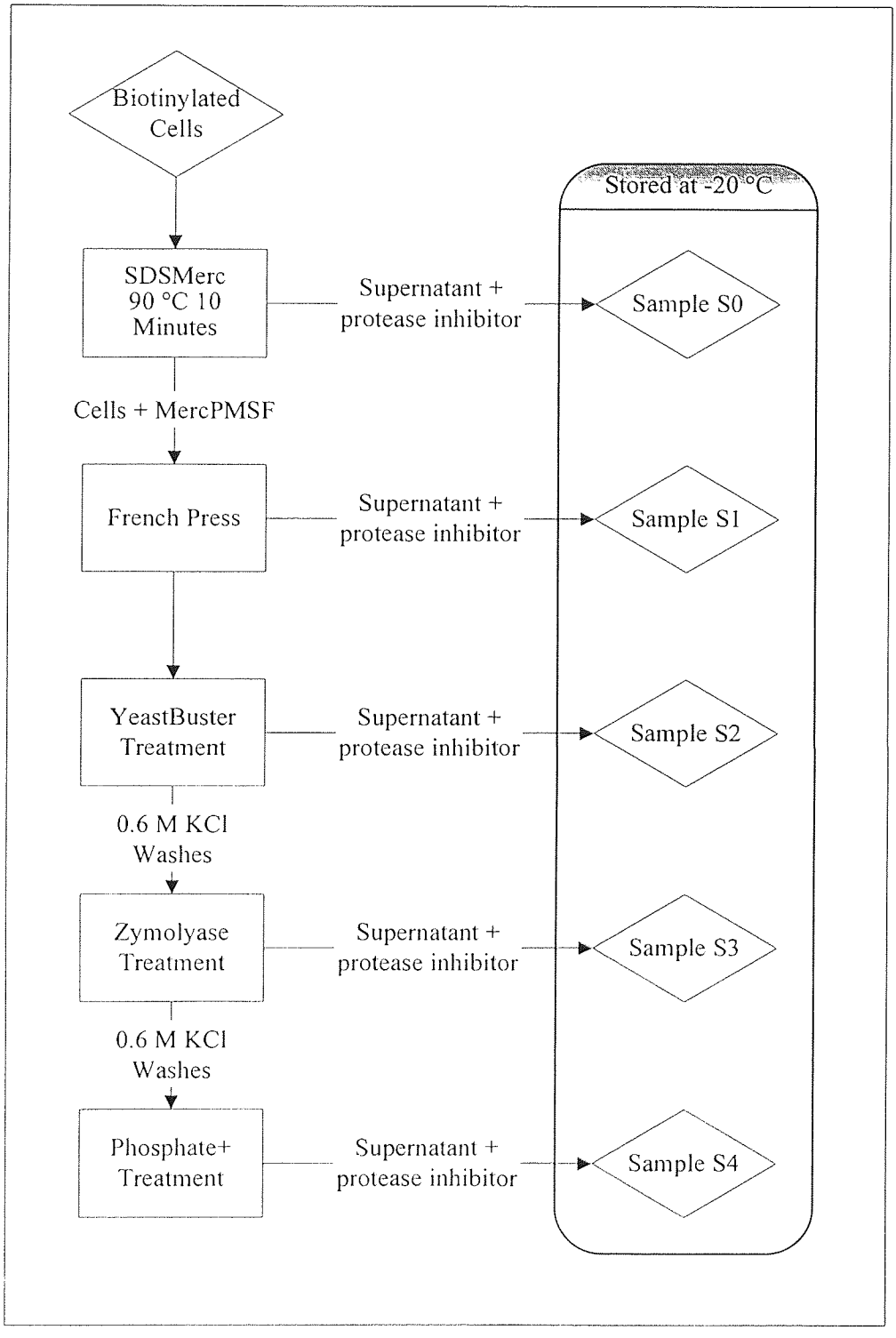


Figure 6.2.3a. A flow diagram illustrating the steps used to extract proteins from *C. neoformans* yeast cells.

the addition of 5 µl fungal protease inhibitor (protease inhibitor cocktail for fungal and yeast cells), the resulting supernatant (Soluble cell surface proteins: S0) was stored at -20 °C. Remaining cells were suspended in fresh MercPMSF (Appendix D). Cells were kept on ice before being repeatedly put through a fridge cooled Nike 43HD 30T French Press (Nike Hydraulics AB, Eskilstuna, Sweden) at 35,000 – 45,000 psi until 90 % cell breakage was observed with light microscopy. Samples were pelleted by centrifugation at 15,000 *g* for 30 minutes and the resulting supernatant (proteins released after mechanical disruption of whole cells: S1) was stored at -20 °C after the addition of 5 µl fungal protease inhibitor. Cell debris was then suspended in freshly prepared '"Yeastbuster" mixture, prepared according to the manufacturers guidelines (Novagen (Merck BioSciences), Nottingham, UK), before being incubated at room temperature for 20 minutes. Samples were pelleted by centrifugation at 15,000 *g* for 30 minutes and, after the addition of 5 µl fungal protease inhibitor, the resulting supernatant (proteins released after mild chemical disruption of cell wall debris: S2) was stored at -20 °C. Remaining cell debris was washed twice in 0.6 M KCl and resuspended in 0.6 M KCl with 1000 units of Zymolyase (Lyticase of *Arthrobacter luteus*), before being incubated at 37 °C for 30 minutes. Samples were pelleted by centrifugation at 15,000 *g* for 30 minutes and, after the addition of 5 µl fungal protease inhibitor, the resulting supernatant (proteins released after enzymatic disruption of cell wall debris: S3) was stored at -20 °C. All remaining cell debris was washed twice in 0.6 M KCl and suspended in Phosphate+ buffer (Appendix E), homogenised and centrifuged at 40,000 *g* for 30 minutes at 4 °C. Resulting supernatant (further chemical treatment of cell wall debris: S4) was stored at -20 °C after the addition of 5 µl fungal protease inhibitor.

Purification of biotinylated proteins was achieved through the use of anti-biotin agarose column-based chromatography. The procedure is illustrated in Figure 6.2.3b. Each batch S0 to S4, from both control and biotinylated cells were dispensed into the top of glass Pasteur pipettes loosely plugged with 0.1 g of glass wool. Each pipette contained 1 ml of PBS-washed goat anti-biotin agarose (Vector Laboratories, Burlingame, USA). After passage of a complete sample, each column was washed with 4 fractions of 2 ml PBS. Combined sample and wash fractions were immediately dialysed against distilled water for 48 hours with four changes of water. Anti-biotin agarose bound proteins were eluted using 4 fractions of 2 ml 200 mM acetic acid.

Elute fractions were combined and dialysed immediately against distilled water for 48 hours with four changes of water. The resulting wash and elute samples, from both control and biotinylated cells, were lyophilised using a Savant Micromodulyo lyophilyser (Savant Instruments, New York, USA), reconstituted with 200 µl sterile distilled water and stored at -20 °C until SDS-PAGE.

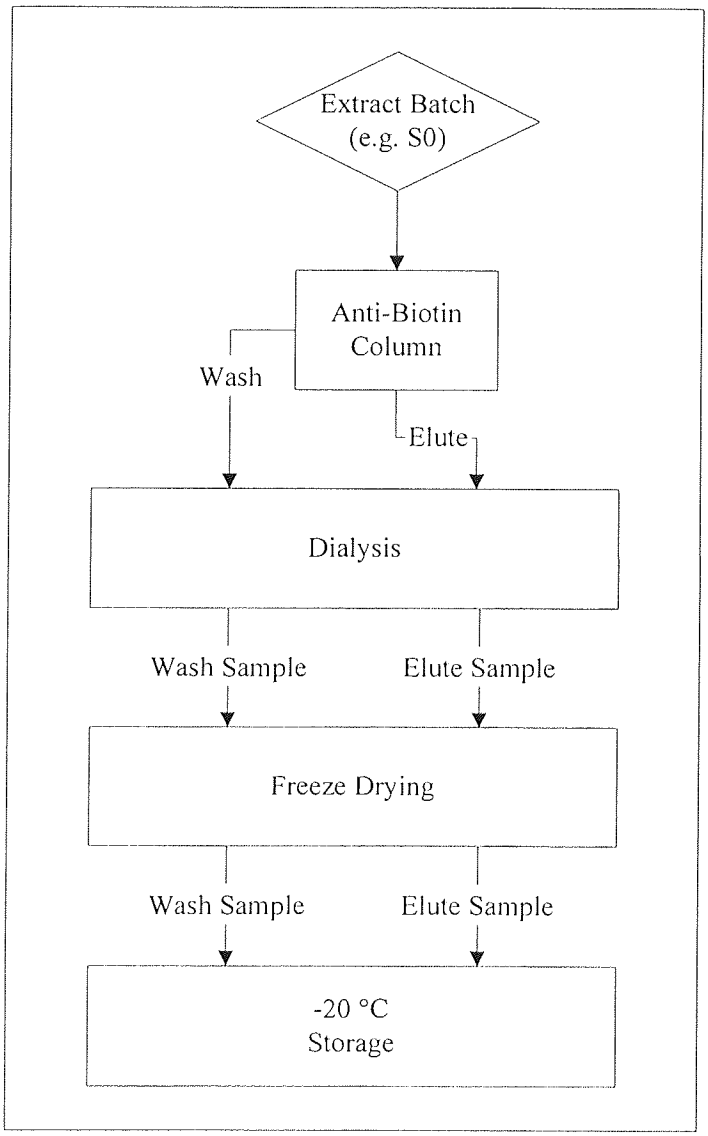


Figure 6.2.3b. A flow diagram illustrating the steps used to purify proteins from *C. neoformans* yeast cells.

SDS-PAGE was adapted from the method of Laemmli (1970), and was used to detect, separate, and partially quantify biotinylated proteins remaining after

purification. SDS-PAGE also served, via the examination of sample washes from the previous step, to ensure that protein extraction had occurred. Formulations used during this procedure are listed in Table 6.2.3.

Table 6.2.3. Formulations of the various constituents of the SDS-PAGE system.

	Separating gel 11 % w/v	Stacking gel 5 % w/v	Sample buffer	Electrode buffer
44 % Acrylamide stock*	5 ml	-	-	-
30 % Acrylamide stock*	-	2.5 ml	-	-
10 % w/v SDS	0.5 ml	0.15 ml	5 ml	10 ml
1.5 M Tris-HCl (pH 8.8)	6 ml	-	-	-
0.5 M Tris-HCl (pH 6.8)	-	3.75 ml	2.5 ml	-
Distilled water	8 ml	8 ml	5 ml	1 L
TEMED	50 µl	40 µl	-	-
10 % w/v amps†	70 µl	50 µl	-	-
Glycerol	-	-	25 ml	-
2-mercaptoethanol	-	-	0.25 ml	-
5% w/v bromophenol blue	-	-	0.2 ml	-
Tris	-	-	-	3 g
Glycine	-	-	-	14.4 g

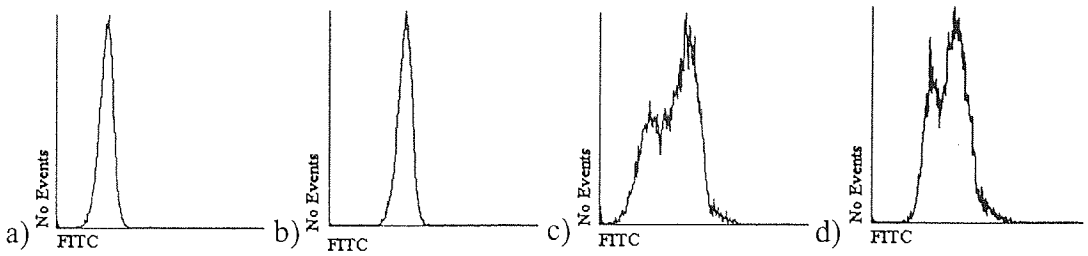
* Acrylamide stocks contain 0.8 % w/v Bis(N,N',-methylene-bis-acrylamide) (Severn Biotech, Kidderminster, UK).
† Freshly prepared ammonium persulphate.

Samples, either wash or elute, in sample buffer (Table 6.2.3) were denatured at 90 °C for five minutes and microfuged at 15,000 *g* for two minutes to pellet any debris. Sample supernatants were loaded, 25 µl per lane, into an 11 % w/v polyacrylamide minigel (Table 6.2.3), which was set up for use within the BioRad Mini Protean II system (Bio-Rad Laboratories, Hemel Hempstead, UK). The tank was filled with electrode buffer (Table 6.2.3) and the gels were run with broad range pre-stained protein markers (New England BioLabs Inc., Hitchin, UK) at 200 V, until the bromophenol blue marker dye reached the bottom of the gel. Gels were stained using Coomassie blue R-250 (Appendix F). Once stained, gels, over a white underlight, were digitally photographed in reverse contrast using the GeneGenius Biomanaging System, utilising Syngene GeneSnap 6.00 software (Synoptics, Cambridge, UK). In order to obtain satisfactory prints, contrast and brightness of images were altered using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, USA).

6.3 Results

6.3.1 Flow cytometry analysis of sulfo-NHS-biotin biotinylation

Representative flow cytometry profiles received from cells treated with sulfo-NHS-biotin and fluorescein-streptavidin, are presented in Figures 6.3.1a-d. The profiles in Figures 6.3.1a-d are of cells treated with 0.5 mg ml^{-1} sulfo-NHS-biotin; this concentration was chosen as it represents the peak of binding, and possible beginning of the binding plateau (see Figures 6.3.1e&f). Figures 6.3.1a-d demonstrate the contrasting nature of the two strains examined. Acapsulate strain ATCC 52817 has a noticeable shoulder of weaker fluorescence below the modal average, this indicates that some members of the population bound less biotin / fluorescein-streptavidin than the modal average, a phenomenon present in both logarithmic and stationary phase cells. Cells of strain CN IRM 5815 display a strongly leptokurtic distribution, regardless of cell maturity, in turn, characteristic of a population with little variation in the level of ligand binding amongst members.



Figures 6.3.1a-d. Flow cytometry population fluorescence profiles of sulfo-NHS-biotin binding to *C. neoformans strains* (at 0.5 mg ml^{-1}): (a) CN IRM 5815 (logarithmic), (b) CN IRM 5815 (stationary), (c) ATCC 52817 (logarithmic) and (d) ATCC 52817 (stationary).

Following the examination of profiles, mean fluorescence values, from each of the various concentrations employed have been collated and used to produce Figures 6.3.1e&f.

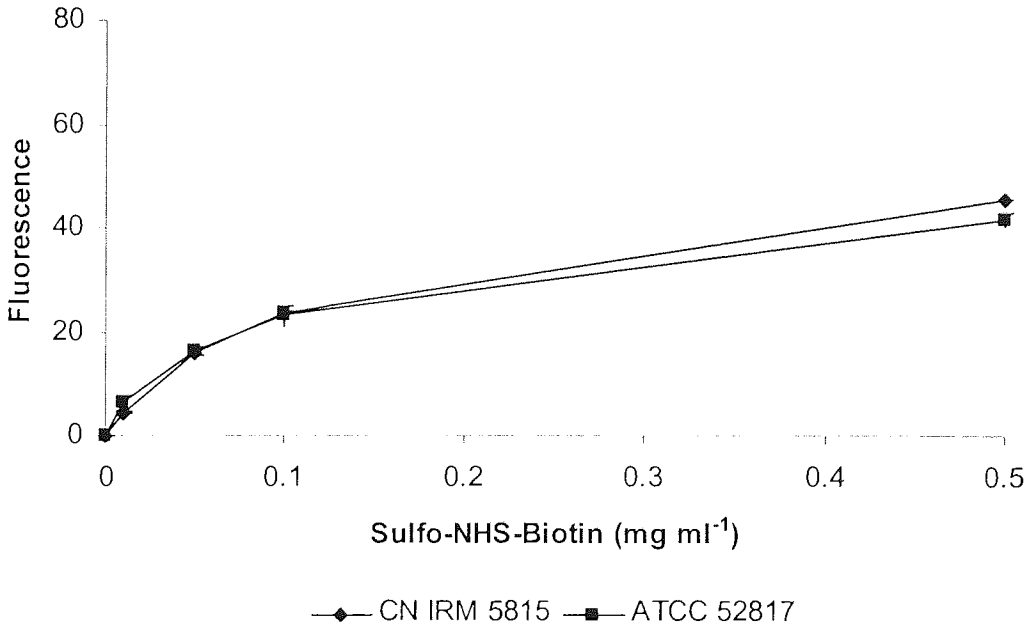


Figure 6.3.1e. The affect of sulfo-NHS-biotin concentration upon biotin / fluorescein-streptavidin binding to proteins on the cell surface of *C. neoformans* strains grown to logarithmic growth phase. Results are means of three experimental readings ± standard error.

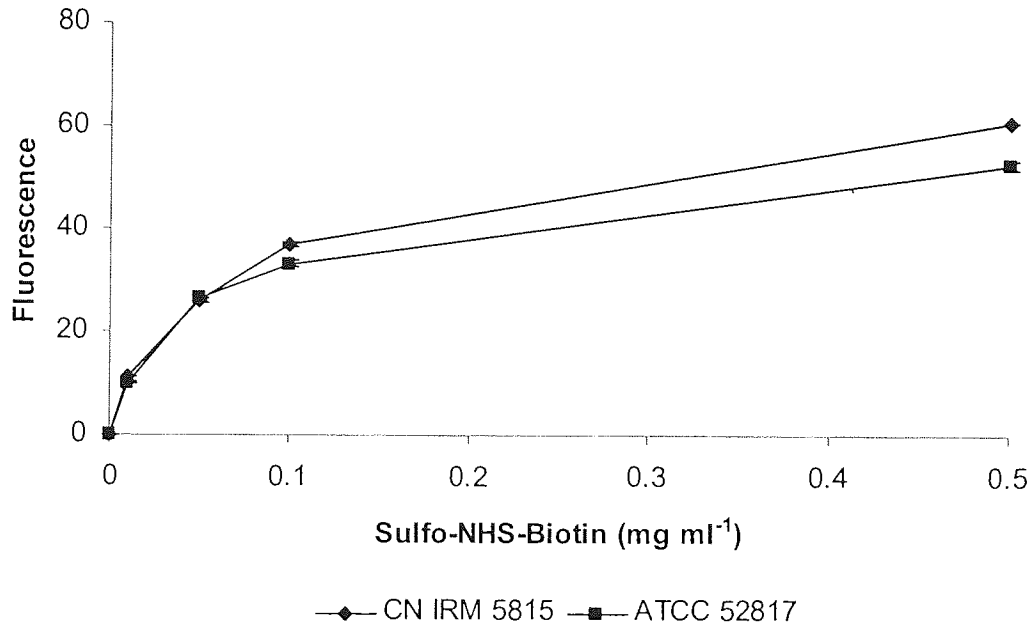


Figure 6.3.1f. The affect of sulfo-NHS-biotin concentration upon biotin / fluorescein-streptavidin binding to proteins on the cell surface of *C. neoformans* strains grown to stationary growth phase. Results are means of three experimental readings ± standard error.

It is apparent from Figures 6.3.1e&f that, regardless of strain or growth phase, levels of fluorescence increased in line with increasing sulfo-NHS-biotin concentration. Figure 6.3.1e describes the binding of sulfo-NHS-biotin to logarithmic phase cells from both strains of *C. neoformans* examined. Each figure describes a significant (ANOVA, $P < 0.001$), though 'gentle', increase in fluorescence with increasing concentrations of sulfo-NHS-biotin. Neither plot reaches a defined plateau, probably a consequence of sulfo-NHS-biotin proving unstable at concentrations higher than 0.5 mg ml^{-1}; an observation similar to that of Cole *et al.* (1987). No significant difference occurred amongst differing logarithmic strains (ANOVA, $P = 0.47$). Contrastingly, a significant difference was found between strains in stationary growth phase (ANOVA, $P < 0.001$), the LSD value of 1.20 achieved in this analysis indicates that values with the greatest influence upon significance are found amongst the higher concentrations utilised.

Non-linear regression analysis gave the data provided in Table 6.3.1. A contrast is clearly visible between growth phases, stationary phase cells show stronger avidity in the form of both lower EC50 values, and greater Fmax values. Fmax values

also indicate that cells of strain CN IRM 5815 expose more protein than cells of strain ATCC 52817 in the same growth phase.

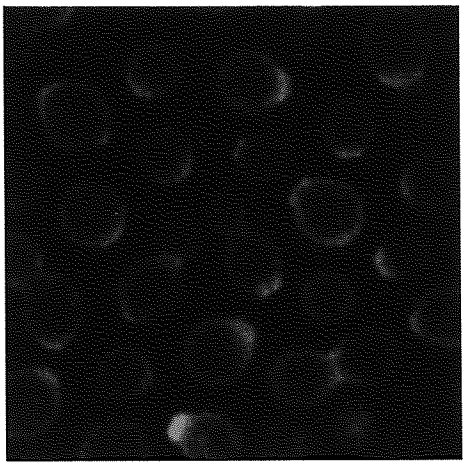
Table 6.3.1. Non-linear regression analysis of sulfo-NHS-biotin / fluorescein-streptavidin binding to proteins on the cell surface of *C. neoformans* (± standard error).

	CN IRM 5815		ATCC 52817	
	Logarithmic	Stationary	Logarithmic	Stationary
Fmax	57.9 (± 0.8)	70.4 (± 1.8)	50.3 (± 2.5)	58.3 (± 1.6)
EC50 (mg ml^{-1})	0.136 (± 0.005)	0.083 (± 0.006)	0.107 (± 0.014)	0.064 (± 0.005)
Hn	0.89 (± 0.04)	0.69 (± 0.029)	0.68 (± 0.16)	0.72 (± 0.05)

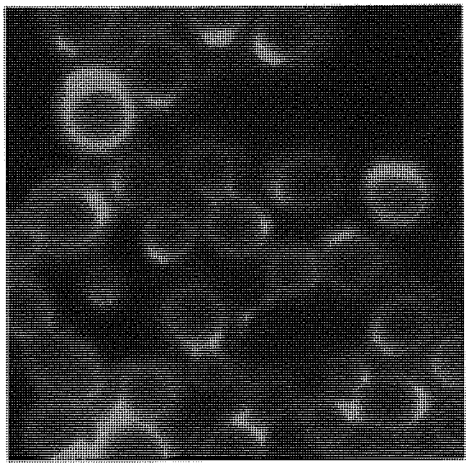
6.3.2 Confocal microscopy

Figures 6.3.2a-d are representative images of sulfo-NHS-biotin / fluorescein-streptavidin biotinylation of selected *C. neoformans* strains. Figure 6.3.2b demonstrates both fulgent and extensive fluorescence amongst mature cells of strain CN IRM 5815, this includes extensive capsule based fluorescence, a characteristic not observed amongst logarithmic phase cells of the same strain (Figure 6.3.2a). Images of acapsulate strain ATCC 52817 illustrate diffuse fluorescence. Regardless of strain or maturity, cells exhibit vivid localisation of fluorescence associated with budding-related tips, scars and collars.

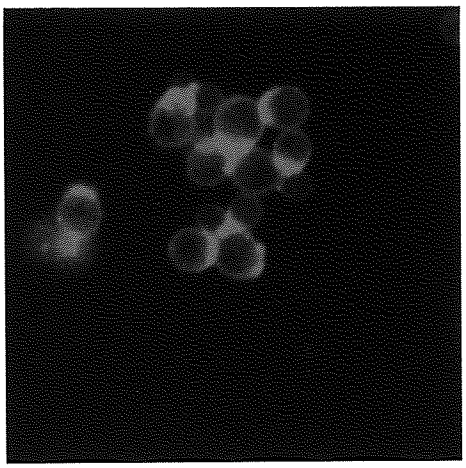
a)



b)



c)



d)

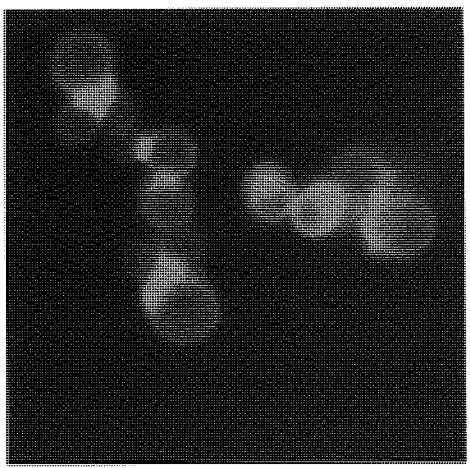


Figure 6.3.2. Confocal images of sulfo-NHS-biotin / fluorescein-streptavidin mediated *C. neoformans* yeast cell fluorescence.
a) CN IRM 5815 (logarithmic). b) CN IRM 5815 (stationary).
c) ATCC 52817 (logarithmic). d) ATCC 52817 (stationary).

6.3.3 Isolation and purification of cell surface proteins

After purification, both wash and elute samples from each batch were subjected to SDS-PAGE. Figure 6.3.3a depicts an SDS-PAGE gel produced using elute samples from biotinylated cells. One batch, S1, lead to proteins visible upon staining with Coomassie blue R250.

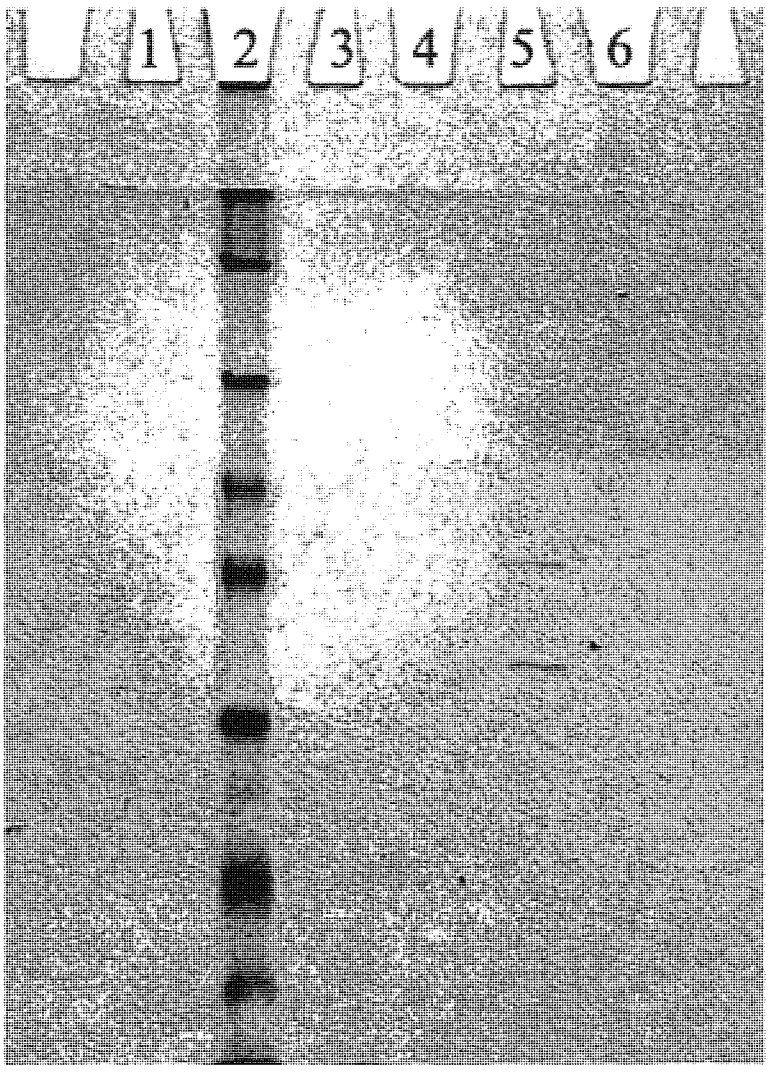


Figure 6.3.3a. An SDS-PAGE gel indicating the protein content of anti-biotin column elute stages from biotinylated cells. Column 1: Elute of sample S4, 2: Protein Standard Markers, 3: Elute of sample S3, 4: Elute of sample S2, 5: Elute of sample S1 (containing two proteins), 6: Elute of sample S0.

Figure 6.3.3b is a gel comparing the proteins stained in batch S1 samples from control and biotinylated cells; columns 2 and 3 are control and biotinylated wash samples respectively, columns 6 and 7 are control and biotinylated elute samples respectively. Both samples from chromatography washes indicate numerous proteins extracted, however, elute samples indicate only two proteins (one of around 57 kDa, and one of 47 kDa), both of which are found only in material from biotinylated cells, thus indicating that these proteins originated from the cell surface.

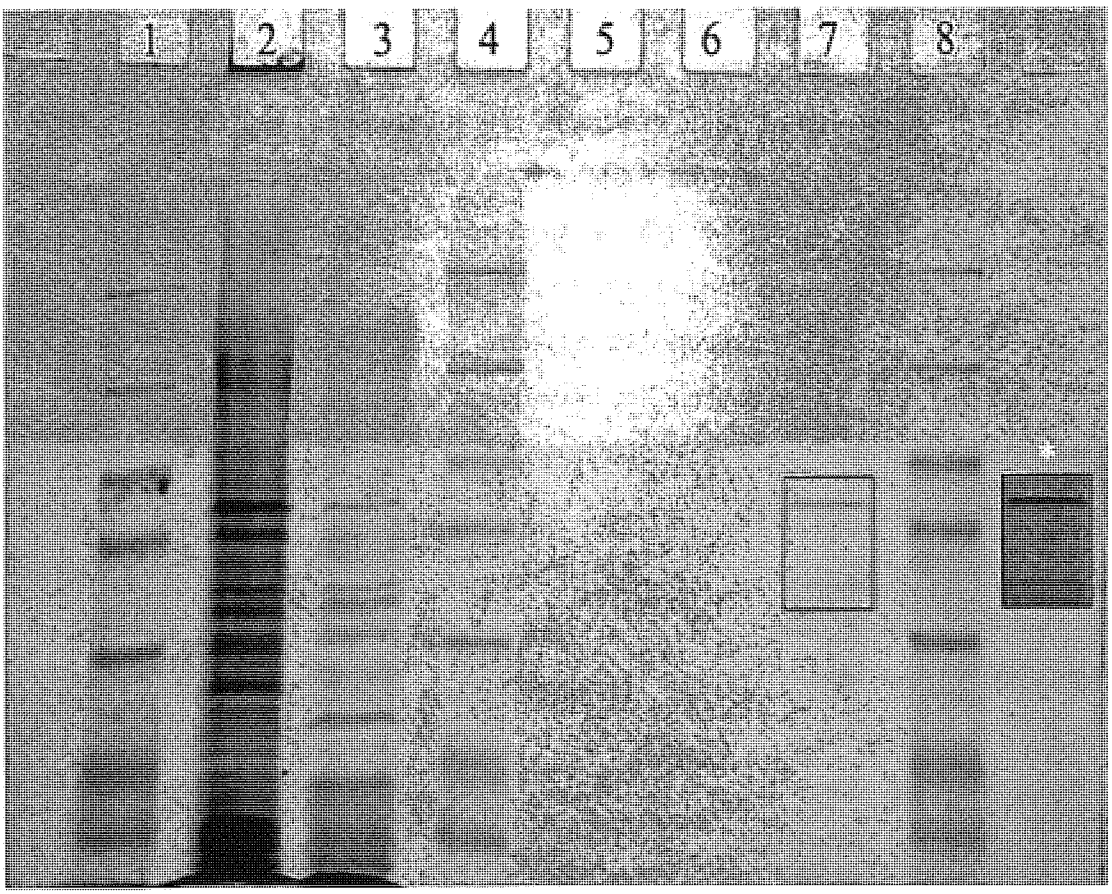


Figure 6.3.3b. An SDS-PAGE gel indicating the protein content of anti-biotin column wash and elute stages from biotinylated and control cell stage 1 samples (S1). Columns 1, 4 & 8: Protein standard markers. 2: Un-biotinylated wash, Column 3: Biotinylated wash 5: No Sample, 6: Un-biotinylated elute, 7: Biotinylated elute containing 2 proteins (*enhanced to the right).

6.4 Discussion

Use of sulfo-NHS-biotin to biotinylate mammalian cell surface proteins is an established technique. In particular, cells involved in the mammalian immune response have been examined in detail (Hurley *et al.*, 1985; Kasinrerk *et al.*, 1992; Meier *et al.*, 1992; Kähne & Ansorge, 1994). More recently, *C. albicans* hyphae and blastospores, as well as *S. cerevisiae* yeast cells, were biotinylated for the purpose of cell surface protein visualisation and extraction (Edwards *et al.*, 1999; Urban *et al.*, 2003). Considering the stark contrast between *C. albicans* and *C. neoformans* with respect to various other cell surface properties investigated, *C. neoformans* was therefore an obvious candidate for similar investigation. In turn, protein biotinylation techniques have been expanded not only to allow protein visualisation and extraction, but also flow cytometry based analysis of the biotinylation reaction itself. As previously, comparison between varying strains is especially useful with regards to *C. neoformans* as such an approach broadens and enhances our understanding of the interaction between cryptococcal capsule and cell surface entities. A further important consideration is the comparison of protein based biotinylation with both the biotin hydrazide and lectin based carbohydrate investigations. Similarities in the cell surface location of protein and carbohydrate may be indicative of glycoprotein presence and exposure. In fact, *C. neoformans* strains were found to have marked amounts of protein exposed on cell surfaces; this protein was observed and quantified in varying amounts on the two strains examined. Although ubiquitous across the cell surface, protein was found to be concentrated at the interface between mother and daughter cells. Of the proteins responsible for the fluorescence observed, two were isolated from crude cell extracts, purified and sized.

6.4.1 Flow cytometry analysis of sulfo-NHS-biotin biotinylation

Flow cytometry work clearly indicated marked levels of protein exposure on surfaces of cells from two *C. neoformans* strains. Population fluorescence profiles were found to differ markedly between strains. Of particular interest is the shoulder of

cells exhibiting weaker fluorescence, observed in profiles of the acapsulate strain ATCC 52817. A similar though much less pronounced shoulder was observed in cells of strain ATCC 52817 after biotinylation with biotin hydrazide. A possible cause of this affect, given in Chapter 4, was the tendency of cells from this strain to aggregate; this in turn, may reduce exposure of cells, and therefore surface moieties, to the binding agents employed, i.e. biotinylating agent and fluorescein-streptavidin; such an explanation may also be valid on this occasion. However, the disparate weakly fluorescing cells, observed amongst the population of strain ATCC 52817, may instead indicate the presence of two distinct cell types within this population. Davey & Kell (1996) suggested that actively growing yeast support flow cytometry profiles characterised by two distinct populations, which are separated by size due to the presence of mother-daughter doublets in addition to single cells. These two distinct populations may prevail amongst the cryptococcal strains under current investigation. If so, doublet presence is also manifest in not only size profiles, but also fluorescence profiles. Should the weakly fluorescing subpopulation – lacking amongst encapsulated cells – be composed of doublets, it could therefore be construed that encapsulation reduces apparent disparity between single and doublet cells. Regardless of whether this occurrence is due to aggregation in acapsulate cells, or indeed greater production of exposed proteins in encapsulated doublets, it appears that encapsulation has a marked affect upon cell surface protein exposure.

Extended investigation, in the form of non-linear regression analysis applied to varying concentration data, indicates disparity between cells of differing maturity. Logarithmic phase cells appear from Fmax data to express fewer exposed proteins than stationary phase cells. This finding is in agreement with the biotin hydrazide based work which was focussed on carbohydrate moieties; it indicates that cells in a senescent state expose a greater amount of protein. This increased protein exposure could be a consequence of cellular antecedence, in that senescent cells have undergone more extensive cellular change than less mature cells, and thus will have incurred greater numbers of bud scars and other cell surface perturbations or traumas. Avidity of sulfo-NHS-biotin for cells also varies between growth phases, with EC50 values indicating weaker avidity amongst logarithmic phase cells. This change in avidity with age may be due to a change in the conformational status of proteins on the cell surface with cell maturation, or possibly the appearance of differing fresh and novel proteins. Although an exact comparison is not feasible, it appears that the

avidity of sulfo-NHS-biotin for *C. neoformans* cells is comparatively weaker than that observed with biotin hydrazide. Certainly a comparison of graphed data indicates that data derived from sulfo-NHS-biotin work supports a less severe gradient, suggestive of less affinity. An observation of this nature suggests that biotin hydrazide may be a better choice, compared to sulfo-NHS-biotin, when attempting to biotinylate carbohydrate and protein rich glycoproteins. However, the most striking information gained from this analysis is the indication from Fmax data that strain CN IRM 5815 appears to express greater levels of protein residue than strain ATCC 52817, thereby leading to the conclusion that capsule production may increase protein exposure. A conclusion which challenges evidence presented in Chapter 5 that carbohydrate, potentially in the form of glycoprotein, may be moderated and tempered by capsular components.

6.4.2 Confocal microscopy

Confocal microscopy has again been of invaluable use with respect to the examination of fluorescent based tagging of *C. neoformans*. Images indicate, as with both biotin hydrazide and lectin based work, that sulfo-NHS-biotin based fluorescence is found in greatest amounts around bud scars, nascent cells, and at the mother-daughter cell interface. Confocal microscopy clearly shows that proteins, bound by sulfo-NHS-biotin, are found on the cell surface in a localised manner. In turn, it is not unreasonable to hypothesise, due to marked co-localisation, that some of the carbohydrate and protein observed may be complexed together as glycoprotein. Images also indicate that, as with mammalian cell membranes (Hurley *et al.*, 1985; Sargiacomo *et al.*, 1989; Meier *et al.*, 1992) and cell walls of Ascomycota fungi (Cappellaro *et al.*, 1998; Kandasamy *et al.*, 2000; Urban *et al.*, 2003), sulfo-NHS-biotin is incapable of binding intracellular proteins of the basidiomycete yeast form of *C. neoformans*. However, sulfo-NHS-biotin is capable of penetrating the *C. neoformans* capsule, allowing contact and binding of this compound to the yeast cell wall surface.

The presence of glycoproteins is of particular importance as they may have considerable biological consequences; both advantageous to a microbe, such as an

adhesive function; and deleterious for instance as immunological modulators (see sections 1.2.1 & 5.4.4). Images of mature encapsulated cells (Figure 6.2.3b) appear to indicate that, in addition to cell surface binding, some binding occurred amongst proteins shed by cells of strain CN IRM 5815 into the capsule matrix. The presence of protein in the capsule matrix is not unexpected, several proteins are known to be secreted from cryptococcal cells; these include amongst others mannoprotein (Vartivarian *et al.*, 1989) (probably in several forms), phospholipases (Cox *et al.*, 2001) and urease (Cox *et al.*, 2000). In particular, mannoprotein, as a result of its protein content, was expected to bind sulfo-NHS-biotin.

The 'extra' proteinaceous material observed in the capsule matrix of stationary cells from strain CN IRM 5815, when combined with that from the cell surface, is the likely reason for the significantly greater Fmax value exhibited by this strain in stationary phase in comparison to stationary cells from strain ATCC 52817. Although not apparent via confocal imaging, logarithmic phase cells from strain CN IRM 5815, in kind with their more mature kin, gave a significantly greater Fmax value than acapsulate cells of a similar age; this phenomenon may also be due to fluorescence emitted by proteins within the capsule matrix. In addition, the appearance of fluorescence in the capsule matrix may explain the observation, by flow cytometry, that populations of encapsulated cells have a more homogenous fluorescence profile; if mother-daughter doublets are sharing the 'extra' capsule based proteinaceous materia observed by confocal microscopy, a more homogenous profile might be expected.

6.4.3 Isolation and purification of cell surface proteins

Isolation and purification of proteins expressed on and from cell surfaces of pathogenic microorganisms, allows investigation, not only into their identity, but also virulence characteristics. Properties most often explored include the function a protein performs and, if derived from a vertebrate pathogen, the effect it has on the mammalian immune system. Furthermore, a proteins function may embody a virulence factor and further research can be focussed upon nullifying the protein and thus reducing microbial virulence. Alternatively, those proteins which stimulate

vertebrate immunity may, if characterised and appropriately formulated, support viable immunisation regimes. The ability to extract and isolate proteins specific to the cell surface, is especially useful in this respect. Cell surface proteins, as a consequence of their exposed location, constantly interact with the external environment which, for a vertebrate pathogen, will often include elements of the mammalian immune system.

This investigation, utilising a combination of protocols adapted from those used upon ascomycetous fungi, successfully demonstrates cell surface-protein isolation from the far broader pool of total *C. neoformans* proteinaceous material. Using such a novel approach, two proteins were purified in significant quantities and sized. Both proteins, one of around 57 kDa and the other of 42 kDa, have yet to be further characterised, however such an aspiration is achievable when proteomics information for the identification of *C. neoformans* protein isolates becomes available.

Using differing isolation techniques, other researchers have succeeded in isolating proteins from *C. neoformans*. The information gained from their work may be of use in examining the possible makeup and role of the 57 and 47 kDa proteins isolated. Their published techniques examined isolated proteins from culture supernatant (Pietrella *et al.*, 2001), material that underwent post-homogenisation separation into cell wall and cytoplasmic proteins (Pitzurra *et al.*, 1997; Mody *et al.*, 1999), or both (Hamilton *et al.*, 1992). When the work described here is compared to these published techniques, it appears to be both more elegant and possibly more focussed, thereby ensuring greater selectivity in cell surface protein isolation.

Of the work performed with *C. neoformans* by other researchers, three reports are of particular interest (Mody *et al.*, 1996; Pitzurra *et al.*, 1997; Pietrella *et al.*, 2001). Pitzurra *et al.* (1997) used similar techniques to those reported here, however, instead of isolation based on the surface tagging of whole cells, they isolated proteins – in the form of glycoprotein – post-homogenisation of cell wall material, and then used a Con A based approach for purification. In comparison to the protocols reported here, those employed by Pitzurra *et al.* (1997) appear less definitive and therefore more likely to isolate proteins other than cell wall surface proteins. Irrespective of such concerns, they fully separated and isolated a Con A binding protein 105 kDa in size and concluded it was a form of *C. neoformans* mannoprotein. In contrast to the technique developed here, the technique employed by Pitzurra *et al.* (1997) appears likely to lead to the isolation of proteins buried deep within the cell wall. The 105 kDa mannoprotein isolated could therefore be that observed by Vartivarian *et al.* (1989),

which is known to occur in greatest amounts within cryptococcal cell walls and is not expressed upon cell wall surfaces. Regardless of this, the work of Pitzurra *et al.* (1997) is still of contrasting value as they also investigated the influence of encapsulation and noted that acapsulate strains of *C. neoformans* furnished greater amounts of protein. Such an observation suggests that capsular absence, or poor capsular representation, may actually increase the levels of mannoprotein present in the cell wall. This does not equate, and even contrasts, with the exhaustive flow cytometry work performed here, in which acapsulate cells were found to express less protein than encapsulated counterparts. Such a disparity potentially confirms that sulfo-NHS-biotin is incapable of penetrating the cell wall. If correct, the use of sulfo-NHS-biotin on acapsulate cells would result in only the selective tagging of proteins that are exclusively cell surface in nature. In addition, the work of Pitzurra *et al.* (1997) indicates that Con A can be used to isolate glycoproteins from mixed protein samples. This concept could be used to further refine the technique described herein, thus leading to the isolation of cell wall surface glycoproteins.

A second group of researchers, Pietrella *et al.* (2001), attempted to isolate proteins from culture supernatants. The proteins Pietrella *et al.* (2001) isolated are unlikely to be the same as those obtained here, this is primarily due to the differing protocols employed, and an obvious mismatch in protein size. Their characterisation identified one protein of 8.2 kDa, and a second of 35.6 kDa. Although unlikely, the larger of the two proteins isolated may be a modified form of, or related to, the 42 kDa protein obtained in this study. Furthermore, it is possible that proteins expressed on the cell surface may be modified and released into the capsule, resulting in proteins of varying size to those isolated on the cell wall surface. Though further work is required to confirm such a supposition, if correct, the work performed here may have isolated proteins pre-modification; another potentially unique aspect of this work with possible vertebrate immunity implications.

Potentially of greater interest are the techniques used by Mody *et al.* (1996) to extract proteins from *C. neoformans* cells. Mody *et al.* (1996) were initially unable to obtain adequate visualisation of proteins isolated from cell walls. However, when they attempted to visualise proteinaceous material using [^{35}S]-methionine labelling – instead of both Coomassie blue R250 and silver staining techniques – they were able to confirm the isolation of 18 or so unique cell wall proteins sized between > 200 and 14 kDa, some of which bound Con A. Of the proteins isolated, a number were sized

between protein markers at 45 and 66.2 kDa. Mody *et al.* (1996) attributed this disparity between staining methods to the inability of both Coomassie blue R250 and silver stains to adequately visualise highly glycosylated proteins. In turn, the relatively few proteins isolated here through the sulfo-NHS-biotin study, as compared to a similar study using *C. albicans* (Urban *et al.*, 2003), may not be due to either a lack of proteins, or indeed, poor extraction, but instead a consequence of poor Coomassie blue R250 sensitivity for *C. neoformans* cell surface proteins.

In their study, Mody *et al.* (1996) did not investigate extracted cell wall proteins separately; instead they analysed the entire protein fraction to assess its lymphoproliferative affect. This fraction produced a proliferative affect on peripheral blood mononuclear cells (PMBC), a result not found with the mannoprotein documented by Reiss (1993), indicating that the most common reported form of mannoprotein, that found in culture supernatants, was not responsible. The work of Mody *et al.* (1996) indicates that at least one protein isolated from *C. neoformans* cell walls has a lymphoproliferative affect and therefore warrants greater analysis. This further analysis may be best performed using the technique developed here, as it may be possible to isolate and characterise individual proteins or specific protein combinations responsible for this proliferative affect, only however, if the relevant proteins are located on the cell surface. If possible, visualisation of proteins in gels may be best performed using the [^{35}S]-methionine labelling technique as performed by Mody *et al.* (1996).

Despite a lack of more complete evaluation and therefore identification, the proteins isolated here are unique as this method greatly improves the confidence that isolated material is not only proteinaceous, but crucially, originates from the cell wall surface and not the capsule or cytoplasm. Evidence has also been presented which indicates that this method excludes proteins localised within the cell wall interior, thus adding further useful specificity. When complete and widely available, *C. neoformans* proteomics information could be used, along with this technique, to further identify cell wall surface proteins, along with the factors which influence their appearance, thus potentially enhancing our insight into various cell characteristics. Example characteristics, known to be mediated by microbial proteins in other microorganisms, include: hydrophobicity (Rosenberg & Doyle, 1990; Doyle, 2000), cell surface charge (James, 1991), adherence (Kanbe & Cutler, 1998) and molecular mimicry (Mims *et al.*, 1995).

6.4.4 Conclusion

Proteins on the cell surface and capsule of *C. neoformans* have been subjected to controlled biotinylation, observation *in situ*, extraction, isolation and purification. Biotinylated proteins were found to be expressed in greatest amounts amongst encapsulated cells, probably a result of considerable protein presence within the capsular matrix. Proteins that were observed on cell surfaces, although ubiquitous, appeared more concentrated and localised to areas where carbohydrate has also been detected in marked amounts. Such an occurrence is indicative of glycoprotein presence.

In addition, a unique technique involving biotinylation of cell surface proteins on acapsulate cells, followed by their extraction and purification, led to the successful isolation of two previously undocumented and distinct proteins. Such success is a potential stimulant for enhancement and refinement of the protocols outlined, allowing for further analysis of cell surface proteins and their interaction with both the vertebrate immune system and with greater and lesser elements of prevailing microbial environments.

CHAPTER 7 – Summary and conclusions

The purpose of this investigation was to increase our knowledge of *C. neoformans* cell surfaces. In particular surface characteristics which might influence the interface between *C. neoformans* cells and their immediate external environment were investigated. Various strains, both encapsulated and acapsulate were examined with specific focus, not upon the extensively studied capsule alone, but rather the cell surface and the capsular-cell surface interface. The presence and absence of capsular material has therefore been carefully considered and contrasted, thus highlighting any differences or variation between strains resulting from capsule production and presence.

In order to ensure full confidence in the work described in subsequent chapters, an initial and careful evaluation was executed to identify and characterise the various *C. neoformans* isolates acquired for experimental investigation. The importance of such work is not to be underestimated as such an approach ensures a robust understanding of both the relationship and variation between differing experimental strains, a fundamental requirement when interpreting, comparing and contrasting results. Detailed study of such a nature demonstrated marked differences between strains with regards to capsule production and flocculatory behaviour. Also of some consequence was the evaluation of individual strain growth over time, which characterised actively growing and stationary phases of growth; knowledge extensively drawn upon in later work.

Interaction with, and subsequent adhesion of cells in a liquid medium and juxtaposed surfaces, is influenced by non-specific phenomena (James, 1991; Marshall, 1991; Van Der Mei *et al.*, 1991). Investigation of cell surface phenomena therefore commenced with examination of cryptococcal cell surface charge and hydrophobicity, both of which have previously received some attention (Kozel, 1983; Nosanchuk & Casadevall, 1997). Any repetition in such an undertaking was justified by ensuring more extensive analysis utilising modern equipment and techniques.

C. albicans yeast manifesting a weak negative charge have been shown to exhibit greater adhesive potential (Klotz *et al.*, 1985). Researchers have postulated that such a weak negative charge may dampen the repellent effect which occurs when two negatively charged entities are placed in close proximity, thus facilitating contact

with, and colonisation of, host tissues. Viewed from another perspective however, a weak negative charge is postulated to enhance contact with, and phagocytosis by, elements of the vertebrate immune system (Nosanchuk & Casadevall, 1997), and possibly protozoa of the environment at large. Results presented from this current investigation, in accordance with previous studies (Nosanchuk & Casadevall, 1997), found that encapsulated *C. neoformans* cells maintain a relatively strong negative charge, thus indicating an 'aloof' or repellent quality amongst such cells. Contrastingly, acapsulate cells manifest a more neutral charge, possibly indicative of an organism better adapted for host tissue colonisation, should speculation associated with other microbes be valid.

Hydrophobicity, in a similar manner to cell surface charge, is considered an integral component of microbial adherence and adhesion. Supposition holds that hydrophobic organisms in a water based environment mediate the removal of water layers between themselves and other hydrophobic entities, thus encouraging contact and potentially subsequent colonisation, of host tissues (Rosenberg & Doyle, 1990; Van Der Mei *et al.*, 1991). However, evidence suggests that relatively hydrophobic microorganisms exhibit an increased likelihood of destruction by predator entities (Absolom, 1986). *C. neoformans* cells were found to be hydrophilic in nature when examined. This hydrophilicity occurred regardless of capsule status, growth phase and the varying analysis techniques employed, and may indicate a survival-enhancing avoidance-centred bias as this hydrophilic characteristic of such cells does not lend them to ready contact with phagocytic entities.

Reports indicate that *C. neoformans* yeast are capable of adhering to host tissues (Jimenez-Lucho *et al.*, 1990; Ibrahim *et al.*, 1995), and yet the non-specific forces examined here appear less than ideal for this purpose, thereby suggesting further research into the interaction of *C. neoformans* yeast cells and host tissues is necessary to identify any further mechanisms which may have a role. In addition to non-specific phenomena, expression of particular cell surface moieties by an organism can support specific recognition forces that are known to mediate the extent and form of not only microbial contact, but also other functions which may enhance both colonisation and growth within a host organism. Various different entities, isolated from cell walls of microorganisms, have been found to underpin processes such as adhesion and nutrient uptake (Braun, 1999; Ngondi-Ekome *et al.*, 2003). Analysis of the relevant literature found that, whilst examination of *C. neoformans* capsule and

cell wall makeup has been performed, more specific examination and analysis of moieties from the cell wall surface alone was poorly reported. In turn, novel and detailed study of carbohydrate and protein moieties associated with the cryptococcal cell wall surface and capsule, was deemed appropriate and therefore carried out.

Of interest to researchers examining a pathogens interaction with its proximal environment is the production of glycosylated structures. This current investigation successfully employed a technique, utilising biotin hydrazide in conjunction with fluorescein-streptavidin, to characterise the presence of carbohydrates which might part constitute glycosylated structures. Such characterisation of carbohydrate exposure on the cell wall surface of *C. neoformans* was particularly successful as carbohydrates revealed by this approach were not only quantified using flow cytometry, but also visualised using confocal microscopy. Carbohydrate was found to be present in marked amounts amongst strains analysed. Senescent cells were found to expose greater amounts of receptive carbohydrate than actively growing cells. In addition, complex saturation binding analyses allowed comparison between encapsulated and capsule free yeast which indicated little variation amongst mature stationary cells, yet interestingly, substantially less fluorescence was observed amongst logarithmic encapsulated cells when compared to acapsulate cells of the same age and phase of growth. Arguably more dramatic were confocal images of biotin hydrazide / fluorescein-streptavidin binding that revealed fluorescein tagged moieties concentrated around mother and daughter cell interfaces.

Investigation of carbohydrate moieties was further developed and enhanced through use of FITC-lectin compounds capable of binding and therefore identifying specific carbohydrate moieties. This detailed study has since been published (see Appendix H, Foster *et al.*, 2004). *C. neoformans* yeast cells were exposed to lectins of known affinity, thereby giving a more defined view of surface carbohydrate – and potentially of glycosylated structures – exposed on cryptococcal cell surfaces. Comparison of the relative carbohydrate avidity for a panel of eight lectins, indicated exposure of considerable amounts of glucose, mannose and N-acetyl-glucosamine on yeast cell wall surfaces of four strains of *C. neoformans*. A relative absence of fluorescence emitted in conjunction with the use of several panel lectins indicated that little if any N-acetyl-galactosamine, galactose and α-fucose were present on experimental strains. Data received in this initial experimentation indicated that a

more detailed examination of mannose / glucose and N-acetyl-glucosamine cell surface exposure would be beneficial, as a result Con A and WGA associated experimentation was extended. In turn, saturation binding analysis investigations along with hapten interference analysis supported a greatly enhanced understanding of mannose / glucose and N-acetyl-glucosamine moiety exposure. Such an approach facilitates examination and comparison of lectin binding characteristics amongst encapsulated and acapsulate cryptococcal cells as well as microorganisms of other species and potentially genera. Tunicamycin interference analysis was also employed to further broaden understanding as to any proteinaceous nature of lectin bound moieties. As a result of using these techniques, variation in Con A and WGA binding between strains, hence surface carbohydrate exposure, was highlighted.

Analysis of FITC-lectin Con A binding revealed some differences amongst experimental strains, possibly due to capsule presence, as strains which manifest a severely reduced capsule, or are acapsulate in form, had greater predicted maximal fluorescence than strains which manifest a capsule as visualised by India ink staining. Capsule material may therefore affect either the number of moieties available to FITC-lectin Con A, their orientation, or possibly ease of Con A access to exposed moieties. Con A saturation binding analysis also revealed that binding of Con A to mannose / glucose moieties – although varying amongst strains – in general occurred in a cooperative fashion. Hapten interference analysis clearly showed the specificity of Con A avidity for proven and known carbohydrate residues, and therefore legitimised the extensive lectin saturation binding analysis. Tunicamycin, which potentially interferes with the production of glycoproteins (Chaffin, 1985), was therefore employed to examine glycoprotein production and subsequent exposure on *C. neoformans* cell surfaces. In turn, exposure of cryptococcal cells to tunicamycin indicated that Con A bound material, if glycoprotein based, does not appear to turnover at a high rate, and therefore may be produced early in cell wall development and remain present throughout the life of the cell.

Investigation and evaluation of Con A binding was further achieved by confocal microscopy and associated digital imaging which, in turn allowed visualisation of fluorescence patterns and localisation. Although diffuse, cell surface fluorescence was concentrated, in much the same way as for biotin hydrazide tagging, around areas associated with bud scars, buds and nascent cells. This localised binding may suggest that mannose / glucose moiety exposure is linked to the process of

asexual reproduction. This could be as a result of physical trauma exposing normally shielded moieties during production of daughter cells and *de novo* cell wall synthesis; however, such exposure may instead fulfil a specific albeit unknown function. Interestingly, images of heavily encapsulated cells manifest Con A mediated fluorescence emanating from capsular material. This capsule-based fluorescence is likely a result of Con A binding mannose residues of cryptococcal mannoprotein, a Con A binding glycoprotein liberated by *C. neoformans* into capsular material (Cherniak & Sundstrom, 1994).

Lectin WGA binds N-acetyl-glucosamine and was examined in the same detailed manner as for Con A. Saturation binding analysis revealed that, relative to Con A, WGA bound *C. neoformans* yeast cells with great avidity, yet fewer N-acetyl-glucosamine moieties were in general exposed. WGA binding was found to be variable between strains; however, cells lacking capsular material gave the highest predicted maximal fluorescence. In addition, stationary phase cells were found to fluoresce more than actively growing logarithmic phase cells, indicating mature cells expose greater amounts of N-acetyl-glucosamine. Hapten interference analysis, although showing fluorescence associated with non-specific binding, also demonstrated marked amounts of specific binding. Similarly to Con A, use of tunicamycin indicated that WGA associated carbohydrate moieties, if glycoprotein associated, do not turnover at a high rate, as only a slight reduction in fluorescence occurred in test populations after tunicamycin exposure. It is likely that WGA bound moieties may not be components of glycoproteins as N-acetyl-glucosamine is generally associated with the polysaccharide chitin, a structural component of many fungal cell walls (Gooday, 1995). Confocal microscopy of WGA bound cells, in common with that of Con A binding, revealed greater levels of fluorescence associated with bud scars, budding collars and nascent cells. Capsular material was found not to fluoresce, indicating, along with previous studies (reviewed in Casadevall & Perfect, 1998), that the capsule of *C. neoformans* lacks N-acetyl-glucosamine.

The quantifiable nature of flow cytometry and the standardised properties of saturation binding analysis, allows valid comparison between the above described lectin binding of *C. neoformans* and that confirmed by Smith *et al.* (2001) amongst *C. albicans* yeast cells. Comparison of this nature suggests that markedly fewer exposed carbohydrate moieties are found on *C. neoformans* yeast cells. *C. albicans* yeast are reported to expose mannose based moieties associated with adhesion (Korting &

Ollert, 1994; Kanbe & Cutler, 1998). Further observation and measurement of mannose exposure on *C. neoformans* yeast cells may indicate a similar function amongst cryptococcal mannose residues; however, flow cytometry work suggests exposure of cryptococcal mannose occurs in a much attenuated manner potentially influencing adherence and adhesion efficacy. This contrastingly weak exposure of carbohydrates may be beneficial, for instance it is likely that such reduced exposure of mannose may in turn reduce the likelihood of opsonophagocytosis. Regardless of the lack of carbohydrate recorded, the concentrated nature of that which was observed, may indicate a localised adhesive function, this may act to enhance colonisation, or alternatively, may have a use in protection of daughter cells.

Although tunicamycin investigations were somewhat inconclusive, cryptococcal cell surface carbohydrates may potentially be associated with protein. The presence of proteinaceous material was therefore examined using a third fluorescein based tagging technique. This technique utilised sulfo-NHS-biotin and fluorescein-streptavidin, employed in a controlled manner, to bind cryptococcal proteinaceous material in a similar fashion as biotin hydrazide was used to bind carbohydrates. Results from this novel work confirmed that substantial levels of protein were exposed on yeast cell wall surfaces of both encapsulated and acapsulate strains of *C. neoformans*. In addition, encapsulated cells were found to expose greatest amounts of protein. Confocal microscopy confirmed that fluorescence emanating from within capsular material was the likely cause of such enhanced fluorescence, which in turn may be associated with mannoprotein (Casadevall & Perfect, 1998) or indeed other secreted proteins such as urease and phospholipase (Cox *et al.*, 2000; Cox *et al.*, 2001). Yeast cells from each of the strains examined exhibited ubiquitous and diffuse fluorescence, however, intense patches of fluorescence were observed in areas where carbohydrate-based fluorescence was also particularly marked, i.e. bud scars and nascent cells. Such co-localisation of carbohydrate – including mannose – and protein is strongly suggestive of glycoprotein presence and exposure. In addition, it suggests that carbohydrate moieties are not revealed simply as a result of the mechanical trauma of daughter cell production, but instead observed exposure may have a more complex basis dynamically associated with active cell wall and capsule synthesis, during which moieties may be obscured or revealed in response to internal and external variables.

The presence of proteinaceous material on the cell wall surface of cryptococcal cells encouraged a further extension of the sulfo-NHS-biotin biotinylation studies, which culminated in the extraction and preliminary characterisation of individual cell surface localised proteins. This novel approach employed isolated proteins from the cell wall surface of *C. neoformans* by tagging intact whole cells with sulfo-NHS-biotin, followed by the extraction of all cellular proteinaceous material. Biotinylated proteins were then isolated from this crude extract and purified using affinity chromatography. Two protein moieties, successfully extracted and purified from the cell wall surface of an acapsulate *C. neoformans* yeast, warranted more detailed characterisation to elucidate their molecular weight and potentially merit even further evaluation as to their structure and function. Cell surface proteins – particularly glycoproteins – may have several specific roles, examples include mediating hydrophobicity, playing a structural role (Gooday, 1995), facilitating adhesion to host tissues and medical implants (Korting & Ollert, 1994; Kanbe & Cutler, 1998; Brandhorst *et al.*, 1999), evading the vertebrate immune system (Mims *et al.*, 1995), and regulating ingress of amino acids (Braun, 1999). Any of these roles may contribute to survival of an organism within a vertebrate host; many may also be beneficial in any greater environment from which *C. neoformans* may be isolated. In addition, isolated cell surface proteinaceous material may also have a therapeutic worth.

In conclusion, an extensive and detailed analysis of the cell wall surface and capsule characteristics of *C. neoformans* yeast has been carried out. This in-depth investigation used a selection of technologically advanced equipment and techniques to quantify non-specific cell based characteristics. In addition, novel applications of robust techniques allowed examination of cell surface carbohydrate exposure. The use of varying cryptococcal strains allowed, by comparison and contrast, an evaluation of capsular presence on surface moiety exposure. Exploitation of procedures with inherently absolute qualities, allowed convincing comparison between species of fungi, and particularly yeast. In further studies, protein exposure on cryptococcal surfaces was also examined producing evidence which suggests that carbohydrate associated with bud scars, buds and nascent cells may be in close and intimate contact with proteinaceous material from the same locality, possibly in the form of glycoprotein. Extension of cryptococcal protein evaluation procedures, facilitated the extraction, isolation and purification of at least two cell wall surface protein moieties,

thereby demonstrating a course for future yeast cell surface protein investigation. Furthermore, such a thorough and meticulous examination of the *C. neoformans* yeast cell wall surface adds much new information to this relatively understudied, yet quintessential aspect of microbiology. In addition, although extensively studied, understanding of the capsule of *C. neoformans* has also been enriched.

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APPENDICES

Appendix A: Canavanine-glycine-bromthymol blue (CGB) agar

Solution A (temperature sensitive do not autoclave):

10 g glycine.

1 g potassium dihydrogen phosphate (KH_2PO_4).

1 g magnesium sulphate.

1 mg thiamine-HCl.

100 ml distilled water.

Adjust pH to 5.6 with NaOH / HCl and filter sterilise through a 0.22 μm filter (Millipore Corporation, Watford, UK).

Solution B:

0.4 g bromthymol blue.

64 ml 0.01 N NaOH.

36 ml distilled water.

One litre of medium was prepared as follows: 20 ml Solution B and 20 g agar (Oxoid, Basingstoke, UK) was added to 880 ml distilled water and autoclaved. Immediately before pouring, 100 ml of Solution A was added to the mixture aseptically. A positive test (indicating variant *gattii*) was recorded if the agar changed from greenish yellow to blue after 48 hours growth at 30 °C.

Appendix B: Phosphate urea magnesium (PUM) buffer

PUM buffer was prepared with the following ingredients:

127 mM dipotassium hydrogen phosphate (K_2HPO_4).
53 mM potassium dihydrogen phosphate (KH_2PO_4).
30 mM urea.
0.8 mM magnesium sulphate (heptahydrate).

PUM buffer was made up with distilled water and was filter sterilised through a 0.22 μm filter (Millipore Corporation, Watford, UK).

Appendix C: HEPES / NaCl buffer

10 mM HEPES / 0.15 M NaCl Buffer was prepared with the following ingredients:

2.38 g HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid).
8.77 g NaCl.
Made up to 1 L with distilled water.

HEPES / NaCl buffer was adjusted to a pH of 7.5 and filter sterilised through a 0.22 μm filter (Millipore Corporation, Watford, UK).

Supplemented HEPES / NaCl buffer was prepared exactly as HEPES buffer and supplemented with 0.1 mM $CaCl_2$ and 0.01 mM $MnCl_2$ before filter sterilising.

Appendix D: MercPMSF

MercPMSF was prepared with the following ingredients:

0.6 % 2-mercaptoethanol.
0.3 mM phenylmethylsulphonyl-fluoride (PMSF).
5 $\mu l\ ml^{-1}$ fungal protease inhibitor.

MercPMSF must be made fresh immediately before use.

Appendix E: Phosphate+ buffer

Phosphate+ buffer was prepared with the following ingredients:

10 mM phosphate buffer pH 7.4 (see below).
1 mM calcium chloride.
1 mM magnesium chloride.
3 mM phenylmethylsulphonyl fluoride.
200 mM N-octylglucoside.

Phosphate buffer (pH 7.4) is prepared by mixing 19 ml of 0.02 M monobasic sodium phosphate with 81 ml of 0.02 M dibasic sodium phosphate, this mixture is then adjusted to pH 7.4 with approximately 200 ml of distilled water.

Appendix F: Coomassie blue R250 staining

Coomassie Blue R250 stain was prepared with the following ingredients:

20 % v/v methanol.
10 % v/v acetic acid.
0.1 % w/v Coomassie blue.

Coomassie Blue R250 destain was prepared with the following ingredients:

20 % v/v methanol.
10 % v/v acetic acid.

Immediately after electrophoresis gels were placed in Coomassie blue stain for 45 minutes with agitation, stain was then discarded and replaced with destain for one hour with agitation. Destain was removed and replaced repeatedly until it remained clear.

Appendix G: Chemicals, materials and equipment

Listed below are details of chemicals, materials and equipment not fully described within the text:

Equipment:

Balances : Sartorius U4800P (Sartorius Instruments, Epsom, UK).
Sartorius 1702 (Sartorius Instruments, Epsom, UK).

Centrifuges: Sigma 3KLS (Sciquip, Appelby Magna, UK).
Beckman J221 (Beckman Coulter, High Wycombe, UK).
Eppendorf 5415C (Eppendorf, Cambridge, UK).

Incubators: Heraeus B6120 (30 °C) (Kendro Laboratory Products, Bishop's Stortford, UK)
JeioTech BS-20 (37 °C) (Lab Companion, Chalgrove, UK).

Microscope: Wild M-20 (Wild, Heerbrugg, Switzerland).

Ph Meter: Corning 240 (Corning B.V., Schiphol-Rijk, The Netherlands).

Vortex: Whirlimixer UM/250/F (Fisons, Leicester, UK).

Chemicals and other materials:

All chemicals and materials used were standard laboratory grade, those not already referenced in the text, unless listed below, were obtained from Sigma-Aldrich (Poole, UK).

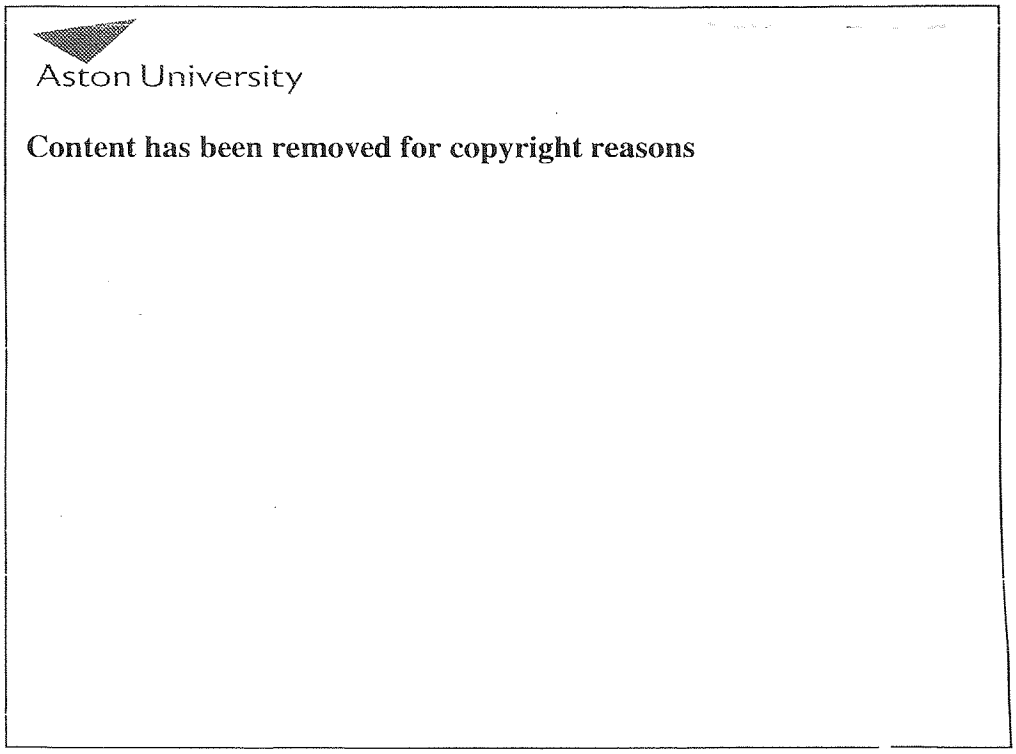
Item	Manufacturer / Supplier
Bromthymol blue	Fisons
Calcium chloride (dihydrate)	BDH
Dipotassium hydrogen phosphate (K_2HPO_4).	Fisher Scientific
Glycerol	Fisher Scientific
Hydrochloric acid (HCl)	Fisher Scientific
India ink	Fisher Scientific
Potassium chloride (KCl)	Fisons
Potassium dihydrogen phosphate (KH_2PO_4).	Fisher Scientific
Potassium hydroxide (KOH)	BDH
Sodium chloride (NaCl)	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Fisher Scientific
Sodium hydroxide (NaOH)	BDH
Sodium periodate ($NaIO_4$)	BDH
Urea	Fisons

Manufacturer / Supplier details:

Fisons, Ipswich, UK.
Fisher Scientific, Pittsburgh, USA.
BDH (VWR International), Poole, UK.

Appendix H: Publications

Foster, A. J., R. A. Bird, S. L. Kelly, K. Nishimura, D. Poyner, S. Taylor, and S. N. Smith. (2004). FITC-lectin avidity of *Cryptococcus neoformans* cell wall and capsular components. Mycologia **96**: 1-8.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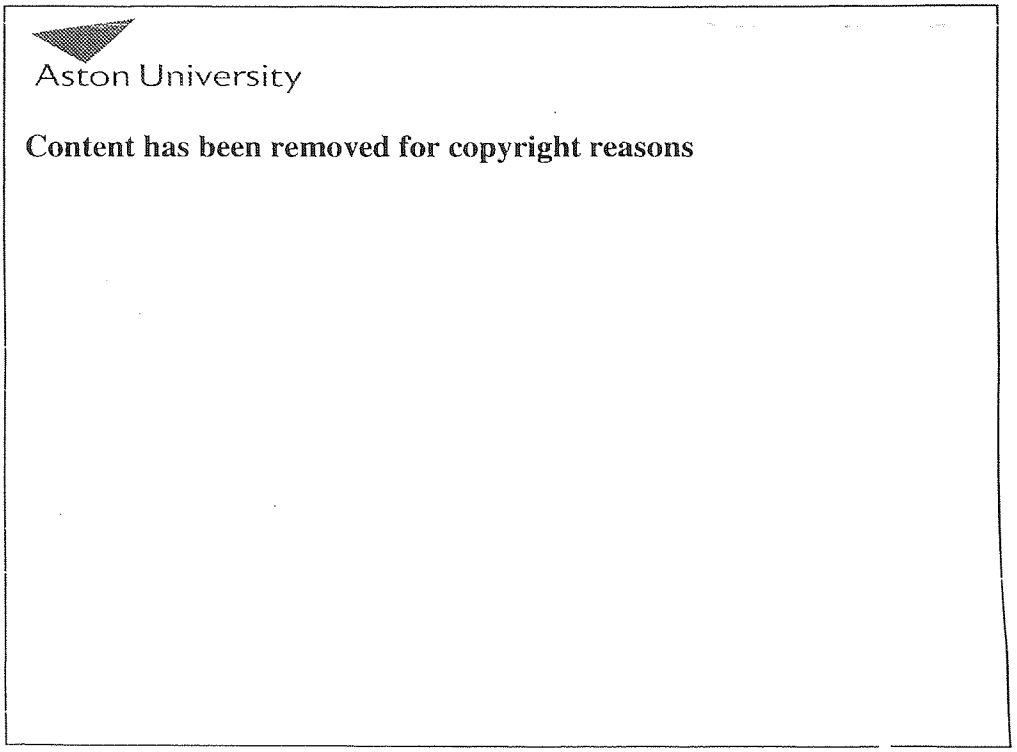
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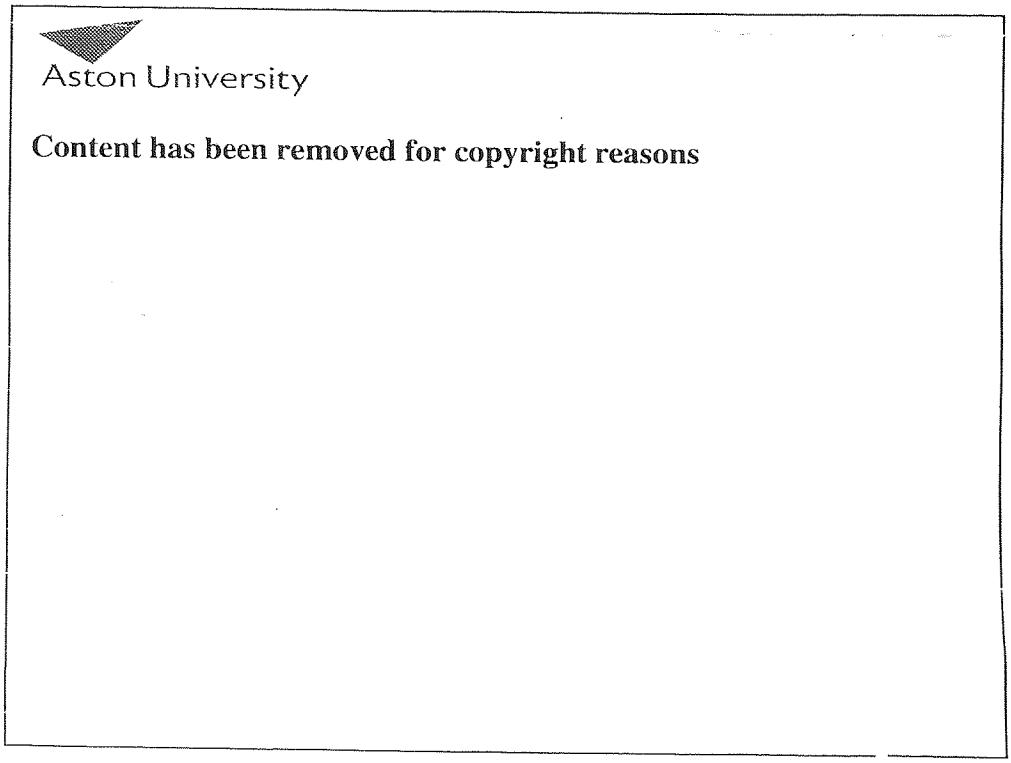
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