INFLUENCE OF IRON DEPRIVATION AND SUB-INHIBITORY CONCENTRATIONS OF ANTIFUNGAL ANTIBIOTICS ON SURFACE ANTIGENS OF *CANDIDA ALBICANS* YEAST CELLS

BY

TERRY RAYMOND PAUL
DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF ASTON IN BIRMINGHAM
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Influence of iron deprivation and sub-inhibitory concentrations of antifungal antibiotics on surface antigens of *Candida albicans* yeast cells

by

Terry Raymond Paul

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Summary

This study examined the effect of iron deprivation and sub-inhibitory concentrations of antifungal agents on yeast cell surface antigen recognition by antibodies from patients with *Candida* infections.

Separation of cell wall surface proteins by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and immunological detection by immunoblotting, revealed that antigenic profiles of yeasts were profoundly influenced by the growth environment. Cells grown under iron-depleted conditions expressed several iron-regulated proteins that were recognized by antibodies from patient sera. An attempt to characterize these proteins by lectin blotting with concanavalin A revealed that some could be glycoprotein in nature. Furthermore, these proteins which were located within cell walls and on yeast surfaces, were barely or not expressed in yeasts cultivated under iron-sufficient conditions. The magnitude and heterogeneity of human antibody responses to these iron-regulated proteins were dependent on the type of *Candida* infection, serum antibody class and yeast strain. Hydroxamate-type siderophores were also detected in supernatants of iron-depleted yeast cultures. This evidence suggests that *Candida albicans* expresses iron-regulated proteins/glycoproteins *in vitro* which may play a role in siderophore-mediated iron uptake in *Candida albicans*.

Sequential monitoring of IgG antibodies directed against yeast surface antigens during immunization of rabbits revealed that different antigens were recognized particularly during early and later stages of immunization in iron-depleted cells compared to iron-sufficient cells.

In vitro and *in vivo* adherence studies demonstrated that growth phase, yeast strain and growth conditions affect adhesion mechanisms. In particular, growth under iron-depletion in the presence of sub-inhibitory concentrations of polyene and azole antifungals enhanced the hydrophobicity of *C.albicans*.

Growth conditions also influenced MICs of antifungals, notably that of ketoconazole. Sub-inhibitory concentrations of amphotericin B and fluconazole had little effect on surface antigens, whereas nystatin induced profound changes in surface antigens of yeast cells. The effects of such drug concentrations on yeast cells coupled with host defence mechanisms may have a significant affect on the course of *Candida* infections.

Key words: *Candida albicans*, iron, adherence, sub-inhibitory concentrations, surface antigens

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ABBREVIATIONS

Ab	antibody
BEC	buccal epithelial cell
C'	complement
CAS	chrome azurol S
CMC	chronic mucocutaneous candidosis
Cw	cell walls
Con A	concanavalin A
CSH	cell surface hydrophobicity
DMSO	dimethyl sulphoxide
EC	epithelial cell
EDTA	ethylenediamine tetra-acetic acid
HDTMA	hexadecyltrimethylammonium bromide
IF	indirect immunofluorescence
IRMP	iron-regulated membrane protein
Kd	kilodaltons
L-DOPA	3-(3,4-dihydroxyphenyl)-L-alanine
MAb	monoclonal antibody
MIC	minimum inhibitory concentration
MPO	myeloperoxidase
MW	molecular weight
Nc	nitrocellulose
NHS	normal human serum
OD	optical density
OP	1'10-phenanthroline
PM	plasma membrane
PMN	polymorphonuclear leukocytes
PMSF	phenylmethylsulphonyl fluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	tris (hydroxymethyl) amino ethane
TEM	transmission electron microscopy
VEC	vaginal epithelial cell
Wc	whole cells

PREFACE

It has been established that bacteria growing *in vivo*, during infection possess different characteristics from those organisms growing *in vitro* (Brown and Williams, 1985b; Dalhoff, 1985). Nutrient limitation, particularly iron, growth rate, biofilm formation and the presence of sub-inhibitory concentrations of antibiotics are factors known to alter the surface composition of bacteria, and consequently influence it's interaction with the host (Brown and Williams, 1985b).

Cell walls of *Candida albicans* are the site of initial contact between the organism and it's environment. The cell wall surface is important in the mediation of adherence in colonization and invasion of tissue and presentation of antigens to the host, however, surface characteristics remain largely undefined. In contrast to extensive studies concerning the role of iron in bacterial infections, little is known about the ability of *Candida* to sequester iron *in vivo*, nor about the effects that an iron-restricted environment may have on its surface characteristics and biological properties.

In an attempt to establish growth conditions *in vitro*, so as to mimic more closely those *in vivo*, this study looks at the effects of iron-deprivation on the surface composition and properties of *C.albicans* and relates the significance of these effects to the host-*Candida* interaction.

1. INTRODUCTION

1.1. Importance of *C.albicans* as a pathogen

1.1.1. Taxonomy

The treatise of Lodder and Kreger-van Rij (1952), and subsequently that of Lodder (1970) are generally accepted as the basis for the taxonomy of *C.albicans*. The genus *Candida* is characterized as including white asporogenous yeasts capable of forming pseudohyphae. These yeasts are frequently found classified among the 'Fungi Imperfecti'.

Until recently, there were few valuable criteria for distinguishing strains of *C.albicans*. Hasenclever and Mitchell (1961) demonstrated the existence of two serological groups of *C.albicans*, serotypes A and B. Within the genus, species are characterized primarily on colonial morphology, carbohydrate utilization, and fermentation patterns (Odds, 1979). Subsequently, Odds and Abbott (1980) reported a method of strain differentiation based on growth characteristics that are variable within the species. Although the system has yielded valuable information with regard to the epidemiology of candidosis, the method has not found widespread acceptance.

Serological methods have often been applied to the taxonomy of pathogenic yeasts (Jonssen, 1955), although they are still far from routine in yeast identification. However, such tests are currently receiving increasing attention (Taguchi *et al.*, 1979) in view of the potential value of monoclonal antibodies for identification and serotyping of *Candida* species (Polonelli and Morace, 1986). Five presumptive serotypes have been reported by

examining the pattern of cross-reaction of a single monoclonal antibody (Polonelli and Morace, 1986) and immunoblotting has been successfully used as a novel sensitive typing method for differentiating *Candida* isolates to allow early identification of an outbreak (Lee *et al.*, 1986).

Although it is known that the medically important *Candida* species may be divergent genetically (Meyer and Phaff, 1972), their identification on the basis of phenotypic properties is not always simple (Rippon, 1982). Several workers have explored methods based on genotypic properties by molecular probing with the *C.albicans* actin gene (Mason *et al.*, 1987; Alsina *et al.*, 1988). This molecular probe provided definitive identification of *Candida* species and suggested its potential use when standard methods do not allow a precise identification.

1.1.2. Morphology

C.albicans is often referred to as a "dimorphic fungus". However, this description oversimplifies the many subtle changes of morphology the fungus can undergo. *C.albicans* cells of several morphologies frequently coexist in any particular environment.

C.albicans yeast cells are ovoid to almost spherical, varying in size from 2.7 x 2μm to 10 x 6μm (Bedell *et al.*, 1980), and reproduce by budding. The daughter buds often remain attached to the mother cells until stationary phase, at which time cells accumulate as singlets (Soll and Bedell, 1978). Pseudohyphae are essentially elongated yeast cells

varying from 2 to 5μm in diameter and 26 to 30μm in length. They arise by an acropetal budding process (distinct from yeast cell budding) and remain unseparated, sometimes giving an appearance of a filamentous cell chain that is often narrow enough to be confused with a true hypha. True hyphae, which conversely elongate continuously and lay down septa at intervals behind the growing tip, are almost perfectly cylindrical cells.

Chlamydospores are thick-walled, highly refractile spheres approximating 10μm in diameter. They emerge from specialized "suspensor cells" which are elongated yeast cells that branch from hyphae or pseudohyphae (Szaniszlo *et al.*, 1983; Odds, 1985). Although yeast cells are the easiest form to grow *in vitro*, pseudohyphae often occur as minority components of yeast and hyphal cultures, and some true hyphae and pseudohyphae may appear in the same mycelium. In addition, hyphal cultures almost always ultimately generate some budding cells. Despite the coexistence of the different morphological forms, the strongest predilection of *C.albicans* is to grow in the yeast form, therefore, mycelia may be considered a transient form *in vitro* (Odds, 1985).

1.1.3. Epidemiology and infection

It is generally considered that *C.albicans* has a world wide distribution although some reports suggest that infection with *C.albicans* occurs more frequently in tropical climates (Hall and Larsh, 1985). *Candida* species are endogenous as normal flora on mucocutaneous regions and in alimentary tracts of humans and other animals (Do Carmo-Sousa, 1969). Yeasts have been recovered from the mouth, gastrointestinal tract and

vagina of humans; with the incidence of *C.albicans* increasing in patients (Table 1.1.).

Table 1.1. Recovery of *C.albicans* from patients and normal individuals.

Site of recovery	Description of subjects	% *C.albicans* recovered from total yeast flora*
Oral cavity	Normal	10.3
	Hospital patients	42.9
Anorectal tract	Normal	14.6
	Patients	22.0
Vagina	Normal	7.8
	Patients without vaginitis	14.9
	Patients with vaginitis	25.7

* Figures compiled from data cited by Odds (1979).

Candidosis is the most opportunistic fungal infection in humans (Odds, 1979; Rippon, 1982). Seven species, *C.albicans*, *C.guilliermondii*, *C.krusei*, *C.parapsilosis*, *C.pseudotropicalis*, *C.stellatoidea*, and *C.tropicalis*, as well as *Torulopsis glabrata*, are recognized as the principal medically important yeasts. The most common aetiological agent is *C.albicans*, however, *C.tropicalis* (Hurley and Winner, 1962) and *C.parapsilosis* (Winner and Hurley, 1964) have been incriminated in systemic candidosis. *C.albicans* is usually present as a harmless asymptomatic commensal but can be manifest as an opportunistic pathogen when the balance between host defences and yeast commensalism is breached. Predisposing factors to infection include mechanical trauma, hormonal changes, prolonged steroid therapy, and underlying immunologically compromised disease (Table 1.2.). Many tissues and organs may be involved in candidosis, thus, clinical manifestations are usually divided into two groups: superficial

and systemic candidosis.

Table 1.2. General classification of localized and systemic factors that predispose humans to candidosis.

Classification of predisposing factors	Explanation	Examples
Natural factors	1. Infectious, idiopathic congenital or other debilitating diseases and disorders.	Microbial infections, endocrine disorders, defects in cell-mediated immunity.
	2. Digressions from normal physiological status.	Pregnancy, infancy
Dietary factors	Excess or deficiency of foodstuffs that may alter the composition of the endogenous flora.	Carbohydrate-rich diets, vitamin deficiencies.
Mechanical factors	1. Trauma	Burns
	2. Local occlusions or maceration of tissues.	Wearing dentures, Thumb-sucking.
Iatrogenic medical factors	1. Treatment with drugs that alter the composition of the endogenous microbial flora or suppress host defences against infection	Antibiotics, cortico-steroids.
	2. Surgical procedures, or introduction of mechanical devices and prostheses.	Bowel resections, heart valve replace-ments, indwelling catheters.

1.1.3.1. *Superficial candidosis*

Superficial candidosis involves epithelial and cutaneous surfaces, in particular the respiratory, uro-genital, and gastrointestinal tracts. Although oral thrush is the best

known form of mouth infection mediated by *Candida*, it is also recognized that *Candida* is an important aetiological agent of denture stomatitis, some cases of angular cheilitis, leukoplakia, and possibly other types of oral disease. The incidence of oral thrush is no more than 5% among neonates in Britain and North America (Taschdjian and Kozinn, 1957; Kozinn *et al.*, 1958; Dunn, 1962), whilst *Candida*-associated denture stomatitis may be found in 60% of elderly denture wearers (Budtz-Jorgensen *et al.*, 1975).

Newborn infants are probably susceptible to *Candida* infection because of their immature immune system (Odds, 1979). Among adults, oral thrush may appear as a consequence of predisposition to infection in a number of clinical situations e.g. cancer or topical oropharyngeal therapy with steroids (Table 1.2.). Oropharyngeal thrush may sometimes spread to adjacent mucosa of the digestive and respiratory tracts, and is often associated with *Candida* infections of cutaneous sites.

Studies prior to 1930 suggested that vaginal thrush was an uncommon and mild infection (Winner and Hurley, 1964), however improved methods of identification for *Candida* have subsequently confirmed that vaginal thrush is a common occurrence. Although there is a marked variation in epidemiological data on vaginal thrush, studies have shown some consistency (Pumpianski and Sheskin, 1965; Jennison, 1966). These studies reveal that the incidence of vaginal thrush is about 20% among women attending venereal disease clinics in England and Wales, but an incidence rate for thrush of 4.4 to 5% is the consensus figure from studies of women outpatients between 1965 and 1973.

Pregnancy is a predisposing cause of vaginal candidosis. Vaginal carriage of yeasts is greater in pregnant than in non-pregnant women, and several studies demonstrate that the

carriage rate increases markedly in the third trimester of pregnancy, with an abrupt decline in the immediate post-partum period (Odds, 1979). Oral contraceptives containing oestrogen enhance the susceptibility of the vagina to yeast overgrowth, which may in turn lead to symptoms of vaginal candidosis (Lapan, 1970; Anyon *et al.*, 1971).

The natural distribution of *Candida* in the body suggests a predilection for moist habitats, hence any situation that involves occlusion or maceration of the skin or mucous surfaces, so as to raise the local humidity, may predispose to yeast overgrowth and infection. The occlusive effects on the vagina of nylon underwear and tights have been cited as factors that contribute to vaginal candidosis (Bull, 1969; Hurley, 1975). Treatment with broad spectrum or multiple narrow spectrum antibiotics have been considered to exacerbate vaginal thrush (McVay and Sprunt, 1951; Caruso, 1964), and to increase vaginal yeast carriage (Barlow and Chattaway, 1969; Davis, 1969). However, despite a strong consensus view in favour of antibiotic enhancement of yeast carriage, the relationship between antibiotic therapy and candidosis is far from proven unequivocally.

Diabetes mellitus predisposes to vaginal candidosis, but the mechanism by which diabetes increases host susceptibility to candidosis remains unclear. It has been proposed that high blood and enhanced tissue glucose levels (Knight and Fletcher, 1971), and the availability of vaginal glycogen (Ryley, 1986), may promote the growth of *Candida* in diabetics. Furthermore, it has been found that polymorphs from diabetics show reduced killing activity towards *C.albicans* (Raith *et al.*, 1983), suggesting impaired phagocytic function.

Little is known about the pathogenesis of recurrent and chronic vaginal candidosis, although several factors are recognized as predisposing to recurrent infection (Sobel, 1982). Nevertheless, most women with recurrent candidal vaginal infections have no identifiable risk factor (Sobel, 1985). It has been suggested that true vaginal relapse due to incomplete eradication of the initial vaginal infection appears to be responsible for a large percentage of recurrent episodes. However, the reason for the specific susceptibility of these women to recurrent symptomatic episodes of vaginitis is unclear. Vaginal reinfection also occurs and may be more important in women with less frequent recurrences (Sobel, 1986).

The majority of cutaneous *Candida* infections tend to arise in intertriginous areas and various factors have been implicated as predisposing to cases of cutaneous candidosis, including diabetes (Arao and Inoue, 1973) and corticosteroid therapy (Borman and Pittlekow, 1967). However, local occlusion and maceration are clearly the most significant and usually the only factors involved in the aetiology of disease.

Although *Candida* infections of the mouth or vagina may occasionally spread to involve skin adjacent to these sites, candidosis of mucous membranes and skin are essentially localized infections. However, chronic mucocutaneous candidosis (CMC) is the exception. CMC is a relatively uncommon manifestation of candidosis characterized by the development of chronic, often widespread, infections of skin, nails, and mucous membranes. Onset is usually in infancy or early childhood, potentially a wide range of underlying disorders and diseases can be associated with cases of CMC; including

idiopathic hypothyroidism and hypoadrenocortism, multiple endocrinopathies and diabetes mellitus (Odds, 1979). Genetic predisposition to CMC has been reported in a number of cases (Miller, 1946; Satenstein, 1946).

Deficient T-cell immunity has been implicated as the major host factor responsible for CMC (Kirkpatrick and Smith, 1974). A particularly interesting feature of CMC is that it rarely spreads to the gastrointestinal or respiratory epithelial surfaces, and has never been found to disseminate to deep organs, yet CMC patients possess defective cellular immunity to *Candida*.

1.1.3.2. *Systemic candidosis*

Systemic candidosis may arise as localized primary diseases, with a single organ affected, or as disseminated candidosis (septicaemia) in which *Candida* is spread via the bloodstream to invade multiple organs. A review of a series of autopsy and clinical studies performed between 1954 and 1978 (Bodey and Fainstein, 1985) (Table.1.3) clearly indicated that the frequency of disseminated candidosis has increased substantially during the past two decades.

Disseminated candidosis is primarily a disease of immunocompromised individuals, such as patients with acquired immunodeficiency syndrome (Mildvan *et al.*, 1982), and debilitated patients (Barrett *et al.*, 1957; Louria *et al.*, 1962; Dennis *et al.*, 1968; Pillay *et al.*, 1968; Triger *et al.*, 1981). It is especially common among patients with haematological malignancies and organ transplants. The majority of systemic infections

Table 1.3. Frequency of systemic candidosis from some autopsy and clinical studies*

Years	Underlying disease	Source	Total population	Percentage with candidosis
1954-1958	Acute leukaemia	Autopsy	157	7
1959-1964	" "	"	297	20
1957-1962	Leukaemia	Multiple	205	1
1959-1969	Burn wound	Blood	783	2
1970-1971	" "	"	284	14
1962-1965	Numerous	"	185	5
1962-1965	Renal transplant	Autopsy	51	24
1963-1974	Numerous	"	2616	1
1963-1970	"	"	2714	0.1
1971-1975	"	"	1325	1.2
1964-1971	Burn wound	Blood	427	5
1966-1972	Acute leukaemia	Multiple	494	7
1968-1970	" "	Autopsy	65	12
1972-1973	Cancer	Blood	364	7
1972-1973	"	Autopsy	88	18
1973-1976	Marrow transplant	Blood	60	66
1975-1977	Numerous	"	500	6
1977	"	"	2316	4.8
1977-1978	Haematological	"	89	21

* Table compiled from data cited by Bodey and Fainstein (1985).

represent disseminated candidosis. The infection may present itself as meningitis, endocarditis, pneumonia, arthritis, peritonitis, laryngitis, endophthalmitis, or urinary tract infections. Single organ infections occur infrequently and usually arises from haematogenous dissemination.

Several authors, including Stone *et al.* (1974) have indicated that the most important route by which candida septicaemia may arise is by persorption of yeasts from the gut into the bloodstream. It is probable that yeasts may also gain direct access to the bloodstream from exogenous sources. In particular, widespread use of in-dwelling intravascular catheters has led to a dramatic increase in the frequency of candida septicaemia (Curtis and Fowler-Bergfeld, 1969; Portnoy *et al.*, 1971; Hill *et al.*, 1974;

Bodey and Fainstein, 1985).

1.2. Cell wall composition and ultrastructure

Although different studies on cell wall (Cw) composition in *C.albicans* have revealed some quantitative variation in constituents, they agree qualitatively that the wall comprises mainly glucan (polymers of glucose) and mannan (polymers of mannose) with lesser amounts of chitin (polymers of acetyl glucosamine), protein and lipid (Kessler and Nickerson, 1959; Chattaway *et al.*, 1968; Odds, 1979).

1.2.1. Mannans

Several groups of investigators have analyzed the *C.albicans* mannan structure, and all have found that mannan is organized into an inner-chain, outer-chain, and base-labile oligomannosides (Fig.1.1.) (Bishop *et al.*, 1960; Sunayama and Suzuki, 1970; Phaff, 1971; Suzuki and Usuyama, 1971).

The mannan backbone is composed of α(1→6)-linked mannose residues with side branches 2-7 sugar residues in length. Antigenic specificity is determined by the outer-chain region (Sakaguchi *et al.*, 1967; Suzuki and Sunayama, 1969; Sunayama and Suzuki, 1970). The side chain mannose residues in the outer-chain region range from mannobiose to mannohexaose in serotype B and to mannoheptaose in serotype A, with some evidence for even longer oligomers in the latter (Bishop *et al.*, 1960; Suzuki and Sunayama, 1969). The predominant linkage in the side chains is α(1→2), but a small proportion of α(1→3)-linkages occur in the mannotetraose type A and in the

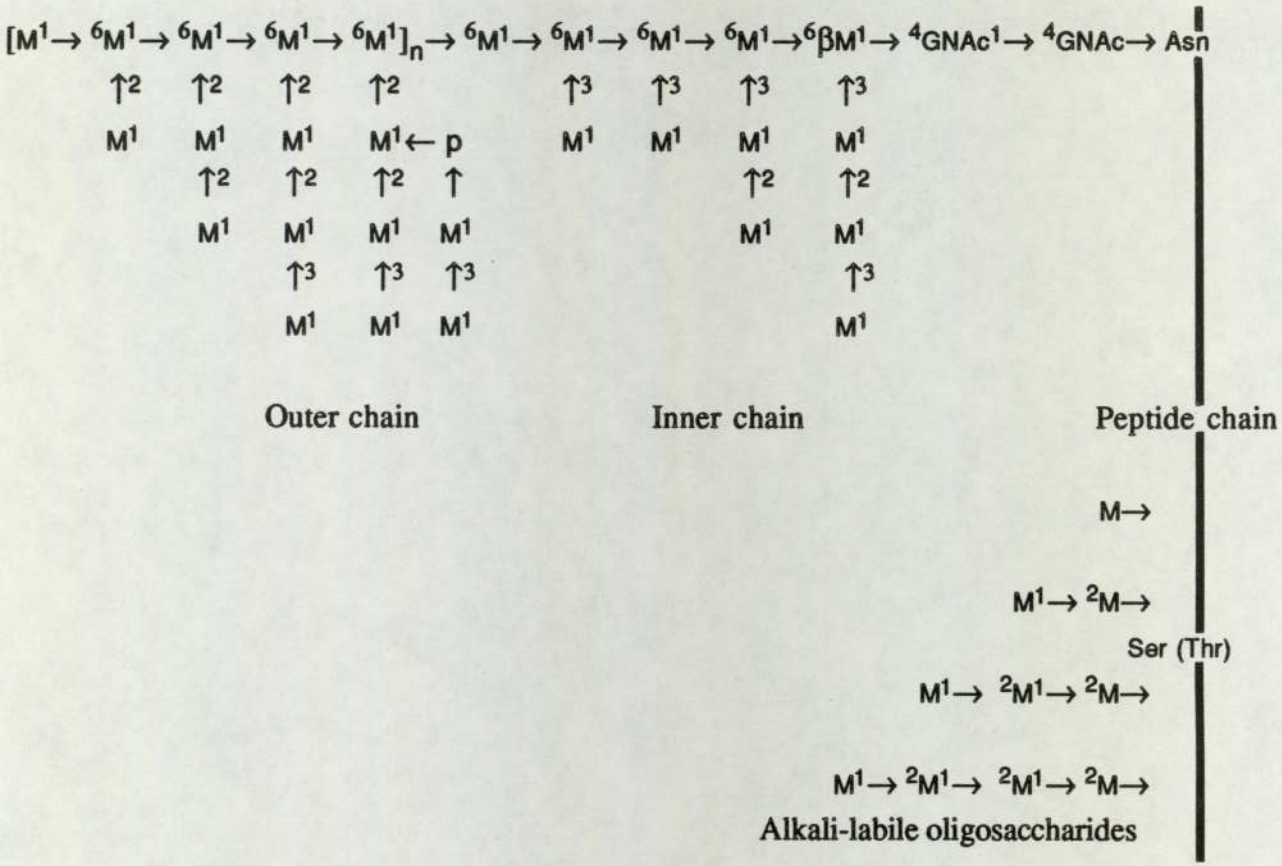


Fig. 1.1. Schematic representation of yeast cell wall mannan structure. M=mannose, GNAc=N-acetylglucosamine, Asn=asparagine, Ser=serine, Thr=threonine, and p=phosphodiester group. The first mannose residue attached to chitobiose is in the ß-configuration. All other mannose residues are in the α-configuration.

mannopentaose and mannohexaose of both serotypes in ratios of α(1→2)-linkages to α(1→3)-linkages of 2:1, 3:1, and 4:1, respectively (Suzuki and Sunayama, 1969; Sunayama and Suzuki, 1970). The inner-chain region connects the α(1→6) backbone to structural proteins through N,N'-diacetylchitobiosyl-asparagine glycopeptide bonds. Alkali-labile oligomannosides are joined directly to the structural protein via hydroxyamino acid ester bonds between mannose residues and serine or threonine of the peptide moiety (Farkas, 1979). Therefore, it appears that differences between mannans of *C.albicans* serotypes A and B reside in the degree of branching, the length of side chains and the proportion and position of α(1→3)-linkages in the side chains (Sunayama and Suzuki, 1970; Suzuki and Usuyama, 1971; Kogan *et al.*, 1988).

The method of preparation influences the molecular weight (MW) of mannan (Peat *et al.*, 1961; Nakajima and Ballou, 1974). Mannan isolated by borate-acetyl trimethyl-ammonium bromide complexation, avoiding high pH, has a MW of 133Kd, while further exposure of this mannan to dilute alkali reduces the size to 40Kd.

1.2.2. Glucans

Glucans are considered to be major constituents of the Cw microfibrillar structure (Farkas, 1979). In *C.albicans* they consist of long chains of β(1→6)-linked glucopyranose residues with β(1→3)-linked side chains (Bishop *et al.*, 1960; Yu *et al.*, 1967). As the linear sequences develop, the β(1→3)-linkages of glucan associate to form fibrils that increase the strength of the wall. Linkages between glucan and protein or between mannan and glucan in the intact wall have been investigated. Kessler and

Nickerson (1959) revealed their association with proteins in an alkali-insoluble fraction and with glucomannoproteins in the soluble fraction. Soluble glucan-mannan complexes have been isolated from defatted walls with dilute alkali, and the glucans present were bound to a peptide moiety by N-glycosidic linkages (Kessler and Nickerson, 1959; Reiss *et al.*, 1974). These complexes have been shown to be antigenic (Masler *et al.*, 1966; Reiss *et al.*, 1974).

1.2.3. Chitin

Chitin exists as insoluble fibrils composed of long, unbranched chains of homopolymers of N-acetyl-D-glucosamine joined by β(1→4)-linkages (Reiss, 1985). Despite being a minor component of the Cw, it plays a critical role in cell division. The importance of wall chitin in maintaining structural integrity of *C.albicans* is demonstrated by the requirement for both glucanases and chitinases for the preparation of protoplasts from this yeast (Domanski and Miller, 1968). This suggests that chitin and β-glucans are more significant structural Cw components than mannans.

1.2.4. Proteins

A structural role for proteins is clearly demonstrated by the impossibility of causing Cw lysis by action of polysaccharases alone, in spite of a large proportion of polysaccharide in the wall. Complete lysis requires previous or simultaneous degradation of wall proteins with proteinases or reducing agents, which split disulphide bridges between protein molecules (Chattaway *et al.*, 1976). Consequently, proteins in Cw are found as

linkages between polysaccharide, as functional enzyme units within a polysaccharide matrix or as autonomous enzymes (Poulain *et al.*, 1985).

Mannans consistently carry a protein moiety whose mode of linkage has been described above (Odds, 1979; Poulain *et al.*, 1985; Reiss, 1985). These glycoproteins, as well as mannans and glucans are immunogenic, however, it is not known to what extent the antigenic specificities are modulated by proteins attached to mannans. In addition to their structural role, some of the Cw proteins have been identified as enzymes; e.g. acid phosphatases and two β-glucanases (Poulain *et al.*, 1985).

1.2.5. Lipids

There are lipid components in the Cw (Bianchi, 1967), but they constitute a small proportion of the wall composition, and their structure and role in the Cw remains to be determined.

The general model of the organization of the Cw seems to be one of superficial layers of protein microfibrils extending from the periphery of the wall which are loosely attached to readily soluble mannans. These mannans are embedded in an interwoven network of fine fibrils of β(1→3) glucans and chitin (Odds, 1985; Reiss, 1985).

1.2.6. Ultrastructure

Several workers have used electron microscopy to reveal the precise location of principal wall components. In electron micrographs, the Cw appears as a structure of variable thickness with several layers of different electron densities. Most investigators have

described five layers in the Cw (Djaczenko and Cassone, 1972; Cassone *et al.*, 1973; Howlett and Squier, 1980), whereas Poulain *et al.* (1978) demonstrated eight layers, but this conclusion was based on examination of cells grown for different times on different media which may exaggerate the number of layers (Szaniszlo *et al.*, 1983; Odds, 1985).

Cw layers alter in number, composition, and thickness with age of *C.albicans* yeast cells, and with growth environment (Djaczenko and Cassone, 1972; Cassone *et al.*, 1979). The layered appearance of the Cw in electron micrographs suggests the wall is composed of stratified material of different chemical composition. However, Odds (1985) supported the view that ultrastructural stratification is partly false and layers observed in electron micrographs reflect proportional, not absolute changes in Cw composition. Conversely, Poulain *et al.* (1978, 1985) revealed by cytochemical staining methods that different layers in the Cw contain different dominant polysaccharides.

The outermost fibrillar layers of the wall appear to consist of mannans and proteins (Cassone *et al.*, 1978; Odds, 1985; Poulain *et al.*, 1985). The middle layers contain predominantly glucans, however mannans and glucans span throughout most of the wall structure. The innermost region of the wall contains chitin and proteins.

1.3. Antigenic variation

There is much evidence that the composition of the bacterial surface is profoundly influenced by growth environment (Ellwood and Tempest, 1972; Gilbert and Brown, 1978; Costerton and Marrie, 1983; Brown and Williams, 1985 a and b). Growth rate and nutrient limitation have a considerable affect on the biochemistry of cell envelopes. These

factors may play an important role in bacterial susceptibility to antibiotics (Finch and Brown, 1975; Dalhoff, 1985) and host defences (Brown and Williams, 1985b), thus influencing pathogenicity. The flexibility in both structure and composition of bacterial cell envelopes confers a distinct survival advantage in a changing environment.

Phenotypic variation induced by changes in the growth environment can alter the antigenicity of bacteria. During infection, organisms are exposed to host immune defences, while host temperature and nutrient availability will vary. Hence, it has been shown that bacteria grown *in vivo* differ profoundly from their counterparts *in vitro* (Brown and Williams, 1985b; Dalhoff, 1985).

By analogy with bacterial cell envelopes, the Cw of *C.albicans* is the site of initial interaction between organism and its environment. The wall maintains the structural shape which characterizes each growth form of the fungus and is part of a permeability barrier. The cell surface is important in the mediation of adherence in colonization and invasion, and presentation of antigens to the host, yet the surface moieties and their antigenic determinants remain largely undefined. Furthermore, the definition of surface components and their antigenic determinants is essential to an understanding of structural and functional relationships of the Cw in host-parasite interactions and in maintenance of morphological states and permeability (Chaffin *et al.*, 1988).

An enhanced understanding of surface epitopes and the production of monoclonal antibodies (MAbs) against surface antigenic determinants could also facilitate the development of serological tests specific for a morphological form of growth.

1.3.1. Antigenic variation in *C.albicans*

Hasenclever and Mitchell (1961), using absorbed polyclonal sera, described two antigenic determinants of *C.albicans* designated serotypes A and B. Subsequently, the differences in antigenicity between serotypes A and B were shown to reside within the mannan component of the mannoproteins (Summers *et al.*, 1964; Guinet and Gabriel, 1980). Twenty years later, the finding of antigenic heterogeneity of surface determinants within a strain, serves as a reminder of the dangers of assuming that the cell surface structure and composition of *C.albicans* grown under apparently uniform conditions is predictable. Thus it would appear that early classifications of *C.albicans* into two or three serological groups may have been oversimplified, since antigenic expression is a dynamic process which may be influenced by several environmental factors (Brawner and Cutler, 1984, 1986a).

Poulain *et al.* (1985) compared six strains of *C.albicans* isolated from patients and subcultured regularly for more than a year, with eight strains freshly isolated from patients with candidosis for their ability to detect antibodies by indirect immunofluorescence with sera from patients. Serum from patients revealed antigenic components on the freshly isolated strains (1 or 2 subcultures) which were absent on the frequently subcultured strains. The same workers compared the reactivity of ten strains isolated from patients with candidosis, with that of ten strains from healthy subjects against sera from the same patients and healthy subjects. It was observed that strains isolated from patients and healthy subjects showed the same reactivity against sera from healthy individuals; whereas strains isolated from patients were significantly more

reactive against sera from patients than strains from healthy subjects (Poulain *et al.*, 1982).

It appears that yeast cells isolated from patients possessed an antigen(s) *in vitro* that was similar to an antigen(s) *in vivo* which induced antibody production during infection. Since this antigen was either absent or expressed in low amounts in strains isolated from healthy subjects, it has been speculated that it may be related to pathogenicity (Poulain *et al.*, 1982, 1985).

Recently, MAbs have been used successfully not only for examining the immunodeterminants that are present on the surface, but also for investigating the regulation of antigen expression. Several studies using MAbs against Cw or whole cells (Wc) of *C.albicans* have produced antibodies with similar characteristics (Brawner and Cutler, 1984, 1986a; Hopwood *et al.*, 1986; Chaffin *et al.*, 1988; Sundstrom *et al.*, 1988). As a class, these MAbs are in the IgM class, with the exception of one IgG-producing clone.

Using concanavalin A, a lectin with a high affinity for mannose polymers, results of cytochemical, ultrastructural and agglutination studies have shown that the mannose-containing constituents are present at the cell surface (Cassone *et al.*, 1978; Tronchin *et al.*, 1981). Surface exposure of protein constituents have also been demonstrated by the accessibility of these substrates to protease digestion (Cassone *et al.*, 1987; Maisch and Calderone, 1981; Lee and King, 1983; Sundstrom and Kenny, 1984). However, in these studies the individual constituents were not identified.

Brawner and Cutler (1984, 1986a) observed the variability in expression of two cell

surface antigenic determinants using MAbs H9 and C6 by agglutination studies, indirect immunofluorescence and immunoelectron microscopy. They and other workers have found that antigen expression in yeast cells varies among different strains of *C.albicans* of undetermined serotypes (Hopwood *et al.*, 1986; Chaffin *et al.*, 1988; Sundstrom *et al.*, 1988). Expression was also influenced by growth phase and morphology (Hopwood *et al.*, 1986; Chaffin *et al.*, 1988), furthermore cells grown in either complex or chemically defined media were more reactive than cells grown on an agar surface (Brawner and Cutler, 1984). Both antigenic determinants, when expressed, were located in the outer fibrillar Cw layer of both yeast cells and hyphae. The determinant detected by H9 antibody was lost from the mother cell surface during germ tube formation and expressed on the hyphae, whereas the antigen detected by antibody C6 was continually expressed on mother cells and germ tubes throughout germination (Brawner and Cutler, 1986 a and b).

Further work by Brawner and Cutler (1987) compared the intracellular and cell surface expression of the same two antigenic determinants during growth of *C.albicans* *in vitro* and *in vivo* (recovered from infected mice) using immunoelectron microscopy. Differences between *in vitro* and *in vivo* antigen expression were noted during early stages of growth. Antigen expression *in vitro* was confined to the surface layers and to the innermost wall layer of mother cells and hyphae. Comparison of antigen expression during *in vivo* growth in early intraperitoneal infection against late disseminated kidney disease suggested that both of the antigens were expressed in greater quantities on the surface of germ tubes than on mother cells in kidney tissue.

Using indirect immunofluorescence and a MAb 24, Chaffin *et al.* (1988) observed a difference in antigen expression between logarithmic and stationary phase yeast cells at 28°C. As cells reached the end of logarithmic phase growth and entered stationary phase, the number of cells that expressed antigen increased. This increase occurred in the last one or two generations rather than after cessation of cell division. Antigen expression on germ tubes was restricted to mother cells and did not appear on germ tubes or hyphae.

Collectively, these studies have examined several different antigenic determinants using similar techniques, therefore observations that are common from their findings are as follows: (i) antigen expression is dependent on growth phase, growth environment, and cell morphology; (ii) there is a heterogeneity of expression among cells in a population; (iii) the epitope is shared by various isolates of *C.albicans*, *C.stellatoidea*, *C.tropicalis*, and *C.guilliermondii*, but not by *C.parapsilosis* or *C.krusei* ; and (iv) the expression of antigen may vary among strains of the same species.

Several observations have suggested that the antigenic determinants recognized by these MAbs are predominantly polysaccharide in nature (Brawner and Cutler, 1986a; Hopwood *et al.*, 1986; Chaffin *et al.*, 1988). These observations are based on (i) interaction with a mannan preparation from the same organism; (ii) sensitivity of antigen to periodate but not proteases; (iii) gel liquid chromatography and mass spectroscopy analysis; and (iv) coincidence of migration of antigen during electrophoresis with material which stained intensely with carbohydrate but not with protein reagents. Since structural polysaccharides are linked to proteins during their synthesis in the cytoplasm and at the cell surface, and glycoproteins represent the majority of yeast antigens, a glycoprotein

nature for antigenic determinants seems probable (Poulain *et al.*, 1985; Hopwood *et al.*, 1986).

In view of this, it appears that the antigenic determinant on the cell surface is most likely part of the carbohydrate component of the mannoprotein (Chaffin *et al.*, 1988; Sundstrom *et al.*, 1988). This does not preclude the presence and significance of protein epitopes at the surface, although further studies are required. It should be noted, however, that hybridomas selected for propagation have been those which were positive for immunofluorescence or agglutination, that is, those most likely directed against mannoprotein. Antibodies against less abundant surface epitopes remain to be found.

Since polysaccharide structure is heterogeneous and is subject to regulation, such regulation could involve mechanisms of shedding of antigen to the environment as cytological evidence suggests (Brawner and Cutler, 1984, 1986a; Tronchin *et al.*, 1984; Hopwood *et al.*, 1986); loss by degradation of surface components or Cw reorganization; or synthesis of new surface components that mask other antigens (Chaffin *et al.*, 1988; Sundstrom *et al.*, 1988).

This variation and shedding of surface antigens may have important implications in pathogenesis of *C.albicans* and in host-parasite interactions. If antibodies are directed against some surface determinants that are readily shed, then this would be one mechanism by which *C.albicans* could evade the host immune response and establish infection, particularly if shedding of surface antigens exposes a moiety which may mediate adherence. Furthermore, shedding of surface antigens during growth may correlate with the mannan antigenemia reported in candidosis (Lehman and Reiss, 1980a

and b; De Repentigny *et al.*, 1984; Brawner and Cutler, 1986a) and result in formation of immune complexes (Weiner and Yount, 1976).

1.4. Iron and infection

A successful pathogen must be able to enter the host; multiply in host tissues; resist, suppress or not stimulate host defences; and in some cases damage the host (Smith, 1977; Mims, 1982). In most cases these properties are greatly influenced by the nature of the bacterial surface. There is evidence to suggest that composition and related biological properties of the bacterial surface are influenced by growth environment, especially specific nutrient deprivation (Brown and Williams, 1985b). In particular, the influence of iron deprivation on the envelope structure and function of pathogenic microorganisms growing *in vitro* and *in vivo* has been well documented.

1.4.1. Significance of iron in infection

Iron plays an essential role in the life processes of both eukaryotic and prokaryotic cells. Early evidence that specific iron-binding proteins present in blood and in the whites of eggs inhibited growth of bacteria (Schade and Caroline, 1944, 1946), was later confirmed when the active component in egg white was shown to be ovotransferrin (Alderton *et al.*, 1946). Schade and Caroline (1946) found a similar iron-binding protein in blood termed transferrin.

Growth of *C.albicans* is inhibited in the presence of iron-binding proteins or serum, such inhibition can be lifted by adding iron (Caroline *et al.*, 1964; Esterly *et al.*, 1967;

Kirkpatrick *et al.*, 1971; Elin and Wolff, 1973). *In vivo*, excess iron promoted proliferation of *Candida* in mice (Abe *et al.*, 1985), furthermore it has been suggested that systemic candidosis in patients with acute leukaemia might be related to increased saturation of their serum transferrin with iron (Caroline *et al.*, 1969).

The amount of free iron available within the body is extremely small. Ferric ions under physiological conditions tend to oxidize, hydrolyze and polymerize, yielding insoluble hydroxide polymers (May and Williams, 1980). Free iron mediates a variety of deleterious effects manifest by release of hydroxyl radicals which subsequently attack and destroy cell membranes and nucleic acids. These effects are, however, normally suppressed as most iron is complexed with a variety of chelating agents. These agents include the intracellular ferritin, haemosiderin and haem while body fluids contain transferrin (Aisen, 1980) and lactoferrin (Bezkorovainy, 1980; Morgan, 1981) which is also commonly found in polymorphonuclear leukocytes (PMNs) (Masson *et al.*, 1969).

Transferrins bind two atoms of ferric iron per molecule of protein and is accompanied by binding of an anion on a 1:1 molar basis. Physiologically, this anion is carbonate and iron is not bound in its absence or in the presence of other anions, although, under laboratory conditions, other anions can be utilized (Schlabach and Bates, 1975). Interestingly, Valenti *et al.* (1985) found that bicarbonate ions did not influence the antifungal activity of ovotransferrin towards *C.albicans*, whereas other workers have shown that its presence is essential for inhibition of candidal growth (Rolling and Lupan, 1988). Furthermore, contrary to that observed with bacterial species, ovotransferrin retained partial inhibitory activity even in the absence of bicarbonate ions.

The affinity constants of lactoferrin and transferrin for iron is 10^{52} and 10^{36} respectively (Griffiths, 1987a). These iron-binding proteins are normally only partially saturated (Weinberg, 1984), thus serving as an important safeguard against the sudden presence of free iron. So, although there is an abundance of iron in the body, the amount of free iron in equilibrium with the proteins is about 10^{-18}M, which is too small to sustain bacterial growth (Bullen *et al.*, 1978; Bullen, 1981; Finkelstein *et al.*, 1983).

During infection and inflammation there is a reduction in serum iron concentration (Cartwright *et al.*, 1946). This decrease, called hypoferraemia, is reported to be a result of the release of lactoferrin from PMNs, which removes iron from transferrin so that the lactoferrin-iron complex is selectively taken up by macrophages and removed rapidly from the circulation by the reticuloendothelial system (RES) (van Snick *et al.*, 1974). Further evidence suggests that in mice, hypoferraemia is due to an altered processing of iron within the RES which limits the supply of iron to the extracellular pool (Letendre and Holbein, 1984). Iron is thought to play a role in modulating the inflammatory response in hypoferraemia. Neutrophils produce superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2). In the presence of Fe^{3+}, the O_2^- is converted to O_2 and Fe^{2+} (Haber and Weiss, 1934). This results in the formation of Fe^{3+} and $OH^\bullet$ (hydroxyl radical); the hydroxyl radical contributing to acute tissue damage during inflammation (Ambrusco and Johnston, 1981).

To adapt to the iron-restricted environment usually found *in vivo*, bacteria induce high-affinity iron transport systems, which consists of low molecular weight iron-chelating compounds, called siderophores, and in the case of Gram-negative bacteria the

production of receptors which are involved in the uptake and release of iron from iron-chelators (Neilands, 1982). So far only one bacterial species, *Neisseria*, is thought to operate an iron-uptake mechanism involving direct interaction of the pathogen with iron-binding proteins, however, the means by which this is accomplished is poorly understood. Among *Candida* species, it has been suggested that the antifungal activity of ovotransferrin and lactoferrin is not only mediated by iron restriction, but may involve more complex mechanisms (Petrou and Rogers, 1988); including a direct interaction of the iron-binding protein with the fungal surface (Valenti *et al.*, 1985, 1986; Rolling and Lupan, 1988).

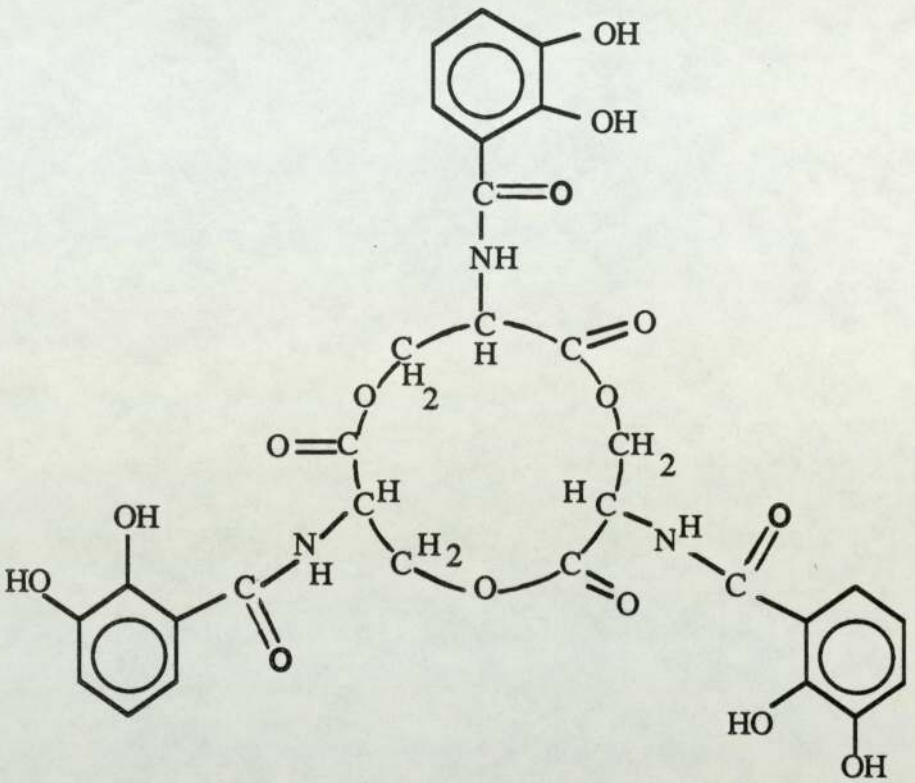
Bacteria of the genera *Salmonella*, *Escherichia* and *Klebsiella* secrete the iron-chelator enterochelin (phenolate siderophore) under conditions of iron restriction *in vitro* (O'Brien and Gibson, 1971; Pollack and Neilands, 1970; Perry and San Clemente, 1979; Williams *et al.*, 1984). This compound is a cyclic triester of 2,3-dihydroxy-N-benzoyl serine, with a high affinity for iron (Fig.1.2). The formation constant for ferric enterobactin is near 10^{52} (Harris *et al.*, 1979), thus organisms using enterochelin-mediated iron transport can remove iron from iron-binding proteins efficiently (Carrano and Raymond, 1979). However enterochelin is not recycled by the cells after use; it is cleaved by a specific esterase when the molecule enters the cell. This hydrolysis is necessary to release iron from the iron-siderophore complex (Cooper *et al.*, 1978; Raymond and Carrano, 1979).

Although enterochelin-mediated iron is the predominant system used for accumulating iron, members of the genus Enterobacteriaceae can also acquire iron by way of

hydroxamate-type siderophores which are not specifically synthesized by this group but produced by other microorganisms as well (Neilands, 1981a and b). Some strains of *E.coli* synthesize a plasmid-encoded hydroxamate-type siderophore called aerobactin, as well as enterochelin. Aerobactin is a conjugate of 6-(N-acetyl-N-hydroxyamino)-2-aminohexanoic acid and citric acid forming an octahedral complex with ferric iron (Harris *et al.*, 1979) (Fig.1.2). Although its production is thought to play a role in the virulence of *E.coli* strains (Williams, 1979), it is not clear why the ability to make aerobactin confers a selective advantage to bacteria already capable of synthesizing enterochelin.

In vivo, the relative ability of these siderophores to remove iron from the iron-binding proteins is influenced by components of the body fluids. Aerobactin has been shown to be more effective than enterochelin at equal molarity in delivering iron transferrin to bacterial cells in human serum (Konopka *et al.*, 1982). This may be due to the fact that enterochelin binds to serum proteins so reducing its activity (Konopka and Neilands, 1984; Williams and Carbonetti, 1986). Unlike enterochelin which has a higher formation constant, aerobactin is recycled by the cell and is more effective at low concentrations; a property that is likely to be advantageous in body fluids (Williams and Carbonetti, 1986). In addition to these factors, the relatively low water solubility (Pollack and Neilands, 1970) and chemical instability of enterochelin (Neilands, 1981a), could further restrict the efficiency of enterochelin *in vivo* and confer a selective advantage on aerobactin-mediated iron transport.

Studies on siderophore production by pathogenic yeasts and fungi are limited. One report demonstrated that several opportunistic fungi, including *C.albicans* secreted



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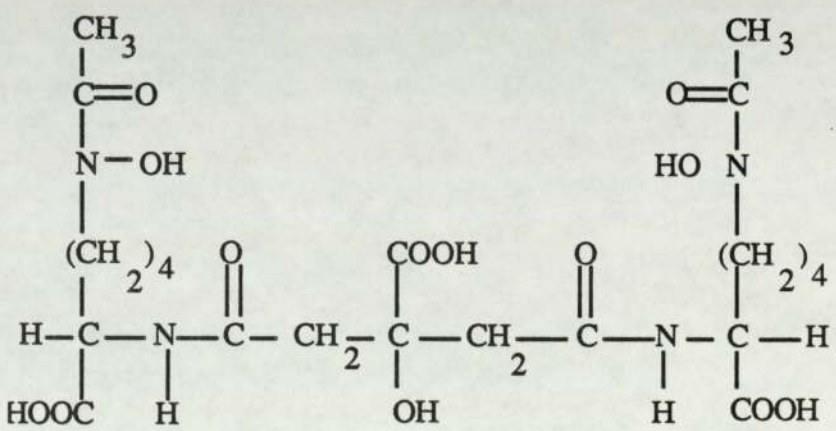


AEROBACTIN

Fig.1.2. Structures of representative siderophores

hydroxamate-type siderophores (Holzberg and Artis, 1983), while 40% of *C.albicans* isolates tested produced both hydroxamate- and phenolate-type siderophores (Ismail *et al.*, 1985a). The isolation and characterization of *Candida* siderophores remains to be determined, as does their role *in vivo* during infection.

An essential part of high affinity iron-uptake systems in addition to siderophores is the induction of iron-regulated protein (IRMP) receptors in Gram-negative bacteria. A need for specific receptors is due, in part, to the molecular weight of iron-siderophore complexes which exceed the limit for diffusion through outer membrane pores (Nikaido, 1979). The ability of bacterial pathogens to synthesize protein receptors under iron restriction therefore appears to be an important virulence factor (Griffiths, 1983). Expression of IRMPs has been demonstrated in bacteria grown *in vitro* and *in vivo*. *E.coli* isolated without subculture from peritoneal cavities of infected guinea pigs (Griffiths *et al.*, 1983) and urine (Lam *et al.*, 1984) produced IRMPs, while *K.pneumoniae* and *P.mirabilis* isolated directly from infected urine (Shand *et al.*, 1985), and *P.aeruginosa* obtained without subculture from the sputum of a patient with cystic fibrosis (Brown *et al.*, 1984) also expressed IRMPs.

Recently, provisional identification and location of a possible iron-regulated membrane receptor in *C.albicans* was reported. The receptor appears to have a stronger affinity for hydroxamate-type than phenolate-type siderophores, and autoradiographic studies suggest that the receptor is of low molecular weight (Ismail and Lupan, 1988).

Changes in composition of the bacterial envelope which occur during adaptation and multiplication *in vivo*, have important implications regarding the sensitivity of pathogens

to antibiotics used in therapy. It is known that iron limitation (and growth rate *per se*) alters the production of penicillin-binding proteins, and influences the sensitivity of the organisms to the action of β-lactam antibiotics (Turnowsky *et al.*, 1983; Brown and Williams, 1985a). In addition, sub-inhibitory concentrations of antibiotics may affect bacterial iron metabolism. Such concentrations have been shown to reduce enterochelin production by *K.pneumoniae* (Kadurugamuwa *et al.*, 1985a and b), and to expose the IRMPs to antibodies.

It is evident that the literature available concerning the role of iron in bacterial infections, especially in those caused by Gram negative bacteria, is increasing. However, it is equally clear that little is known about the mechanisms involved in the acquisition of iron by *C.albicans* when growing *in vivo*. Similarly, it is not known what effects, if any, iron restriction has on properties of *C.albicans*, nor whether alterations in the envelope occur *in vitro* or *in vivo*. The fact that bacteria undergo specific phenotypic changes both in their metabolism and in the composition of their outer membrane proteins to adapt to an iron-restricted environment, adds another dimension to a complex pattern of virulence determinants already found in iron-replete pathogens. However these same bacterial characteristics may provide a valuable framework in which to view the response of *C.albicans* to an iron-restricted environment.

1.5. Adherence

Extensive investigations have elucidated many of the mechanisms that mediate attachment of bacteria to host cells. Bacterial adhesins and host cell receptors have been characterized

at a molecular level, as have factors governing their expression (Beachey, 1981; Freter and Jones, 1983; Sparling, 1983). In particular, bacterial adhesion to host tissues is regarded as an important step in the pathogenesis of infection (Ofek and Beachey, 1980), such knowledge of bacterial attachment mechanisms has been further translated into approaches for modification or prevention of infection in humans. By comparison, an understanding of the mechanisms of candida adherence is at an early stage, however, a rapidly increasing literature demonstrates the potential importance of adherence in the pathogenesis of candidosis.

1.5.1. Adherence of *C.albicans* to host tissues and plastic surfaces

Attachment of *Candida* to epithelial cells (ECs) has been studied extensively to define factors relevant to the pathogenesis of oral, gastrointestinal, and vaginal candidosis. Adherence of the organism to fibrin-platelet matrices (Maisch and Calderone, 1981) and to vascular endothelial cells (Barnes *et al.*, 1983) has been investigated to elucidate early events in the induction of candidal endocarditis and haematogeneously spread disseminated candidosis. In addition, attachment to plastic surfaces has been demonstrated and may be important in infections involving dental prostheses, intravascular and urinary catheters, and prosthetic valves (Zimmerli *et al.*, 1982; Rotrosen *et al.*, 1983).

The adhesin(s) that promotes attachment to mammalian cells have not been identified. However, indirect evidence which was based on results of; (i) enzymatic treatment of Wc or purified Cw to inhibit adherence; (ii) attempts to promote adherence; (iii) the affects of

lectins and antibodies on *Candida* adherence; and (iv) attempts to isolate and characterize the adhesin, suggests that candidal adhesion may be mediated by a mannan or mannoprotein.

Isolated Cw fragments of *C.albicans* adhered to vaginal epithelial cells (VEC), but their adherence was diminished following degradation with α-mannosidase or proteases (Lee and King, 1983). Similarly, Maisch and Calderone (1981) found that sheep red blood cells (SRBC) conjugated with an alkali-soluble Cw extract of *C.albicans* readily attached to a fibrin-platelet matrix formed *in vitro*. This alkali-soluble extract contained 72% polysaccharide and less than 1% protein. Treatment of the extract with α-mannosidase or degradation by acetolysis removed the ability to promote adherence of the SRBC suggesting that cell surface mannan, probably as an intact molecule or as mannoprotein, may play a role in the adherence mechanism.

McCourtie and Douglas (1984) demonstrated that *C.albicans* strains grown in sugar solutions revealed enhanced virulence as measured in a mouse model of disseminated candidosis. Preincubation of viable *Candida* cells with sucrose or galactose promoted adherence of stationary phase organisms to buccal epithelial cells (BEC) and acrylic surfaces, and resulted in the appearance of a fibrillar layer on the yeast cell surface (Samaranayake and MacFarlane, 1980, 1981; McCourtie and Douglas, 1981, 1984). This fibrillar structure, believed by Tronchin *et al.* (1984) to mediate attachment of *Candida* to human epithelial cells has a high mannose content and is probably mannoprotein in nature (McCourtie and Douglas, 1985). Furthermore, its presence has been observed by electron microscopy of *in vivo* grown cells recovered from tongue and

buccal scrapings of patients with oral candidosis (Montes and Wilborn, 1968; Marrie and Costerton, 1981). In the presence of tunicamycin, in addition to galactose, no enhancement of adherence to BECs was observed. The inference being that since tunicamycin inhibits mannoprotein synthesis but not that of glucan or chitin synthesis, mannoprotein is of primary importance in the interaction with ECs (Douglas and McCourtie, 1983).

Other workers have utilized the specific affinity of the lectin concanavalin A (Con A) for yeast surface mannans to study adherence inhibition (Sandin and Rogers, 1982; Sandin *et al.*, 1982; Calderone and Wadsworth, 1987). Pretreatment of yeasts or germ tubes with Con A resulted in diminished adherence to BECs, once more suggesting that candidal adhesion is mediated by a mannan-containing moiety (Sandin, 1987). Adherence was also diminished by pretreating BECs rather than yeasts or germ tubes with Con A.

Despite evidence for mannan or mannoprotein-mediated adherence of *C.albicans* to host ECs, some workers have suggested that chitin, chitin derivatives and amino sugars may be involved in the adherence to VECs (Segal *et al.*, 1982, 1984; Lehrer *et al.*, 1983). This view has been further supported by studies of Lehrer *et al.* (1988) and Segal *et al.* (1988) who demonstrated that chitin isolated from *C.albicans* inhibited *in vitro* adherence of *C.albicans* to vaginal mucosal cells, and prevented development of candidal vaginitis in a murine model.

1.5.2. Host factors and characteristics of human epithelial cells that influence candidal adhesion

Most studies on candidal adhesion have focussed on attributes of yeast cells or their growth environment which might influence attachment (Kennedy and Sandin, 1988), yet, identification of mucosal cell traits that might affect adhesion and offer an explanation as to why some body sites are more susceptible to candidal colonization *in vivo* than others is currently lacking (Persi *et al.*, 1985; Sandin *et al.*, 1987b). Nonetheless, a few studies have attempted to address this point. The *in vitro* adherence of *C.albicans* to mucosal cells from the mouth, vagina and urinary tract were found to be significantly different both within and between individuals although some subjects manifested larger variations than others. A trend was observed in yeast adherence to the cells i.e. BECs > VECs > urinary tract epithelial cells. There appeared to exist distinct sub-populations of ECs with high and low affinity for attachment by *C.albicans* (Sandin *et al.*, 1987a and b), thus suggesting the presence of EC receptors.

A controlled-environment membrane model has been developed in an attempt to mimic the environment of the vagina in order to study yeast-VEC adhesion. Adhesion was affected by strain, pH and level of carbon dioxide present in the vagina. In addition, of the VECs that had yeast cells attached to them, approximately 95% of the total yeasts were attached to the microridge side of VECs, suggesting that the microridge side has receptors that may be recognized by candida adhesins (Persi *et al.*, 1985).

Since fibronectin may serve as the mammalian receptor for some pathogenic bacteria (Woods *et al.*, 1981; Proctor *et al.*, 1983; Simpson and Beachey, 1983), the role of

fibronectin in colonization of mucosal ECs by *Candida* has been investigated (Skerl *et al.*, 1984; Calderone and Scheld, 1987). *In vitro* binding of *Candida* species to fibronectin correlated directly with the ability of organisms to attach to mammalian cells, yet fibronectins' role in the pathogenesis of candidosis, with the exception of *Candida* endocarditis, has not been established. However, Kalo *et al.* (1988) separated VECs into subpopulations rich in either superficial or intermediate cells and demonstrated that *C.albicans* bound preferentially to subpopulations of intermediate cells that possessed high levels of fibronectin than to superficial cells with low levels of fibronectin. These results suggested that fibronectin acts as a receptor for binding of *C.albicans*.

Absence of specific adhesin-receptor interactions in some studies (Reinhart *et al.*, 1985; Kennedy *et al.*, 1987; Klotz and Penn, 1987) may reflect the possibility that widespread epidemiological distribution of *Candida* and its almost indiscriminate attachment to many surfaces favours involvement of multiple mechanisms of adhesion; although a specific adhesin may still be required for attachment.

With few exceptions (Barnes *et al.*, 1983; Scheld *et al.*, 1983), studies demonstrating *in vitro* adherence of *Candida* have failed to determine its role during colonization or infection. Given that bacterial surface structures, including adhesins, may be influenced by host factors *in vivo* and by growth conditions *in vitro*, it would appear relevant to consider these factors when designing *in vitro* assays or models in order to mimic more closely *in vivo* adherence mechanisms of *C.albicans* (Persi *et al.*, 1985).

1.5.3. Cell surface hydrophobicity (CSH)

Forces that have been considered as being important in cell adhesion processes include electrostatic, Van der Waal's and hydrophobic interactions. Plastic surfaces and all living cells possess various degrees of net negative surface charge. Hence, negative-negative charge interactions, e.g. the interactions that would occur between plastic and yeast, and between yeast and yeast, would be expected to repel each other, creating a condition of electrostatic repulsion. Such electrical forces are, however, relatively minor and tend to be overcome by hydrophobic forces, since adherence occurs to a considerable extent even in the presence of this repulsive force (Klotz *et al.*, 1985). The minor nature of this force may also be overshadowed by the presence of ions in the aqueous environment of yeast cells which alters charge characteristics of cell surfaces, thereby enhancing the adherence of yeasts to polystyrene (Klotz *et al.*, 1985; Klotz and Penn, 1987).

Hydrophobicity of microbial surfaces can be measured indirectly by partition of a microbial suspension in a biphasic aqueous system (Rosenberg *et al.*, 1980), by relative adsorption to hydrophobic gels (Smythe *et al.*, 1978), and by measurement of the contact angles between an aqueous phase and the surface in question (Gerson and Scheer, 1980). Peres *et al.* (1977); Beachey, (1981) and Miörner *et al.* (1983) have demonstrated by these techniques, the potential importance of hydrophobic forces in certain bacterium-host cell interactions, however, relatively little is known regarding the role of hydrophobicity in adherence of *Candida.*

Candida species have been shown to vary in their hydrophobic affinity as measured by the contact angle method and yeast adherence to a liquid hydrocarbon (Klotz *et al.*,

1985; Minagi *et al.*, 1985, 1986). In addition, both methods correlated well with the adherence of yeasts to plastic surfaces (Klotz *et al.*, 1985). Hazen *et al.* (1986) examined 18 isolates of *C.albicans* and demonstrated that cells grown in liquid media at 25°C were more hydrophobic than cells grown at 37°C. Hydrophobicity also varied with strain, growth phase and growth medium. Furthermore recent studies by Kennedy *et al.* (1988) using *C.albicans* yeasts cultivated on agar surfaces at 24°C demonstrated that the phenotypic state of organisms also influenced hydrophobicity.

A *C.albicans* mutant possessing a structural defect in Cw mannan expressed markedly increased cell surface hydrophobicity compared to the parent (Shimokawa and Nakayama, 1986). However, the relationship between surface hydrophobicity and defective mannan remains to be elucidated. Recently, a polystyrene microsphere assay for detecting cell surface hydrophobicity of populations of individual cells of *C.albicans*, demonstrated a sequence of strongly, moderately and weakly hydrophobic cells within a population (Hazen and Hazen, 1987). The same assay revealed that regardless of the hydrophobic status of mother cells, germ tubes and hyphae were also hydrophobic.

Phagocytosis is a multi-step surface phenomenon that can be described in physico-chemical terms (Mudd and Mudd, 1933). Determination of the degree of hydrophobicity of bacteria by means of penetration measurements using various two-phase systems, showed at least quantitatively, that degree of hydrophobicity in bacteria correlates well with their susceptibility to phagocytosis. A quantitative approach to the hypothesis that a correlation exists between surface energy of particles and bacteria, and the degree to which they become phagocytized was made possible by contact angle measurements on

bacteria in conjunction with *in vitro* measurements of phagocytosis of the same bacteria by human neutrophils or guinea pig macrophages (van Oss *et al.*, 1975).

The role of antibody and complement on increasing surface hydrophobicity and phagocytosis of pathogenic bacteria, as well as effects of various antibiotics, have been studied (van Oss *et al.*, 1975). However, it is not known whether relative cell surface hydrophobicity of *C.albicans* correlates with the degree to which it becomes phagocytized.

1.6. Virulence factors

In host-parasite relationships expression of disease depends on a balance between virulence of a microorganism and host defence. In candidosis some impairment of the host response must be present for disease to occur, hence virulence of *Candida* for humans is determined more by the host than by the fungus (Odds, 1979). Nevertheless, *C.albicans* possesses several determinants of virulence that can contribute to establishment of infection. The ability to form hyphae, adherence to epithelial surfaces (discussed in section 1.5.), and secretion of hydrolytic enzymes has received most attention.

1.6.1. Enzyme secretion

Considerable attention has been focussed on extracellular proteinase as a virulence factor of *C.albicans*. *C.albicans* and some other *Candida* species secrete a carboxyl proteinase when they are grown in medium containing protein as a sole nitrogen source

(Macdonald and Odds, 1980; Rüchel, 1981; Rüchel *et al.*, 1982). At least three isoenzymes secreted by different *C.albicans* strains have been isolated and characterized (Rüchel, 1981; Rüchel *et al.*, 1985). They have similar antigenic structures but differ biochemically (Rüchel *et al.*, 1982). The pH optima for these enzymes ranges from 2.2 to 3.2, and they digest, *in vitro*, several physiologically important substrates such as albumin, immunoglobulins and skin proteins (Rüchel, 1981; Rüchel *et al.*, 1982; Negi *et al.*, 1984). These enzymes are denatured above pH 8, and their molecular weights are in the range 40 to 45 Kd (Rüchel, 1981).

Most strains of *C.albicans*, as well as *C.tropicalis* and *C.parapsilosis* produce proteinase while *C.glabrata*, *C.guilliermondii*, *C.krusei* and *C.pseudotropicalis* do not (Rüchel *et al.*, 1983; Macdonald, 1984). This rank order of proteinase secretion correlates well with the rank order of virulence established for different *Candida* species (Odds, 1979). In addition, an early observation by Staib (1965) found that a non-proteolytic strain of *C.albicans* was a less virulent pathogen of mice than a proteolytic strain. Further studies following an equivalent approach showed similar differences between proteinase-secreting strains and proteinase-deficient mutants derived from equivalent strains. The former more readily colonized deep organs compared to proteinase-deficient mutants resulting in greater mortality amongst experimental mice (Macdonald and Odds, 1983; Kwon-Chung *et al.*, 1985).

C.albicans proteinases are produced *in vivo*, and are demonstrated by immunofluorescence techniques to be located within infected tissue (Macdonald and Odds, 1980; Rüchel, 1983; Shimizu *et al.*, 1987). The acid environment required for

their activity may be achieved on mucosal membranes as well as in the acidic milieu of damaged tissue (Rüchel, 1984). Macdonald and Odds (1980) and Rüchel (1981) have demonstrated that these enzymes stimulate production of antibodies which circulate in sera of patients with systemic candidosis. These antibodies are, however, in turn susceptible to proteinases which are capable of degrading serum IgA as well as secretory IgA *in vitro* (Rüchel *et al.*, 1982; Reinholdt *et al.*, 1987) possibly explaining the apparent failure of IgA to protect individuals from candidosis.

Two recent studies have provided further evidence for a correlation between proteinase secretion and pathogenicity. Shimizu *et al.* (1987) demonstrated that the ability of *C.albicans* strains to secrete proteinase *in vitro* correlated well with the degree of invasiveness into the chorio-allantoic membrane (CAM) of developing chicks. Furthermore, *C.albicans* isolates from patients with vaginitis produced significantly more proteinase than isolates from patients without vaginitis symptoms (Cassone *et al.*, 1987). Although both studies suggested that proteinase may play a role in the pathogenesis of candidosis, they also acknowledged that other virulence factors are involved.

The evidence therefore appears to suggest that *C.albicans* proteinase can act as a virulence factor, yet there are also indications to the contrary. In the same study that revealed a correlation between proteinase secretion and the degree of invasiveness into the CAM of developing chicks, Shimizu *et al.* (1987) also reported that mortality rates of chick embryos did not correlate with the extent of proteinase production. In a study of denture stomatitis, Budtz-Jörgensen (1971) found no relationship between severity of

mucosal inflammation in infection and proteolytic activity of 62 *C.albicans* strains. Similarly, there was an absence of correlation between gross proteolysis in *C.albicans* biotyping tests and association of biotypes with clinical forms of candidosis (Odds *et al.*, 1983; Burnie *et al.*, 1985). Germaine *et al.* (1978) considered that the low optimum pH of the enzyme and its inhibition by salivary proteins (Germaine and Tellefson, 1981) made it unlikely to be a significant virulence factor in the pathogenesis of oral candidosis.

Overall, evidence to date suggests that proteinase secretion may play a role in the virulence of *C.albicans*. However, its secretion may be one of several virulence properties that influence the pathogenicity of some strains of *C.albicans*. In contrast to proteinase secretion, relatively little is known about phospholipase production of *C.albicans*, and its role in pathogenicity.

Phospholipase activity has been investigated by several workers (Pugh and Cawson, 1975; Price and Cawson, 1977; Banno *et al.*, 1985). Yeast forms of *C.albicans* have been shown to secrete three different types of phospholipases into the culture medium, with lysophospholipase and lysophospholipase-transacylase activities being three times greater than that of phospholipase B. In comparison all phospholipase activities in hyphal forms were extremely low (Banno *et al.*, 1985). Phospholipase A and lysophospholipase have been isolated from bud sites of yeasts and apical tips of germ tubes and pseudohyphae, and from cell membranes and walls of non-dividing cells. Phospholipase A damages cell membranes, whereas lysophospholipase protects yeasts from the damaging effects of their own secreted phospholipase. These enzymes may assist yeast invasion of host tissues by disrupting epithelial cell membranes, thus

permitting mycelial invasion of host cells (Pugh and Cawson, 1975; Price and Cawson, 1977). Samaranayake *et al.* (1984) demonstrated that of 28 isolates of *C.albicans* tested, 22 (79%) were phospholipase-positive. Enzyme production was limited to a pH range of 3.6 to 4.7, suggesting that the enzyme, like proteinases, would be active in acid niches, thus contributing to the disease process.

There is therefore considerable evidence to suggest that in *C.albicans* there exists both constitutive and inducible hydrolytic enzymes whose presence at the cell surface may be considered a contributing factor in the pathogenicity of the organism.

1.6.2. Dimorphism (Mycelium formation)

Numerous studies have suggested that mycelial forms of *C.albicans* are associated with tissue invasion during infection. However, evidence furnished by most studies is based either on the fact that mycelia are constantly visualized in clinical material, or that yeast cells produce mycelia shortly after inoculation into experimental animals; or both.

Yeast, hyphal, and pseudohyphal morphologies are all associated with host lesions (Young, 1958; Gresham and Whittle, 1961; Winner and Hurley, 1964; Simonetti and Strippoli, 1973). Those few reports in which mycelia devoid of yeasts have been observed in infected tissues are matched by studies of tissue infected with yeast free of mycelia (Pluss and Kadas, 1954; Rebora *et al.*, 1973).

Animals and humans have been experimentally inoculated by various routes with both yeast cells and mycelia, and the comparative effects of the two forms assessed. Four studies showed no difference in virulence of the two forms (O'Grady *et al.*, 1967;

Dastidar *et al.*, 1971; Russell and Jones, 1973a; Strippoli and Simonetti, 1973), one found that mycelia inocula persisted longer than yeast cells in mouths' of rats (Russell and Jones, 1973b) and three studies demonstrated a more profound pathological response in animals infected with yeast cells than with mycelia (Mankowski, 1963; Mardon *et al.*, 1969; Simonetti and Strippoli, 1973). Therefore, presence of mycelia in tissues of infected animals or human lesions, fails to indicate the exclusive involvement of this form in the pathogenesis of candidosis. Nevertheless, there remain several studies which provide evidence that mycelium formation is a contributory factor in candidosis.

Taschdjian and Kozinn (1957) prepared smears from mouths of more than 2000 infants for the first seven days of their lives. In 3.8% of babies, *C.albicans* yeast cells were seen by direct microscopy of smears until clinical lesions of thrush became apparent, at which time hyphae were observed as well as yeasts. Similarly, Budtz-Jörgensen *et al.* (1975) found a high correlation of hyphae in oral smears with lesions of denture stomatitis. Asymptomatic denture wearers from whom *C.albicans* was isolated showed primarily yeast cells in smears. More recently, a variant strain of *C.albicans* incapable of hyphal production *in vivo* still successfully colonized rat vaginas, although a higher inoculum was required and the infection tended to be milder than the wild-type strain. Thus, ability to produce hyphae may be a virulence factor but is not absolutely essential in the pathogenesis of candidal vaginitis (Sobel *et al.*, 1984).

Clearly then, it is impossible to deny an association of mycelium production in *C.albicans* with enhanced virulence of the species, however, it would be premature to suggest that mycelia and yeast forms are respectively pathogenic and non-pathogenic, or

invasive and non-invasive.

1.7. Influence of antifungal antibiotics on the interaction between host and *C.albicans*

It is well established that conditions under which an organism is grown may influence its susceptibility to the action of antibiotics (Lorian *et al.*, 1982; Brown and Williams, 1985a; Kadurugamuwa *et al.*, 1985), including sub-inhibitory concentrations (Lorian, 1980). Concomitantly, whilst an organism's surface is in direct contact with the host immune system, the additional presence of antibiotics can influence this interaction to the further detriment of an invading organism (Lorian, 1980).

There are two principal groups of antibiotics which provide compounds most widely used in current antifungal therapy: polyene macrolide antibiotics (polyenes) e.g. nystatin and amphotericin B, and azole antibiotics e.g. miconazole, ketoconazole and fluconazole.

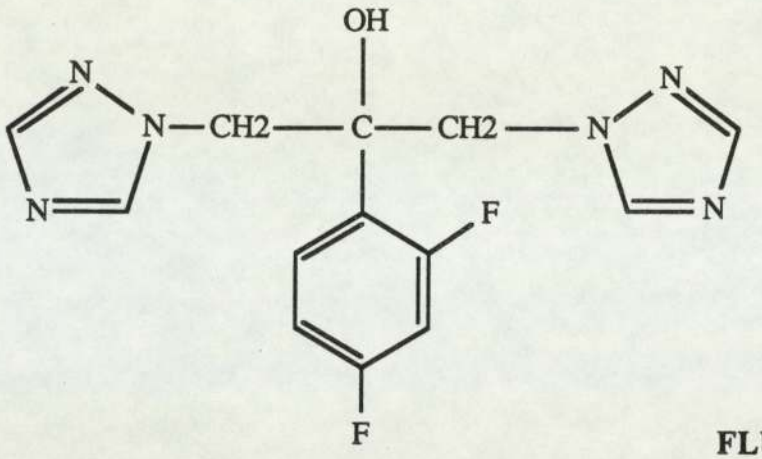
1.7.1. Structure and mechanisms of drug action

Polyenes are characterized by a ring of carbon atoms containing a system of conjugated double bonds and a hydrophilic region characterized by a number of hydroxyl groups; the ring is closed by lactonisation (Fig.1.3). Polyenes differ by number of carbon atoms in the ring, number of conjugated double bonds, number of hydroxyl groups and presence or absence of a carbohydrate moiety attached to the ring (Hamilton-Miller, 1973a; Gale *et al.*, 1981; Ryley *et al.*, 1981). Amphotericin B (Amp B) is a rigid rod-shaped molecule, with opposing hydrophobic and hydrophilic faces. The length of the molecule

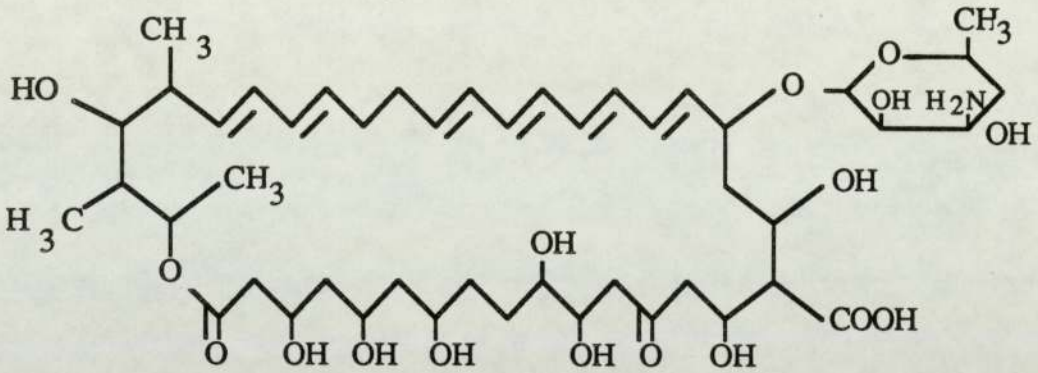
is similar to that of a plasma membrane (PM) phospholipid molecule; a factor of some importance in Amp B's mode of action.

The mechanism of action of polyenes is essentially the same. At minimum inhibitory concentrations (MIC), these compounds interact with sterol components in the PM of sensitive organisms and alter membrane permeability. Binding of polyenes primarily results in a leakage of cytoplasmic constituents, associated metabolic disruption and cell death are considered to be secondary consequences of membrane alterations (Hamilton-Miller, 1973a; Borgers, 1980; Gale *et al.*, 1981). Interaction of polyenes with membrane constituents is complex. Freeze-fracture analysis of yeast exposed to nystatin and Amp B have shown a clustering of PM-associated particles, confined to outer membrane regions suggesting that these antibiotics do not penetrate the PM but remain within the outer lipid layer (Kerridge and Nicholas, 1986). Molecular models predict that polyenes form an annulus in the surface region of PMs giving rise to aqueous pores through which loss of cytoplasmic metabolites can occur.

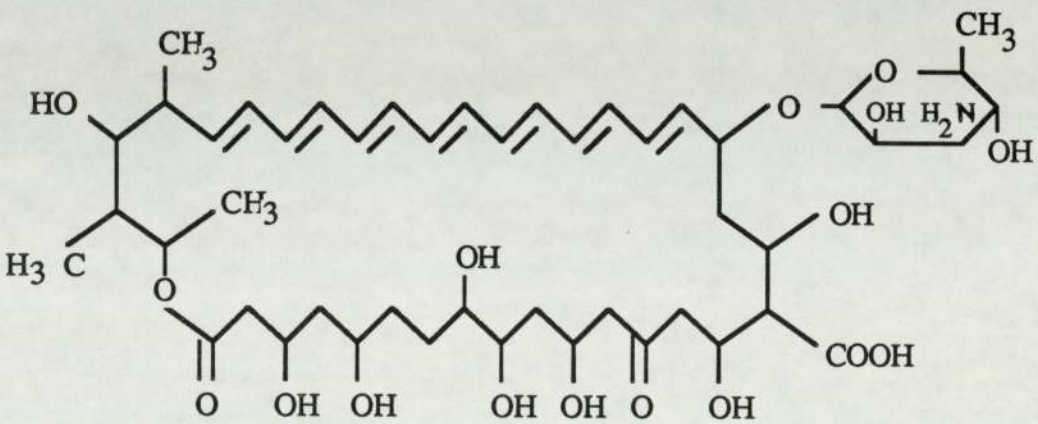
Fungal mutants resistant to polyenes can be readily obtained in the laboratory and has been studied to provide information on molecular mechanisms for drug action. Analysis of the lipid composition of these mutants has shown that resistance may or may not be associated with qualitative and/or quantitative changes in sterol composition (Pierce *et al.*, 1978; Levchenko *et al.*, 1984; Sokol-Anderson *et al.*, 1988). Although the nature of membrane sterols and lipids is important in polyene resistance, they do not completely explain resistance. Hence, this has led to the conclusion that an additional mechanism of polyene resistance must be present.



FLUCONAZOLE



NYSTATIN



AMPHOTERICIN B

Fig.1.3. Structures of three antifungal antibiotics.

Hsu Chen and Feingold (1974) studied fungal mutants that were resistant to permeabilizing effects of Amp B but not to its lethal effects and proposed two types of polyene resistance in *C.albicans*: (i) resistance to polyene-induced leakage of intracellular contents and killing, and (ii) resistance only to polyene-induced killing. The first type of resistance is a result of reduced interaction between PM and polyene. Since it has been demonstrated that oxidative damage plays a role in the mechanism of fungal cell killing by Amp B, the second type of resistance and the additional mechanism of resistance to polyenes is believed to be due to oxidative-dependent cell damage (Sokol-Anderson *et al.*, 1986, 1988).

Despite the findings described above, resistance to polyenes in *C.albicans* is not recognized as a clinical problem (Medoff and Kobayashi, 1980). *C.albicans* is apparently diploid and lacks a haploid sexual stage in its life cycle . Unless mutations are introduced in each of two alleles present for a given locus by a mutagen (modification of which will confer polyene resistance), or resistance is a dominant characteristic, then the frequency with which both mutational events give rise to resistance in diploid organisms will be extremely low compared with isolation of recessive mutants from haploid cell lines (Olaiya and Sogin, 1979). The second factor relates to the complex nature of antibiotic interaction with PMs. Mutations affecting this interaction could also impair membrane function which may lead to reduction in growth rate and hence pathogenicity of *C.albicans* (Kerridge and Nicholas, 1986).

The principal azole antifungals are imidazoles and the newer triazole compounds. The basic structural unit of all azoles is a five-membered azole ring which is attached by a

carbon nitrogen bond to other aromatic rings (Fig.1.3). Imidazole and triazole compounds contain two and three nitrogen atoms respectively in the azole ring (Saag and Dismukes, 1988).

Molecular mechanisms for the action of imidazoles and triazoles appear to be similar. Fungistatic concentrations inhibit ergosterol biosynthesis with subsequent accumulation of 14 α-methylsterols, the precursor intermediates of ergosterols (van den Bossche *et al.*, 1978), which in turn are the principal sterol of fungal membranes and are essential for functional integrity of these membranes. A demethylation step is required to convert 14 α-methylsterols to ergosterol which is dependent on the microsomal cytochrome P450 sterol C14 demethylase. At a molecular level, one of the nitrogen atoms (N-3 in the imidazoles; N-4 in the triazoles) binds to a heme iron of cytochrome P450, thereby inhibiting cytochrome activation and enzyme function (van den Bossche *et al.*, 1983). Azoles may also inhibit the synthesis or functioning of cytochrome c oxidase and peroxidative enzymes, with a resultant increase in intracellular peroxide generation (Shigematsu *et al.*, 1982). Fungicidal effects, which could not be explained by inhibition of sterol synthesis alone, are believed to be due to direct membrane damage associated with primary action of drug on membrane phospholipids (Kerridge and Nicholas, 1986; Saag and Dismukes, 1988).

As with polyenes, emergence of strains resistant to azoles has not proved a clinical problem. Studies on azole-resistant mutants isolated from treatment failures have suggested that resistance could result from changes in phospholipid : sterol ratio of the PM, rendering it permeable to the drug (Ryley *et al.*, 1984; Hitchcock *et al.*, 1986,

1987). It is clear that for this group of antifungal agents, there is no single primary target responsible for all observable effects (Borgers, 1980; Kerridge and Nicholas, 1986). Several potentially lethal targets are affected and the relative importance of each will depend on strain, drug being used, and host environment.

1.7.2. Effects of sub-inhibitory concentrations on *C.albicans*

Antibiotic dose is usually calculated with respect to MIC to achieve therapeutic levels in the body. However, the concentration of antibiotic to which microbial pathogens are exposed *in vivo* will be governed by several factors; including the pharmacology and pharmacokinetic properties of the drug, and site of infection. Therefore, it is likely that microorganisms will be exposed to concentrations well below the MIC, i.e. sub-inhibitory concentrations. Such concentrations, although not microbicidal, may render an organism more susceptible to host defence mechanisms.

Evidence has accumulated that sub-inhibitory concentrations of antibiotics may have multiple effects on bacteria (Lorian, 1980). These effects include decreased growth rate, morphological alterations, enhanced leukocyte ingestion and killing, as well as reduced adhesion to epithelial cells (Lorian, 1980; Shibl, 1985). Only recently have the effects of sub-inhibitory concentrations of antifungal agents on *C.albicans* received attention.

For microorganisms to survive and multiply during infection, they must first have the ability to adhere to host mucosal surfaces. The production of germ tubes by *C.albicans* results in an increase in adherence to buccal and vaginal epithelial cells and , as well as an increase in its pathogenicity (Kimura and Pearsall, 1978; Sobel *et al.*, 1984). Sub-

inhibitory concentrations of azoles (Sobel and Obedeanu, 1983; Plempel and Berg, 1984) and polyenes (Shibl, 1985; Nugent and Couchot, 1986) have been shown to reduce germ tube formation. Inhibition of germ tube formation was associated with decreased adherence (Shibl, 1985). Exposure to sub-inhibitory concentrations of ketoconazole produced profound clumping of yeast cells resulting in large aggregates of somewhat dystrophic-looking yeasts (Sobel and Obedeanu, 1983). In addition, yeast cells pretreated with sub-inhibitory concentrations of bifonazole or clotrimazole resulted in reduced pathogenicity amongst mice compared to untreated cells (Plempel and Berg, 1984), while prophylactic treatment of rats with low-dosage ketoconazole was effective in preventing experimental candidal vaginitis (Sobel and Muller, 1984).

Although the primary target site of Amp B is the PM, sub-inhibitory doses induce significant Cw changes which involve mannoproteins. Using immunofluorescence techniques, Al-Bassam *et al.* (1985) demonstrated that these alterations resulted in a loss of surface antigen immunogenicity. Brief exposures to sub-inhibitory concentrations of Amp B accelerated clearance of *C.albicans* from peritoneal surfaces of mice and reduced the inflammatory stimulus associated with this clearance, as measured by the neutrophil influx. However, pretreatment of yeast cells did not facilitate killing of *C.albicans* by either neutrophils or monocytes *in vitro* (Nugent and Couchot, 1986). In comparison, pretreatment of *C.albicans* with a sub-inhibitory dose of ketoconazole followed by incubation with PMNs and macrophages resulted in eradication of the fungus. This observation suggested a synergistic interaction between ketoconazole and phagocytic cells, whereby inhibition of hyphal formation by the drug facilitated engulfment and

killing of the organism by phagocytic cells (Borgers, 1980). Thus, a subtle combination of effects mediated by antibiotics combined with the host response may influence course and severity of infection. Furthermore, in the absence of direct microbial interaction, sub-inhibitory doses of Amp B may also show immunoadjuvant properties which may in turn influence its *in vivo* effects and play a role in therapy.

In vitro, low doses of Amp B increases phagocytosis, while higher doses result in decreasing phagocytic capacity until a lethal concentration is attained. Low doses appear to activate or prime surfaces of macrophages. These effects may complement the lethal action of Amp B on suppressor T-cells and produce immunoadjuvant properties (Little *et al.*, 1978; Medoff and Kobayashi, 1983). *In vivo* studies have confirmed *in vitro* efficacy of Amp B against a variety of fungi at tolerable drug levels. Sub-inhibitory doses of Amp B resulting in undetectable blood levels have been effective therapeutically in several animal models of infection. This observation was supported by the findings of Little *et al.* (1978) who demonstrated that single intraperitoneal injections of Amp B significantly enhanced the quantity of antibody-producing cells in the spleens and lymph nodes of mice. The effect of Amp B on the seroconversion from IgM to IgG; its reduced effectiveness in thymectomized mice; and its toxicity *in vitro* for thymocytes and a subpopulation of T-cells, have led to the hypothesis that the immunological effects of Amp B may require interaction with a specific T-cell population. In addition to its effect on humoral immunity, Amp B can augment delayed-type hypersensitivity reactions and cell-mediated immunity (Medoff and Kobayashi, 1983).

In contrast to the studies outlined above, observations of Petrou and Rogers (1988)

suggested that sub-inhibitory concentrations of polyenes and imidazoles may protect *Candida* species from inhibitory serum components. Inhibitory factors consisted of a combination of transferrin, IgM and an uncharacterized serum protein. The mechanism of drug action is unclear, but it may be via (i) direct interaction with inhibitory factor; (ii) interference with inhibitory factor's substrate, such as iron; or (iii) action of the drug on the organisms' surface, altering the Cw composition in such a manner that binding of IgM is inhibited.

Low-dosage regimens of Amp B have been particularly successful in patients with infections limited to mucosal surfaces of the esophagous or urinary tract (Medoff *et al.*, 1972, 1983), and evaluated in an attempt to reduce the incidence of invasive candida infection in immunosuppressed liver transplant patients (Husberg *et al.*, 1988). The basis for effectiveness of these low-dose regimens may be a result of non-lethal effects of Amp B on fungi, or immunoadjuvant properties which act on the host immune system; or both.

Occasionally, large vegetations on prosthetic valves observed in *Candida* as well as bacterial endocarditis are unresponsive to antifungal therapy, even though the aetiological agents are susceptible *in vitro* (Rubenstein *et al.*, 1975). This particular phenomenon serves to remind us of the phenotypic plasticity of microorganisms and their ability to form biofilms to protect themselves from antibiotics and host defence mechanisms (Costerton *et al.*, 1987).

1.8. Host immune responses

The outcome of fungal infections are determined largely by interactions between fungi and host defence mechanisms. Induction and expression of cellular immunity is dependent upon a complex sequence of interactions among antigen, macrophages and lymphocytes. Although the mechanisms involved remain to be elucidated, it appears that a deficiency in T-cell function markedly reduces resistance to most fungal diseases (Cox, 1983). In addition to cellular immunity, exposure to fungi evokes a range of specific and non-specific humoral responses within a normal host. Production of specific antibodies in response to infection has received increasing attention as potential markers for serodiagnosis, however, interaction of antibodies with other host defence mechanisms may be of greater significance (Diamond, 1983).

Besides antibodies, complement and other non-specific host humoral systems can interact with other host defence mechanisms in ways that may profoundly influence the course of infection. For example, an ability of pathogenic microorganisms to acquire iron within a host is an important determinant of pathogenicity (See section.1.4.). Although availability of iron is restricted by iron-binding glycoproteins in the host, these glycoproteins may interact with fungi directly and influence their growth and survival *in vivo*.

1.8.1. Humoral responses of the host

The major circulating type of antibody (Ab) is IgG. This molecule is composed of two heavy (γ) and two light chains (χ or λ) held together by disulphide bonds to give the shape of a Y with a molecular weight of 150Kd (Fig.1.4.). IgG is split into three regions;

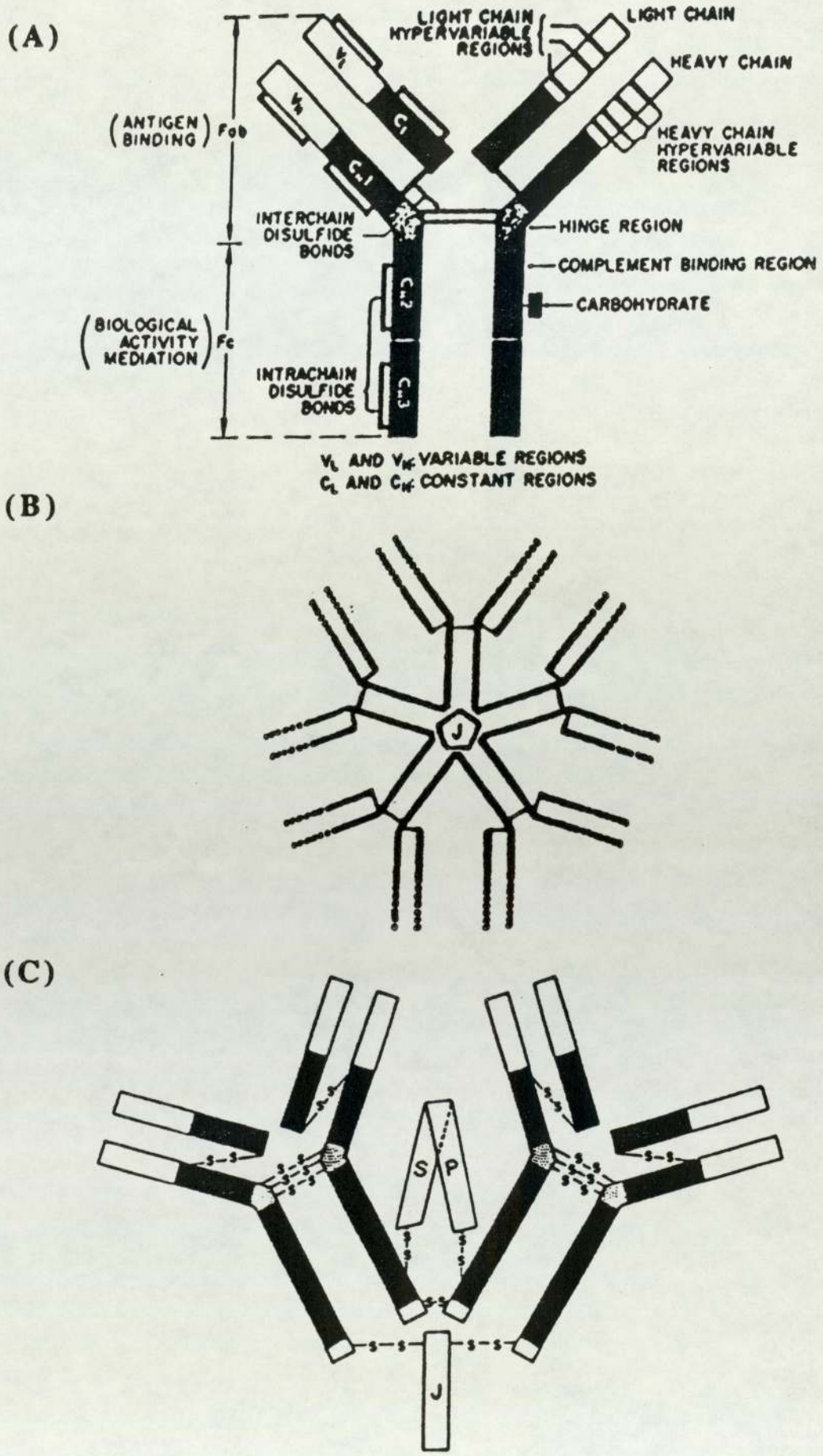


Fig.1.4A, B and C. Structure of immunoglobulins (A) IgG, (B) IgM and (C) IgA.

two of these represent the arms of the Y (Fab) and contain antigen-reactive sites, while a third part (Fc) has no antigen-reactive sites but activates complement (C') and binds to the surface of polymorphs and macrophages. There are four subclasses of IgG in man which differ in their heavy chains and in their biological properties, such as complement fixation and binding to phagocytes (Mims, 1982).

Most normal individuals have circulating IgG Abs directed against surface mannan antigens of *Candida* (Weiner and Yount, 1976). These serum Ab titres tend to be higher in association with local invasive infections, such as in chronic mucocutaneous candidosis (Solomkin *et al.*, 1978), recurrent oral (Lehner, 1970) and vaginal candidosis (Warnock *et al.*, 1978). In addition, anti-*Candida* IgG partially derived from the circulation is detectable in vaginal secretions of approximately 20-25% of women with or without active vaginal candidosis or detectable *Candida* colonization (Milne and Warnock, 1977). In invasive, disseminated candidosis, the level of the specific IgG Ab response depends on the capacity of the host immune mechanisms.

IgM is a polymer of five subunits (Fig.1.4.) with a basic four-chain structure but with a different heavy chain (μ), and a large molecular weight of 900Kd confining it to the vascular system. It has five times the number of Fc and Fab sites as IgG, hence, has high avidity and complement-activating capacity. IgM is formed early in the immune response and may influence the course of infection, promoting rapid recovery and reduction in pathological changes (Mims, 1982). Early production of IgM antibodies is soon replaced by IgG antibodies, therefore the presence of IgM indicates either recent or persistent infection.

IgA consists of two subunits of the basic four-chain structure termed α heavy chains (Fig.1.4.). The molecule acquires a "secretory piece" when secreted locally onto mucus membranes, however, this piece is lost when IgA circulates systemically. Increased titres of IgA have been detected in patients with oral candidosis, and secretory IgA (SIgA) is thought to be important in limiting oral infections. IgA and SIgA appear to have little ability to activate complement, but SIgA may activate complement through the alternative pathway (Epstein *et al.*, 1984; Mims, 1982). In the oral cavity, IgA may function by aggregating yeast cells and/or preventing their adherence to mucosal epithelial cells (Kimura and Pearsall, 1978).

1.8.2. Opsonization

Opsonization is a process whereby serum components (opsonins) bind with surfaces of microorganisms and promote phagocytosis. Two types of opsonins have been described: one is represented by C3b and C3bi fragments of the third component of C' (C3), and the other is represented by specific Abs (usually IgG).

Opsonization can occur in three modes. In the first, only Abs participate by reacting via their $(Fab)_2$ binding sites with specific antigenic determinants on microbial surfaces. The Fc portion of IgG binds microorganisms to Fc receptors on the phagocyte surfaces. In the second mode C3b and C3bi fragments are generated from C3 as a result of its activation through contact with certain microbial surface constituents. C3b or C3bi binds to the microbial surface covalently via CR1 or CR3 receptors, respectively. In the third mode, both Ab and C3b or C3bi participate. Ab binds to microbial surface antigens

(Ags), and the Ab-Ag complex generated activates complement, followed by deposition of C3b or C3bi onto the microbial surface. Microorganisms then bind to phagocytes via receptors for both ligands. This binding is stronger than with either IgG or the fragments of C3 alone (Winkelstein, 1973; Solomkin *et al.*, 1978; Ofek and Sharon, 1988).

Complement activation accounts for most of the serum opsonic activity induced by *C.albicans* (Solomkin *et al.*, 1978). However, specific Abs may be necessary to provide optimum kinetics of phagocytosis (Root *et al.*, 1972; Periera and Hosking, 1984). When *Candida* invades host tissues, pseudohyphal and hyphal forms develop which are too large to be completely engulfed by phagocytic cells. *In vitro*, neutrophils attach themselves to these morphological forms and damage them without ingestion. Anti-*Candida* IgG Abs enhance this process, whereas complement components do not (Diamond *et al.*, 1978).

1.8.3. Complement (C')

Phagocytosis of *C.albicans* yeast cells is critically dependent on opsonization by components of the C' cascade. In common with other Ab-Ag complexes, Abs with whole fungi or fungal products may activate the classical C' pathway. However, strong evidence indicates that an alternative C' pathway is the mechanism for opsonization of *C.albicans* by normal serum (Solomkin *et al.*, 1978; Ray *et al.*, 1979; Kagaya and Fukazawa, 1981).

In opsonization of *Candida*, C' activation appears to play a greater role in promoting phagocytosis by human neutrophils (Lehner, 1970; Solomkin *et al.*, 1978) than by

monocytes (Cline and Lehrer, 1968). When soluble surface mannans from *Candida* are added to normal human serum, the alternative C' pathway is activated as revealed by conversion of properdin factor B and activation of C3 (Weiner and Yount, 1976). In addition to generation of opsonins, local C' activation can yield chemotactic factors at infectious foci. Alone, neither whole *Candida* cells nor metabolic products are chemotactic, however, *Candida* activation of the alternative C' pathway in serum generates products chemotactic for neutrophils (Ray and Wuepper, 1976). C'-mediated accumulation of neutrophils in lesions appears to occur *in vivo* (Sohnle *et al.*, 1976a). Furthermore, lesions from human patients with chronic mucocutaneous candidosis often contain deposits of properdin B or C3 (Sohnle *et al.*, 1976b). Generation of chemotactic factors may therefore be responsible for the intense inflammatory response seen in this disease; the cellular response may prevent deeper spread of infection but is insufficient to clear all fungi from local lesions.

Human peripheral blood cells, such as natural killer cells, neutrophils, lymphocytes and monocytes possess glycoprotein membrane receptors (CR1, CR2, CR3, and CR4) that bind with the cleavage fragments of C3 (C3b, C3bi and C3d). C' component C3 is important for phagocytosis of *C.albicans* and plays a key role in amplification of the C' cascade (Yamamura and Valdimarsson, 1977; Morrison and Cutler, 1981; Kozel *et al.*, 1987). Degradation of covalently bound C3b to C3bi by serum proteases during opsonization generates an active ligand that interacts with the CR3 receptor on phagocytic cells to promote phagocytosis and stimulate release of intracellular microbicidal agents such as superoxide or myeloperoxidase (Gordon *et al.*, 1986).

Noncovalent attachment of C3b and its fragments inhibits opsonization, abolishes phagocytosis (Hostetter *et al.*, 1984), and may reorientate C3 fragments so that their binding sites or phagocytic receptors are masked. Recently, it has been shown that surface receptors for the C3bi fragment are present on *C.albicans*, and that this receptor circumvents phagocytosis, both by noncovalent binding of C3bi and by molecular mimicry of a phagocytic receptor on the neutrophil (Fig.1.4.) (Gilmore *et al.*, 1988). Furthermore, an increase in receptor expression was observed in response to elevated concentrations of glucose or induction of mycelium production. Existence of such surface receptors for C3bi is believed to function as a virulence factor preventing opsonization and in turn phagocytosis of the organism.

Receptors for fragment C3d on *C.albicans* have also been described (Heidenreich and Dierich, 1985; Edwards *et al.*, 1986; Calderone *et al.*, 1988; Linehan *et al.*, 1988), however, the mechanisms by which they may participate in pathogenesis have not been defined.

1.8.4. Cell-mediated immunity (CMI)

Induction and expression of CMI in fungal diseases is dependent upon a complex sequence of interactions between antigens and phagocytic cells. T-cell stimulation requires antigen presentation on the macrophage surface. Specifically sensitized T-cells respond to antigen by proliferation and release of lymphokines, which function to (i) localize and activate macrophages (migration inhibitory or activating factor); (ii) act

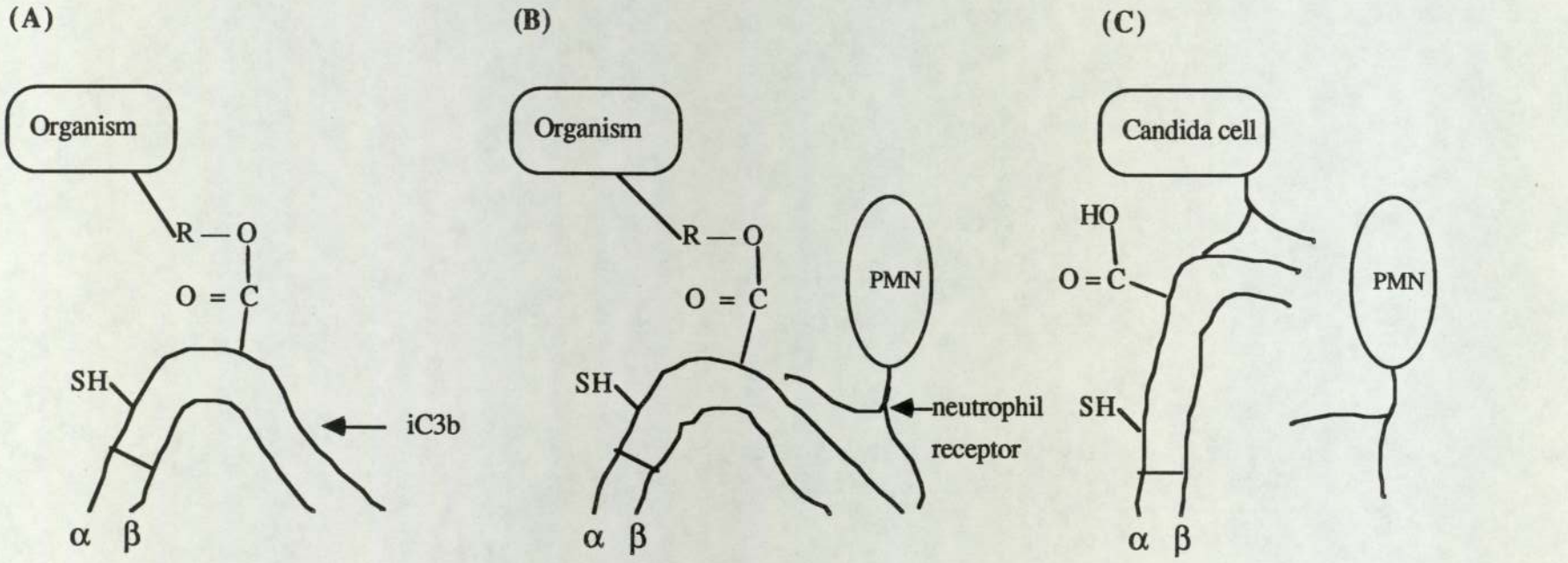


Fig.1.5. Schematic representation of the mechanism by which the candidal receptor binds iC3b noncovalently and prevents recognition by neutrophil CR3. (A) iC3b is shown covalently attached to the organism. In this form, iC3b can trigger phagocytic recognition by neutrophil receptors (B). When iC3b is noncovalently attached to the candidal receptor (C), the recognition site for neutrophil CR3 is masked.

directly on lymphocytes (lymphocyte chemotactic factor); or (iii) mediate killing or inactivation of target cells. Although much remains to be elucidated about the mechanisms involved, there exists extensive clinical evidence that links T-cell deficiency with superficial and systemic candidosis (Odds, 1979; Cox, 1983).

1.8.5. Phagocytic mechanisms in host response

Phagocytic cells express two types of antimicrobial mechanisms: oxygen-dependent and oxygen-independent. In the former mechanism, phagocytosis results in activation of NAD(P)H oxidase (a membrane-associated enzyme system) (Klebanoff, 1980), which catalyzes oxidation of a pyridine nucleotide and reduction of two oxygen molecules to yield the superoxide anion (O_2-). Superoxide anions are relatively unstable and undergo rapid dismutation to H_2O_2. H_2O_2 and O_2^- undergo additional reactions, especially when trace amounts of transition metal ions are present. For example, more powerful oxidizing species such as hydroxyl radicals ($OH^\bullet$) are generated by the reaction:

$$H_2O_2 + Fe^{2+} \rightarrow OH^\bullet + Fe^{3+}$$

Iron bound to lactoferrin, a constituent of neutrophil granules, can support $OH^\bullet$ production in this process (Ambrusco and Johnston, 1981). In addition to greatly augmenting their oxygen consumption to produce molecules such as O_2^-, H_2O_2 and $OH^\bullet$ that can in turn be directed against the fungal target cell, phagocytic cells may contain an enzyme, myeloperoxidase (MPO), that markedly potentiates antimicrobial effects of H_2O_2.

MPO-mediated activity requires H_2O_2 and an oxidizable cofactor such as iodide or

chloride ions in order to kill yeast cells and hyphae *in vitro* (Lehrer, 1969; Diamond *et al.*, 1980). Neutrophils appear to attach and spread over surfaces of pseudohyphae, then degranulate and damage the organisms (Diamond *et al.*, 1978). Neutrophils from patients with hereditary MPO deficiency phagocytose *Candida* normally, but fail to kill hyphae. MPO and H_2O_2 appear essential for effective killing of *C.albicans* by human neutrophils and monocytes (Lehrer and Cline, 1969; Lehrer, 1975; Diamond *et al.*, 1978), whereas other *Candida* species such as *C.parapsilosis*, *C.pseudotropicalis*, and *C.krusei* are killed effectively by MPO-deficient neutrophils (Lehrer, 1972). Thus, these species are killed by oxygen-independent mechanisms that do not require either MPO or H_2O_2.

Studies with intraperitoneally placed diffusion chambers in mice point to the important contribution of phagocytes to clearing sites of *C.albicans in vivo* (Cutler and Poor, 1981). The increased incidence of fungal infection among patients with phagocytic disorders suggests that intact phagocyte function plays a critical role in the defence against candidosis.

Considerable evidence has accumulated demonstrating that specific recognition between phagocytes and microbial surfaces may be accomplished by interaction of carbohydrate (CHO)-binding proteins. This lectin-CHO interaction , which may act as a non-opsonic mechanism of phagocytosis and function *in vivo*, has been termed lectinophagocytosis (Ofek and Sharon, 1988). It has been postulated that lectino-phagocytosis of bacteria can occur when bacteria which carry surface lectins bind to complementary CHOs on the surfaces of phagocytic cells; or conversely when lectins that

are also integral components of the phagocytic cell surface bind to CHOs on the bacterial surface.

Lectinophagocytosis of *E.coli* mediated by mannose-specific lectin associated with type 1 fimbriae has been thoroughly investigated (Ofek and Sharon, 1988). Subsequently, Sharon (1984) and Sharon and Ofek (1986) demonstrated that the phagocyte receptor for mannose-specific bacteria appears to be glycoprotein in nature. Lectinophagocytosis may be important in the defence against bacteria which evade opsonization. In particular, this may be the case for bacteria that do not activate the alternative complement pathway (Peterson and Quie, 1981; Fearon and Austen, 1980), or for bacteria that invade serum-poor sites (Goldstein *et al.*, 1974; Silverblatt *et al.*, 1979) or a complement-deficient host (Aguello, 1978).

In view of the glycoprotein nature of *C.albicans* surfaces, it remains to be determined whether future studies on lectinophagocytosis *in vitro* and *in vivo*, will lead to a better understanding of the mechanisms of interaction between *C.albicans* and host immune systems.

2. MATERIALS

2.1. Organisms

All strains of *Candida albicans* were clinical isolates except C406, and were kindly donated by the individuals named in Table 2.1. Isolates AU1, AU2, OMC3 and 4208 were identified by Dr. C.M. Fraser (Candidal Typing Unit, Mycology Reference Laboratory, Public Health Laboratory Service, Colindale Ave, London.). Isolates C55 and C406 were identified by Dr. D. Loebenberg (Schering-Plough Corporation, New Jersey, USA.). All strains were maintained on Sabouraud Dextrose Agar (Oxoid, Basingstoke, Hants.) slopes at 4°C and subcultured at monthly intervals.

Table 2.1. *C.albicans* strains employed in study.

Isolates	Site of body isolated	Source of isolates
AU1	Vagina and cervix	General Hospital, Birmingham.
AU2	Wounds, feeding line, intestine, urine and sputum	Mr.D.Sinfield, Pathology Department, Hackney Hospital, London.
OMC3	Oral cavity	Mr.J.Hamburger, Oral Medicine Clinic, General Hospital, Birmingham.
4208	Vagina and cervix	Dr.J.Clay, Venereal Diseases Clinic, General Hospital, Birmingham.
C55	Urinary catheter	Dr.D.Loebenberg, Schering Corporation, New Jersey, USA.
C406	Laboratory strain	" " "

2.2. Chemicals

All chemicals and reagents not specified in the text were of analytical grade or equivalent and supplied by BDH Chemicals Ltd. (Poole, Dorset), Sigma Chemical Company (Poole, Dorset) or DakoPatts (DAKO Ltd.) Immunochemical Reagents (High Wycombe, Bucks.). Amphotericin B and Nystatin were supplied by E.R. Squibb & Sons Ltd. (Wirral, Merseyside) and Fluconazole was kindly donated by Dr.P.F.Troke (Pfizer Central Research, Sandwich, Kent.).

2.3. Human serum

Blood was collected by venepuncture from patients and serum was separated and stored at -20°C. Blood was similarly obtained from three male and four female healthy volunteers of age range 23 to 45 years, with no history of *C.albicans* infection (Table 2.2).

Table 2.2. Human sera employed in study.

	Candida infection in patient	Supplier of sera
1	Systemic	Mr.D.Sinfield, Pathology Department, Hackney Hospital, London.
2	Oral	Mr.J.Hamburger, Oral Medicine Clinic, General Hospital, Birmingham.
3	Vaginal	Dr.J.Clay, Venereal Diseases Clinic, General Hospital, Birmingham.

2.4. Equipment

Balances: Sartorius 1702 (Sartorius Instruments Ltd., Belmont, Surrey), Oertling HC22 (Oertling, Orpington, Kent), Mettler P 165 and Type H 16 (Gallenkamp, Loughborough, Leicester).

Centrifuges: Beckman J2-21 (Beckman Instruments Inc., High Wycombe, Bucks), Eppendorf S 412 bench centrifuge (Baird and Tatlock Ltd., Atherstone, Leicester) , MSE Minor and Centaur 2 (MSE Ltd., Crawley, Sussex.) and model HN-S centrifuge (International Equipment Company, Mass., USA)

ELISA equipment: Microtitre plates (L.I.P. Ltd., Shipley, West Yorkshire), Plate washer model 120 and Plate reader, Titertek Multiscan model 310 C (Flow Laboratories Ltd., Irvine, Scotland).

Freeze dryer: Edwards Modylo freeze dryer (Edwards High Vacuum Ltd Crawley, Sussex).

Gel electrophoresis: Mini-Protean system 125 model II (Bio-Rad Laboratories Ltd., Watford, Herts.).

Immunoblotting apparatus: Transblot Cell (Bio-Rad Laboratories Ltd., Watford, Herts.).

Incubators: Size one incubator model 1H-150, G-25 shaking water bath and an orbital shaker (Gallenkamp Ltd., Loughborough, Leicester).

Membrane filters: Gelman Acrodisc 0.22µm and 0.45µm (Gelman Sciences, Brackmills, Northampton).

Microscope:	Olympus BH (Olympus Optical Company Ltd., Japan).
Microtitre plates for agglutination assays:	U-bottomed polystyrene plates (Becton-Dickenson Labware, California, USA).
Peristalsis pumps:	Model P3 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and Gilson Minipuls 2 (Anachem Ltd., Luton, Beds.).
pH meters:	PTI-15 digital pH meter (Fisons Scientific Apparatus, Loughborough, Leicester) and a HI 8417 Microprocessor bench meter (Hanna Instruments, Limena, Italy).
Pipettes:	Gilson pipetman P-20, P-200, P-1000 and P-5000 (Anachem Ltd., Luton, Beds.).
Power packs:	Bio-Rad model 500/200 for electrophoresis and model 250/2.5 for immunoblotting (Bio-Rad Laboratories Ltd., Watford, Herts.).
Scintillation counter:	LS 5801 (Beckman Instruments Incorporated, Palo Alto, California, USA).
Scintillation vials:	Borosilicate 20ml disposable vials (Kimble Division of Owens, Ohio, USA).
Spectrophoto-meters:	LKB Novaspec model 4049 (LKB Biochrom Ltd., Cambridge), Beckmann DB-GT model (Beckmann Instruments Incorporated, Palo Alto, California, USA) and an atomic absorption model 560 fitted with a deuterium background corrector, a model HGA graphite furnace with argon gas flow, a model HGA 500 programmer and a

model 56 chart recorder (Perkin-Elmer Ltd., Beaconsfield, Bucks.).

Whirlimixers : Fisons Scientific Apparatus, Loughborough, Leicester

3. EXPERIMENTAL METHODS

3.1. Preparation of growth medium

The removal of iron from growth medium was either by chelex treatment of a solution of glucose and asparagine, or by use of the chelator 1'10-Phenanthroline (OP).

3.1.1. Chelex treatment

Chelex 100 (Bio-Rad Laboratories Ltd.) is a chelating ion-exchange resin with a high affinity for divalent cations. A glass column (20 x 400 mm) was packed with 100 ml of chelex resin. The chelex was regenerated by passing 200 ml of 1M HCl at 80°C down the column, then washed with 500 ml of double distilled water followed by 200 ml of 1M NaOH. Following another wash with double distilled water, 0.66M sodium phosphate buffer was passed down the chelex until pH 7.4 was obtained. Asparagine and glucose were dissolved in double distilled water to 1L and the solution pumped through the column at a rate of 2.45 ml/min for 36h.

To the iron-depleted eluate the following concentrations were added: 45mM $(NH_4)_2SO_4$, 85mM NaCl, 1.4mM K_2HPO4, 0.8mM $MgSO_4.7H_2O$ and 0.67mM L-Methionine. The medium was adjusted to pH 4.5 with 1M HCl. After autoclaving at 115°C for 20 min, the medium was supplemented with 0.4mM Biotin from a filter-sterilized stock solution. The iron content of the complete iron-depleted simple salts medium (SSM-Fe) is shown in Table 3.1. (section 3.3.). Iron-sufficient simple salts medium (SSM+Fe) was the same as SSM-Fe with the addition of 100 mM $FeSO_4.7H_2O$.

C.albicans is capable of converting from the budding to the mycelial mode of growth, and conversely, and transitions between these phenotypes can be regulated *in vitro* by pH (Mitchell and Soll, 1979; Buffo *et al.*, 1984). Throughout this study cells were grown in the budding mode in SSM at 25°C to stationary phase, and these cells were released into fresh medium at 25°C or 37°C at pH 4.5 to induce bud formation. At pH 4.5 cells evaginate synchronously after a lag period of approximately 135min and each evagination expands into an ellipsoidal bud.

3.1.2. 1'10-Phenanthroline treatment

1'10-Phenanthroline (OP) is a chelator with a high affinity for ferrous ions but it also binds zinc and other cations (Schulman and Dwyer, 1964). Stock solutions at a concentration of 1mM were prepared in double distilled water and sterilized by autoclaving at 115°C for 15 min. Various concentrations of the chelator were aseptically added to SSM prior to cell inoculation.

3.2. Preparation of Glassware

All glassware was immersed overnight in 5% (v/v) Extran 300, once in single distilled water followed by three rinses in double distilled water, then soaked overnight in 0.01% (v/v) ethylenediaminetetra-acetic acid (EDTA). The glassware was then rinsed six times in double distilled water (Kadurugamuwa *et al.*,1987).

3.3. Growth studies

3.3.1. Measurement of yeast cell concentration

Spectrophotometric measurement of the optical density (OD) of a bacterial suspension was used to measure yeast cell concentration. At low cell concentrations, a linear relationship exists between OD and cell concentration, as expressed by the Beer-Lambert law :

$$OD \propto Io/I$$

where Io = incident light and I = emergent light

This relationship does not hold at higher cell concentrations due to secondary light scattering (Meynell and Meynell, 1970). In this study, the Beer-Lambert law was obeyed up to an OD 0.25. Above this, OD values were less than expected for a given yeast population. Linearity was restored by dilution of the suspension to an OD below 0.25. A wavelength scan of the cell-free supernatants from iron-depleted and iron-sufficient stationary phase cultures revealed that absorption by media constituents and yeast metabolic products was negligible at 660 nm. This wavelength was selected for measurements of OD.

3.3.2. Growth measurements

Yeast growth was measured by observing changes in OD with time. Cells collected from a slope were washed with double distilled water then inoculated into 125 ml Erlenmeyer flasks containing 25ml SSM-Fe. After shaking at 150 rpm for 48h at 25°C, an aliquot of culture was transferred to similar flasks containing fresh SSM-Fe. Cultures serially

transferred from this second culture were used for experimental purposes as follows: stationary phase cells were harvested by centrifugation at 2500 g for 5 min, washed twice and resuspended in double distilled water. The cells were reinoculated into fresh SSM-Fe, prewarmed in a flask at a concentration of 2 x 10^6 cells/ml. Yeast phase organisms were obtained by shaking flasks at 150 rpm at 25°C or 37°C. Samples for measurement of OD were removed aseptically at appropriate intervals and diluted when necessary.

3.4. Antifungal antibiotic susceptibility test

3.4.1. Determination of minimum inhibitory concentration (MIC) of antifungal antibiotics

Drugs were dissolved in dimethyl sulphoxide (DMSO) to prepare stock solutions then dispensed in 5ml aliquots and stored at -20°C. DMSO provides a suitable medium in which to dissolve drugs, and abolishes any problems associated with water insolubility of these compounds, particularly with high concentrations. MICs of antibiotics were determined by the standard two-fold dilution method (Doern *et al.*, 1986). From stock solutions of drug, two-fold serial dilutions were prepared in 1ml volumes using SSM-Fe, SSM+Fe or untreated SSM, to give concentrations ranging from 8 to 0.06µg/ml. Tubes were inoculated with 1ml of yeast suspension prepared from an overnight culture grown in SSM-Fe at 37°C to give a final concentration of 1-3 x 10^5 cells/ml in each tube. Tubes, including growth and sterility controls, were mixed, then incubated at 37°C for 48h and examined for macroscopic evidence of turbidity after 24h and 48h incubation. All tube

dilutions were prepared in duplicate. MICs were defined as the lowest concentration of antibiotic tested which yielded no turbidity.

3.4.2. Preparation of sub-inhibitory concentrations of antifungal antibiotics

Sub-inhibitory concentrations of antibiotics were prepared in SSM-Fe, SSM+Fe and untreated SSM, and growth monitored by optical density measurements. Aliquots of culture were removed at intervals and transferred to 1% (v/v) formalin in normal saline for morphological examination. Three sub-inhibitory concentrations were chosen for each antibiotic based on the following criteria: the first concentration chosen for each antibiotic did not affect growth rate whereas the second and third concentrations did alter growth rate compared to control cultures. Although no visible evidence of morphological changes were observed during growth in the presence of these concentrations of drugs, it does not preclude the possibility that ultrastructural changes may have occurred.

3.5. Atomic absorption spectroscopy for the determination of iron

An atomic absorption spectrophotometer was used to measure the iron concentration in complete SSM. A calibration curve was constructed using serial dilutions of a standard iron solution (Spectrosol grade, BDH Ltd.) and the iron concentration determined. Chelex treatment of SSM reduced the iron concentration by approximately 26% (Kadurugamuwa *et al.*, 1987) (Table 3.1.).

Table 3.1. Iron concentration of SSM.

Treatment	Mean iron concentration (μM)*	Percentage (%) reduction in iron
Before chelexing	0.47±0.18	-
After chelexing	0.35±0.15	25.53

* values represent means ± SD of six experiments.

3.6. Adherence assays

3.6.1. Adherence to a water-hydrocarbon interface

The method of Rosenberg *et al.* (1980) was modified and used to determine the relative cell surface hydrophobicity (CSH). Cells were harvested, washed and resuspended in cold phosphate-urea-magnesium buffer (PUM buffer), pH 7 containing 97mM K_2HPO_4. $3H_2O$, 53mM KH_2PO_4, 30mM urea and 0.8mM $MgSO_4$. $7H_2O$. Cells were adjusted to an absorbance of 0.4, then 1.2 ml of the suspension was placed in acid-washed glass tubes (12 x 75 mm) and covered with 0.3 ml of hydrocarbon. After incubation at 37°C for 15 min, the phases were mixed by vortexing for 2 min and allowed to stand for 15 min. Once the phases were separated, the aqueous lower layer was removed and its absorbance at 660 nm (A_{660}) determined. The relative CSH was obtained from the following equation:

$$\text{Percentage change in } A_{660} = \frac{(A_{660} \text{ of controls}) - (A_{660} \text{ of treated cells})}{A_{660} \text{ of controls}} \times 100$$

In agreement with Klotz *et al.* (1985), similar trends were observed in hydrophobic affinity of the *Candida* strains to four hydrocarbons tested, i.e. toluene > xylene > hexadecane > octane; studies were therefore restricted to toluene.

3.6.2. Concanavalin A agglutination assay

Concanavalin A (Con A) is a lectin with a high affinity and specificity for α-D-mannopyranosyl residues, including yeast mannan. Con A was supplied immobilized on sepharose-4B beads (Sigma) in a solution containing 1M NaCl, 1mM $MnCl_2$, 1mM $MgCl_2$ and $CaCl_2$ (saline P). The beads were resuspended in 50% (v/v) saline P prior to use.

Yeasts grown to logarithmic and stationary phase were harvested by centrifugation at 2500 g for 10 min, washed in saline P and the OD_{660} of a 1 in 10 dilution in saline P adjusted to read 0.8. To each well in a microtitre plate was dispensed 20µl saline P, 40µl Con A sepharose beads, 40µl saline P and 20µl of an OD_{660} 0.8 yeast suspension, in the order prescribed. 60µl of 1M α-methyl-D-mannoside was added to appropiate wells as a control for inhibition of agglutination. Visualization of binding was carried out by light microscopy.

To quantify yeasts bound to the beads, 5 ml of a yeast suspension, prepared as described above, was pulse-labelled by adding 25µl of D-^{14}C (U) glucose (specific activity 1mCi per mM, CFB-96, Amersham Corporation, Illinois, USA) then shaken at 30°C for 45 min. Cells were washed twice with saline P and to duplicate tubes the

following was added in the order stated; 40µl saline P, 20µl of OD_{660} 0.8 pulse-labelled cells and 40µl Con A sepharose beads. Each tube was gently shaken and the mixture allowed to stand at 4°C for 15 min. The liquid above the beads was removed by aspiration, washed three times with saline P, then transferred to vials containing Aquasol universal liquid scintillation cocktail (NEN Research Products, Boston, USA) for radioactive analysis. Each sample was performed in triplicate. Control vials containing labelled cells without beads were included to assess non-specific binding.

3.6.3. *In vivo* adherence model

A hamster vaginal infection model was established by Dr. D. Loebenberg, Schering Corporation, New Jersey, USA. Strains AU1, C55 and C406 were grown to stationary phase in SSM containing 50µM 1'10-Phenanthroline then harvested by centrifugation at 2500 g for 10 min and washed in sterile normal saline. Washed cells were resuspended in Sabourauds' Dextrose broth to yield 10^8 organisms/ml. Oestrogenized hamsters were inoculated on three consecutive days by inserting a blunt-end needle into the vagina and irrigating the area with 50µl of the inoculum. 48h after the last inoculation, cotton swabs impregnated in normal saline containing cycloheximide and chloramphenicol were used to scrape the vaginal walls. The swab was immersed in 10ml of the same solution and vortexed for 60s to remove all organisms from the swab. Organisms were separated by passing 2ml of suspension through a 0.45µm membrane filter, then the filter rinsed in saline and placed on the surface of a mycosil agar plate. Plates were examined for

colonies after incubation at 37°C for 48h. Ten hamsters per yeast strain were included for statistical validity.

3.7. Siderophore assays

3.7.1. Detection of siderophores

A highly sensitive chemical assay for the detection of siderophores, which is based on their affinity for iron (III) and is therefore independent of the structure was used as described by Schwyn and Neilands (1987).

When a strong chelator is added to a highly coloured iron-dye complex, the chelator removes the iron from the dye, releasing free dye, which manifests as a colour change (blue to orange). The chrome azurol S (CAS) (Sigma) assay solution was prepared as follows : 6ml of hexadecyltrimethylammoniumbromide (HDTMA) (Sigma) was placed in a 100ml volumetric flask and diluted with double distilled water. A mixture of 1.5ml iron (III) solution (1mM $FeCl_3$. $6H_2O$ and 1mM HCl) and 7.5ml 2mM aqueous CAS solution was slowly added while stirring in water. 4.3g anhydrous piperazine (Sigma) was dissolved in water and 6.25ml of 12M HCl was carefully added. This solution was rinsed into the volumetric flask and the volume made up to 100ml with water. The CAS shuttle solution was obtained by adding 5-sulfosalicylic acid (Sigma) to the above solution at a concentration of 4mM. The solution was stored in plastic bottles in the dark and used within one week.

For determination of siderophores in solution, cultures were incubated for a period of

20 days, during which 1ml volumes of culture were removed at intervals. Cells were separated by centrifugation at 2500 g for 5 min and the supernatant tested immediately for siderophores or stored at -20°C. 0.5ml of culture supernatant was mixed with 0.5ml CAS shuttle solution. After reaching equilibrium by allowing the mixture to stand for 1h, the absorbance was measured at 630 nm. Desferal (Ciba-Geigy, Basel, Switzerland) was used as a positive control and from the equation:

$$\text{Percentage reduction in } A_{630} = 100 - \frac{A_{630} \text{ sample}}{A_{630} \text{ uninoculated medium}} \times 100$$

a calibration curve was plotted of percentage reduction in A_{630} against Desferal concentration to quantify siderophores in terms of Desferal equivalents.

3.7.2. Ferric perchlorate test for the detection of hydroxamate-type siderophores

The production of hydroxamate-type siderophores was detected by mixing 1ml of culture supernatant with 1ml of 5mM ferric perchlorate in 0.14M perchloric acid as described by Atkin and Neilands (1968). The reaction between hydroxamate-type siderophores and ferric perchlorate forms an iron complex, the absorbance of which is measured at 480nm. 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) (Sigma), aceto-hydroxamic acid (Sigma), Desferal (Ciba-Geigy, Basel, Switzerland) and catechol (Sigma) were used as controls.

3.7.3. Detection of phenolate-type siderophores

The method of Arnow (1937) was used to quantify phenolate-type siderophores. 1ml of cell-free supernatant or standard phenolate concentrations (L-DOPA) was placed in a test tube and the following reagents added in the order given: 1ml 0.5M HCl, 1ml nitrate-molybdate reagent (10% w/v sodium nitrate and 10% w/v sodium molybdate) and 1ml NaOH. After mixing the absorbance of the solution was measured at 515nm. A red colour indicated the presence of phenolate-type compounds.

3.8. Preparative techniques

3.8.1. Preparation of whole cells and cell walls

Yeast cells were grown to stationary phase then harvested by centrifugation at 2500 g for 5 min. After washing twice in cold distilled water, whole cells (Wc) were stored at -20°C.

Cells grown and harvested as above, were washed in cold distilled water and resuspended in 1mM phenylmethylsulphonyl fluoride (PMSF). 5ml of this suspension was added to an equal volume of glass beads (425-600 mm, Sigma) and cells were disrupted by vortexing for 3 min, with 30s intervals on ice. Approximately 90% cell breakage was observed by light microscopy. Cell walls (Cw) were sedimented by centrifugation and washed several times in 1mM PMSF until the supernatant was clear. The washed pellet was resuspended in 0.5ml PMSF and stored at -20°C (Chaffin and Stocco, 1983).

3.8.2. Absorption of sera

Stationary phase yeast cells grown in SSM−Fe were washed three times with saline and suspended at 10^{10} cells/ml. Cells were divided into three portions and absorption carried out by resuspending the washed pellet in 10ml of serum then incubated with agitation for 2h at room temperature. After the organisms were removed by centrifugation and the serum recovered, the absorption process was repeated with another portion of cells. The third volume of yeast cells was resuspended in serum and incubated with agitation at 4°C overnight. Finally the absorbed serum was sterilized by filtration through a 0.22μm membrane filter (Millipore) then stored at -20°C.

3.8.3. Production of hyperimmune antiserum

Yeasts were grown to stationary phase in SSM-Fe and washed in normal saline. Formaldehyde was added to a final concentration of 2% (v/v) and the culture incubated for 5h at 4°C. The yeast were harvested by centrifugation at 2500 g for 5 min, washed twice in normal saline and freeze-dried.

1mg of whole cell antigen was mixed with 1ml Freund's incomplete adjuvant (Sigma) and injected subcutaneously, twice a week for 8 weeks into male half-lop rabbits (New Zealand white, 4.0 kg). 4ml aliquots of blood were collected from the marginal ear vein after each week of immunizations and serum separated then examined for anti-*Candida* antibodies by slide agglutination, Western blotting and enzyme-linked immunosorbent assay (ELISA).

3.9. Analytical techniques

3.9.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Wc or Cw proteins were separated by gel electrophoresis on 12% gels in a discontinuous buffer system (Table 3.2.) as described by Lugtenberg *et al.* (1975).

Table 3.2. Composition of 12% gel for SDS-PAGE.

Solution	Running gel (ml)	Stacking gel (ml)	Sample buffer (ml)
Stock 1	3.15	-	-
Stock 2	-	1.0	-
10% (w/v) SDS	0.3	0.06	5.0
Tris-HCl (pH 8.8)	3.75	-	-
0.5M Tris-HCl (pH 6.8)	-	1.5	2.5
Distilled water	4.3	3.2	5.0
TEMED	0.028	0.16	-
10% APS	0.04	0.02	-
2-mercaptoethanol	-	-	0.3
5% (w/v) Bromophenol blue	-	-	0.2
Glycerol	-	-	2.5

Stock 1- 44% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bis acrylamide (Bis).

Stock 2- 30% (w/v) acrylamide and 0.8% (w/v) Bis.

TEMED- NNN'N' Tetramethylethylenediamine.

APS - Ammonium persulphate (freshly prepared).

The Mini Protean II gel system (Bio-Rad) with 0.5mm spacers was used. Samples were denatured by mixing with an equal volume of sample buffer (Table 3.2.) and heating to 100°C for 10 min. Samples were loaded onto the gel and electrophoresis

performed at a constant voltage of 200V until the tracking dye had arrived at the base of the gel. Gels were used either for Western blotting or stained for protein by immersing in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid. Destaining was carried over a period of 2-4h out in 5% (v/v) methanol and 10% (v/v) acetic acid, and finally rinsed in distilled water.

Molecular weights (MW) of proteins separated by SDS-PAGE were estimated from a calibration curve of standard MW markers (Sigma). The markers used were myosin (205Kd), B-galactosidase (116Kd), phosphorylase B (97Kd), bovine albumin (66Kd), ovalbumin (45Kd), B-lactoglobulin (18.4Kd) and lysozyme (14.3Kd).

3.9.2. Immunoblotting

Proteins separated by SDS-PAGE were transferred on to nitrocellulose (Nc) paper (pore size 0.45μm, Bio-Rad) as described by Towbin *et al.* (1979).

Nc paper was soaked in ice-cold transfer buffer containing 25mM Tris, 192mM glycine and 20% (v/v) methanol (pH 8.3), and positioned on a sheet of chromatography paper (Whatman Ltd., Maidstone, Kent). Following electrophoresis, the gel was placed on top of the Nc paper and overlayed with another sheet of chromatography paper. The Nc paper, gel and chromatography paper were sandwiched between two Scotch-brite pads (Bio-Rad) in a Transblot cassette and inserted in a Transblot cell (Bio-Rad) filled with transfer buffer. Efficiency of transfer is reported to be a function of MW (Burnette, 1981; Vaessen *et al.*, 1981), so these particular conditions were necessary to ensure complete transfer of high MW proteins. Electrophoretic transfer of proteins was carried

out at a constant voltage of 90V for 1h then at 50V overnight. Qualitative transfer of proteins was determined by staining with Aurodye forte (Janssen Life Sciences Products, Wantage, Oxon).

Following transfer, the Nc paper was incubated with gentle shaking for 1h at 37°C in a solution containing 0.3% (v/v)Tween 20, 0.9% (v/v) NaCl and 0.01M Tris-HCl (pH 7.4) (TTBS) to saturate non-specific binding sites on the Nc . The paper was rinsed six times in 0.01M Tris-HCl buffer (pH 7.4) containing 0.9% (v/v) NaCl (TBS) then incubated for 4h at 37°C in either rabbit or human serum at a dilution of 1 in 25 in TTBS with gentle shaking. Following further rinsing, the paper was incubated for 1h at 37°C in alkaline phosphatase-conjugated rabbit anti-human IgG, IgM or IgA (DAKO Ltd.) or alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), at a dilution of 1 in 1000 in TTBS with gentle shaking.

After rinsing in TBS as above, the paper was developed by adding a freshly prepared solution containing nitroblue-tetrazolium (0.33mg/ml) (Bio-Rad) and 5-bromo-4-chloro-3-indoyl phosphate (0.165mg/ml) (Bio-Rad) in alkaline phosphate buffer containing 100mM Tris, 100mM NaCl and 5mM $MgCl_2$ (pH 9.5). After 15 min the reaction was stopped by immersing the paper in a stop solution of 20mM Tris and 5mM EDTA (pH 8.0).

Immunoblotting of antigens with a variety of sera was performed by cutting the Nc paper into 5mm wide strips before incubating with serum. This technique allowed strips supporting replicate patterns of antigens to be probed with a range of sera.

3.9.3. Lectin blotting

Glycosylated components on the Nc paper were detected with concanavalin A (Con A) by the method of Hawkes (1982). The procedure was essentially the same as that described for immunoblotting. Instead of incubation in serum for 4h, the washed paper was gently shaken for 2h at 37°C in peroxidase-labelled Con A (Sigma) in TTBS (0.5mg/ml). After rinsing in TBS, the paper was visualized by adding a freshly prepared solution of 0.01% (w/v) H_2O_2 and 4-chloro-1-napthol (Sigma) (25µg/ml) in 10mM Tris-HCl (pH 7.4). The 4-chloro-1-napthol was first dissolved in a small amount of methanol and then added to the H_2O_2-Tris-HCl solution. The reaction was stopped after 10 min by rinsing with distilled water.

3.9.4. Transmission electron microscopy (TEM) with immunogold labelling

Yeast cells grown to stationary phase were harvested by centrifugation at 2500 g for 5 min, then washed twice in cold distilled water and fixed in 4% (w/v) paraformaldehyde (Sigma) in phosphate buffered saline (PBS) pH 7.4 , at 4°C. Cells were rinsed five times in PBS containing 0.1% (w/v) ovalbumin (Sigma) and 0.15% (v/v) Tween 20 (PBS-OT), then incubated with serum from a patient with systemic candidosis diluted 1 in 50 in PBS-OT for 4h at room temperature. Rinsing the cells in PBS-OT was followed by incubation with protein A gold 20 (Sigma) diluted 1 in 10 in PBS-OT for 90 min at room temperature. The cells were fixed after washing in Karnovskys fixative (Agar Scientific,

Stansted, Essex) containing 4% (v/v) formaldehyde and 5% (v/v) glutaraldehyde in cacodylate buffer (0.1M sodium cacodylate, pH 7.3).

Following rinsing in cacodylate buffer, cells were refixed in 2% (v/v) osmium tetroxide (Sigma) in cacodylate buffer for 2h at room temperature. Dehydration was performed in graded ethanol solutions and embedding carried out in Spurrs resin (Agar Scientific, Stansted, Essex). Sections were cut, mounted, then stained with a saturated solution of uranyl acetate (BDH Ltd.) and lead citrate (BDH Ltd.). Sections were viewed using a Philips 301 transmission electron microscope (Philips Scientific, Watford, Herts.) with an aperture of 50μm and at a voltage of 60KV by Geoff Cope (Department of Anatomy and Cell Biology, University of Sheffield, Sheffield). Electron micrographs were taken with Ilford electron microscope film, developed with a Kodak D19 developer and printed on Kentmere resin coated paper.

3.9.5. Enzyme-linked immunosorbent assay (ELISA)

Antibodies against cell surface antigens were measured by indirect enzyme-immunoassay (Engvall *et al.*, 1971; Kostiala and Kostiala, 1981).

Freeze-dried whole cells of *C.albicans* were dissolved in carbonate buffer (0.05M sodium carbonate and sodium bicarbonate, pH 9.6) to give 100μg/ml. Wells of polystyrene microtitre plates were coated with 200μl antigen solution by passive adsorption overnight at 4°C. Plates were washed twice with PBS containing 0.05% (w/v) Tween 20 (PBS-T). Non-specific binding sites were saturated by adding 250μl PBS to

each well for 1h at room temperature. After washing as described above, serial two-fold dilutions of sera were prepared in PBS-T and 100µl of each dilution added to separate wells, in triplicate. Plates were then incubated for 2h at room temperature and washed three times. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) or alkaline phosphatase-conjugated rabbit anti-human IgG, IgM or IgA (DAKO Ltd.), diluted 1 in 1000 in PBS-T was added to each well, and the plates incubated for 2h at room temperature.

After washing, 200µl p-nitrophenyl phosphate substrate solution (Sigma) (1mg/ml in 9.7% (v/v) diethylamine adjusted to pH 9.8 with HCl), was added to each well. Plates were incubated for 30 min at room temperature, then the enzyme-substrate reaction stopped by the addition of 50µl 3M NaOH. Absorbance of each well was measured at 405nm. The antibody titre was the highest dilution that resulted in an absorbance at least twice the mean of serial dilutions of negative controls.

3.10. Statistical analysis of experimental data

Adherence to a water-hydrocarbon interface

Percentage changes in absorbance were transformed by arc sine transformation (Bishop, 1966) to conform to a normal distribution. The effects of growth conditions, growth phase, or the presence of antibiotics on the adherence of yeasts to a water-hydrocarbon interface were analysed by an A x B factorial analysis of variance in a randomised design.

Concanavalin A agglutination assay

Adherence was expressed as the mean percentage binding of yeasts to Con A sepharose beads and subsequently transformed by arc sine transformation (Bishop, 1966). The influence of growth conditions, growth phase, or yeast strain on adherence were analysed by a 2 x 5 factorial split-plot analysis of variance in a randomised design (Ridgman, 1975).

Siderophore assays

The effects of growth conditions, time, or yeast strain on siderophore production was assessed by a factorial split-plot analysis of variance in a randomised design (Ridgman, 1975).

The following statistical notation was used throughout this study:

$p > 0.05$	NS (not significant)
$p < 0.05$	Significant
$p < 0.025$	"
$p < 0.01$	"
$p < 0.005$	Highly Significant
$p < 0.001$	" "

4.-9. RESULTS AND DISCUSSION

4. Influence of iron-deprivation on growth and siderophore production by *C.albicans*

4.1. Growth characteristics of *C.albicans* under iron-sufficient and iron-depleted conditions

Growth of four *C.albicans* isolates (AU1, AU2, OMC3 and 4208) was determined by monitoring OD_{660} measurements of yeasts cultured in untreated SSM, SSM+Fe and SSM-Fe at 25°C and 37°C. Growth of AU2, OMC3 and 4208 were essentially similar to that obtained with AU1, hence AU1 was chosen as representative of these strains. Figs. 4.1A. and 4.1B. show growth curves of AU1 cultured at 25°C and 37°C. Growth in the presence or absence of iron at 25°C or 37°C did not alter the doubling time, 2.0h and 1.6h respectively. However, onset of stationary phase in SSM+Fe and SSM-Fe at 25°C occurred at an OD_{660} of 7.0 and 5.4 respectively, whereas at 37°C onset occurred at an OD_{660} of 9.8 and 5.9 respectively.

When increasing concentrations of iron were added to SSM-Fe, onset of stationary phase was altered and an increase in the final cell density was observed (Fig. 4.2.) A linear relationship appeared to exist between the concentration of iron added and onset of stationary phase up to an OD_{660} of 10 (Fig. 4.3.). Beyond this point no further increase in growth was observed with an increase in iron concentration. Furthermore, when no

iron was added to SSM-Fe, cells grew to an OD_{660} of 5 suggesting that the level of contaminating iron (0.35μM, determined by atomic absorption spectrophotometry) supported growth of *C.albicans* to a lower cell density than cultures supplemented with iron. There were no apparent differences in iron requirements between the four strains examined.

4.2. Growth characteristics of *C.albicans* in the presence of an iron chelator

Preliminary investigations revealed that the minimum concentration of 1,10-phenanthroline (OP) required to inhibit growth of *C.albicans* was 50μM, and that addition of 10μM iron completely reversed this inhibition. This confirmed that the concentration of OP used was not toxic to yeast cells and that the inhibitory action of OP can be attributed to iron chelation. Growth of AU1, C55 and C406 was determined by monitoring OD_{660} measurements of yeasts cultured in untreated SSM, SSM+Fe and SSM+OP at 37°C (Fig. 4.4.). Growth of all three isolates was markedly inhibited in the presence of OP and the laboratory isolate, C406, grew poorly in untreated SSM and SSM+Fe compared to clinical isolates.

4.3. Siderophore production by *C.albicans*

Chemical assays for non-specific and specific classes of siderophores were carried out with culture supernatants from iron-sufficient and iron-depleted cultures of *C.albicans*

AU1 and AU2. Supernatants were assayed from cultures grown at 25°C or 37°C, and at pH 4.5 or 7.0. Cultures were maintained in the yeast form at pH 4.5 as described in section 3.1.1., however, increasing the pH to 7.0, so as to mimic physiological values, produced cultures with approximately 30% pseudohyphae and hyphae, and 70% yeasts. Regardless of temperature, pH, duration of incubation, or iron-depletion, phenolate-type siderophores were not detected in supernatants from cultures of either strain using Arnows' assay (1937), suggesting that any siderophores present were not of the phenolate class or were produced in undetectable concentrations in SSM (data not shown).

4.3.1. Detection of non-specific siderophores

A highly sensitive method for the detection of siderophores, which is based on their affinity for iron (III) and is therefore independent of structure, was utilised as described by Schwyn and Neilands (1987). In order to quantify siderophore production in terms of desferal equivalents, a plot of the relationship between desferal concentration and reduction in absorbance was constructed as shown in Fig.4.5. The plot demonstrates that a linear relationship ($r=0.88$, $P<0.005$) exists between desferal concentration and reduction in absorbance up to 20μM desferal, beyond which this relationship did not hold.

Non specific siderophore production by cultures of strain AU1 grown at 25°C (pH 4.5) was somewhat variable and did not appear to follow a particularly marked trend over

the incubation period utilised, except in two instances, was production greatly influenced by iron status (Fig.4.6A). A lack of obvious differences in siderophore levels may in turn be reflected by Fig.4.6B which demonstrates that iron-depletion reduces growth. When growth temperature was increased to 37°C (pH 4.5) siderophore levels were significantly enhanced, furthermore although levels continued to increase with time ($P<0.001$) iron status did not have a marked influence.

Siderophore production by strain AU2 (Fig.4.7A) was significantly affected by growth in SSM-Fe and SSM+Fe (pH 4.5) at 25°C ($P<0.01$), and with time ($P<0.001$). Prior to day 18, a fall in production was observed in SSM-Fe, whereas detection was negligible after 6 days growth in SSM+Fe. By analogy with AU1, siderophore production by AU2 grown at 37°C was significantly affected by time ($P<0.001$), but not by the presence or absence of iron (Fig.4.7A).

In summary regardless of growth conditions, both AU1 ($P<0.001$) and AU2 ($P<0.001$) achieved greater levels of siderophores during growth at 37°C than at 25°C. Growth of AU1 (Fig.4.6B) and AU2 (Fig.4.7B) was not affected by temperature, however growth in SSM-Fe yielded lower cell densities than in SSM+Fe.

4.3.2. Detection of hydroxamate-type siderophores

Detection of hydroxamate-type siderophores was based on absorbance of iron complexes formed by a reaction between siderophores and iron perchlorate. Concentrations of siderophores were expressed as desferal equivalents after determining the relationship

between desferal concentration and absorbance (Fig.4.8.). A linear relationship existed between desferal concentration and absorbance ($r=0.999$, $P<0.001$).

Hydroxamate siderophore production by AU1 was markedly enhanced under iron-depleted conditions (pH 4.5) at 25°C ($P<0.001$), and with time ($P<0.005$) (Fig.4.9A). Similarly, incubation time ($P<0.001$) influenced those siderophore levels associated with cultures maintained at 37°C, however, iron status did not (Fig.4.9A). Siderophore concentrations of filtrates from cultures of AU2 increased with time ($P<0.001$) irrespective of growth temperature and were further enhanced by growth in SSM+Fe ($P<0.05$) at 37°C (Fig.4.10A). When the pH was adjusted to 7.0, greater levels of siderophores were achieved by AU1 during growth in SSM+Fe ($P<0.001$) than in SSM-Fe, and with time ($P<0.001$) (Fig.4.11A). A similar pattern was observed during growth of AU2 at pH 7.0 in SSM+Fe and SSM-Fe ($P<0.025$), and with time ($P<0.001$) (Fig.4.11A).

In summary regardless of growth conditions, significantly greater levels of hydroxamate-type siderophores ($P<0.001$) were secreted by AU1 and AU2 during growth at 37°C than at 25°C, however, no significant increase was demonstrated by AU2 during growth in SSM-Fe. Temperature or pH did not affect growth characteristics of either strain, however, growth in SSM-Fe was characterized by lower final cell densities than in SSM+Fe (Figs.4.9B to 4.11B).

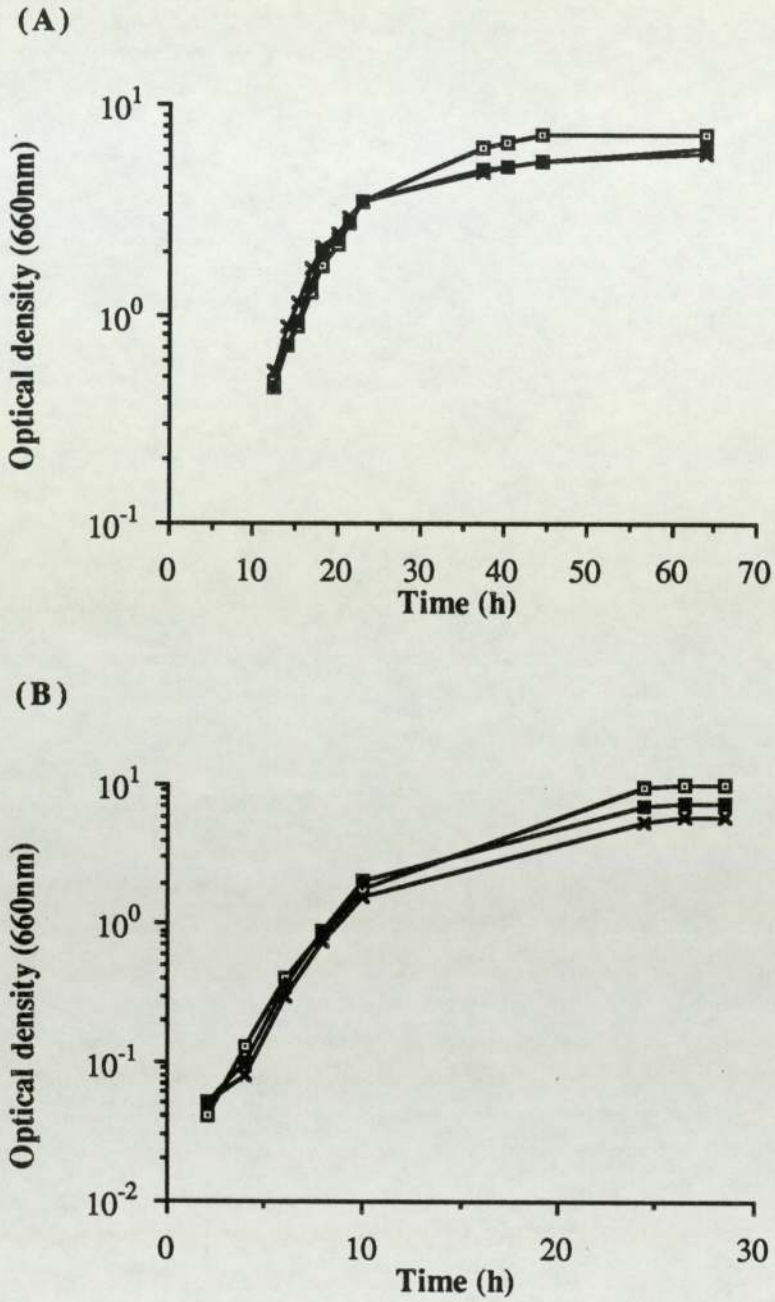


Fig.4.1A and B. Effect of iron-depletion on the growth of *C.albicans* AU1 at 25℃ (A) and 37℃ (B). Yeast cells were grown in (▫) SSM+Fe; (×) SSM-Fe; and (■) untreated SSM. Similar curves were obtained for isolates AU2, OMC3 and 4208.

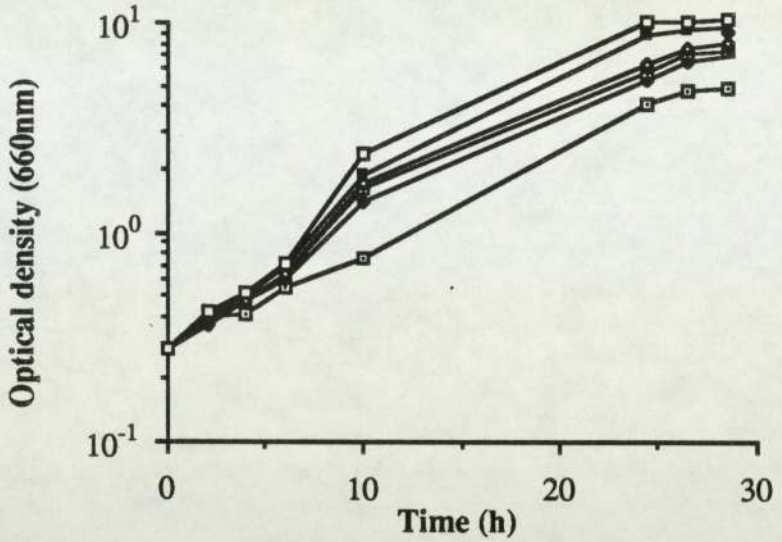


Fig.4.2. Effect of iron concentration on the growth of *C.albicans* AU1 at 37 C. Cells were grown in (▫) SSM-Fe; (◆) SSM-Fe plus 10μM Fe; (■) SSM-Fe plus 50μM Fe; (◆) SSM-Fe plus 100μM Fe; (▫) SSM-Fe plus 200μM Fe; (■). SSM-Fe plus 400μM Fe. Iron was added as ferrous sulphate.

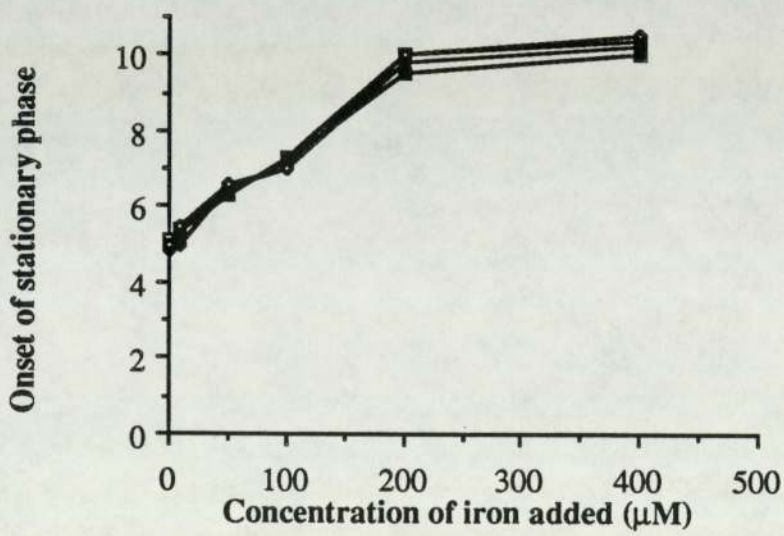


Fig.4.3. Relationship between onset of stationary phase and iron concentration. Comparison of iron requirements between *C.albicans* (▫) AU1; (◆) AU2; (■) OMC3; and (◆) 4208.

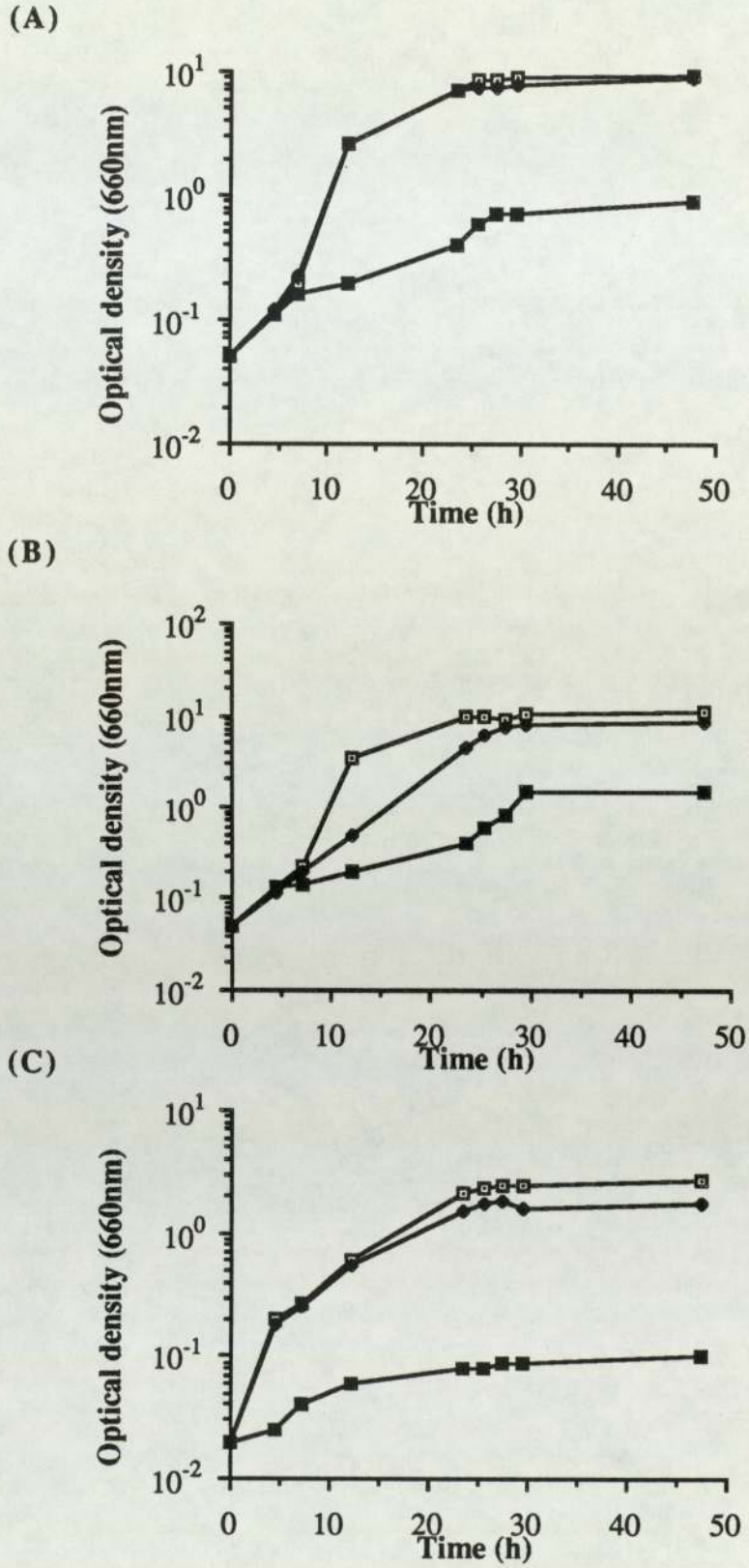


Fig.4.4A, B and C. Effect of iron restriction on the growth of *C.albicans* AU1 (A), C55 (B) and C406 (C). Yeast cells were grown in (▫) SSM+Fe; (◆) untreated SSM; and (■) SSM+OP.

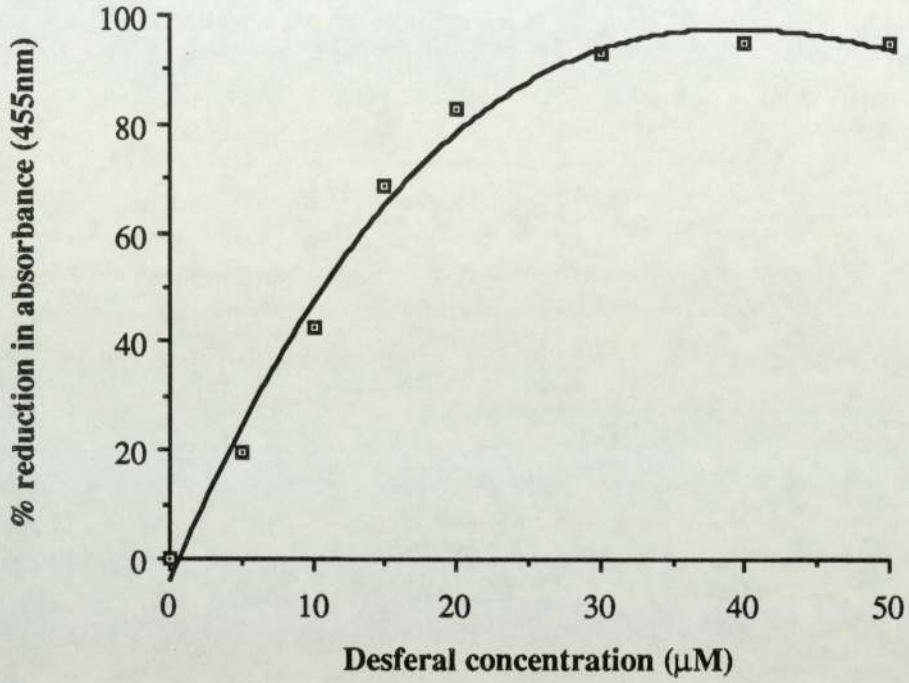


Fig.4.5. Standard curve of the relationship between siderophore concentration and reduction in absorbance using Schwyn and Neilands assay (1987).

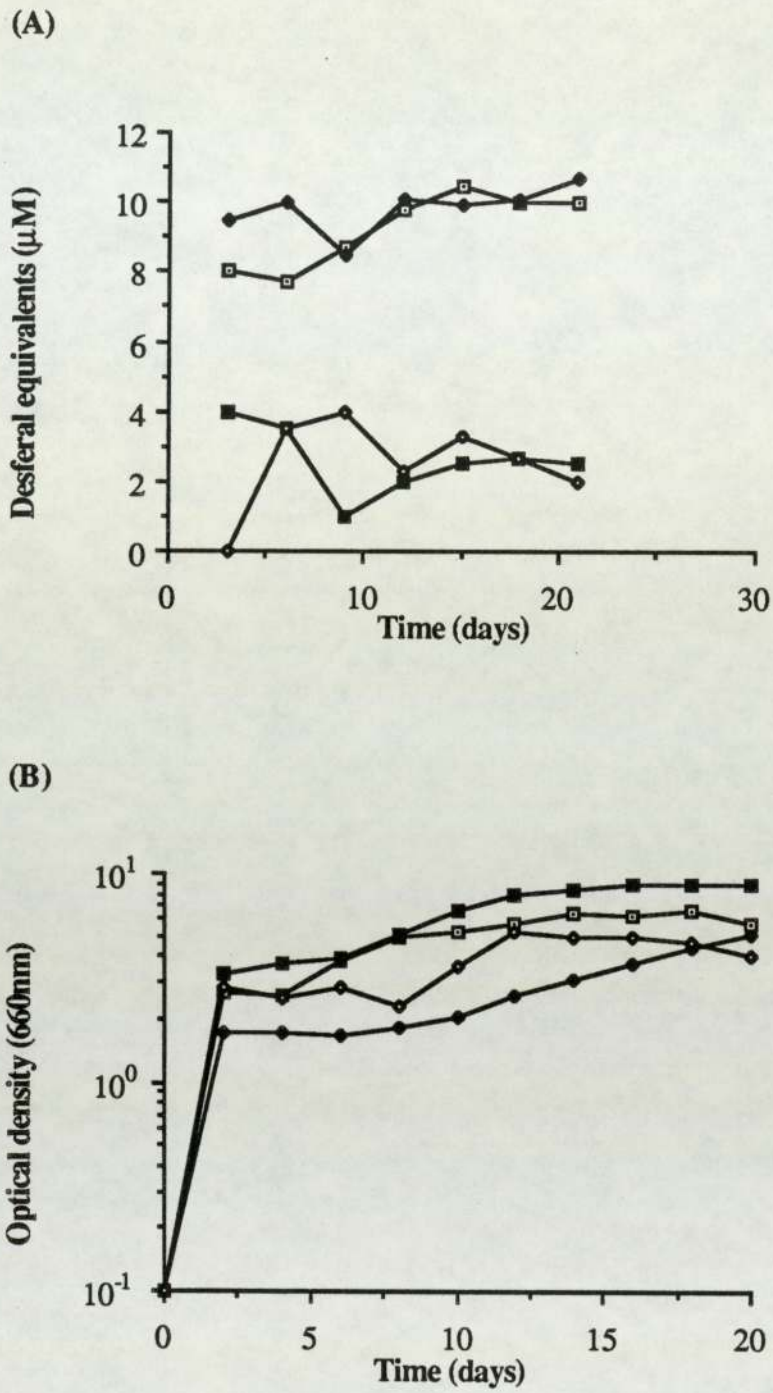
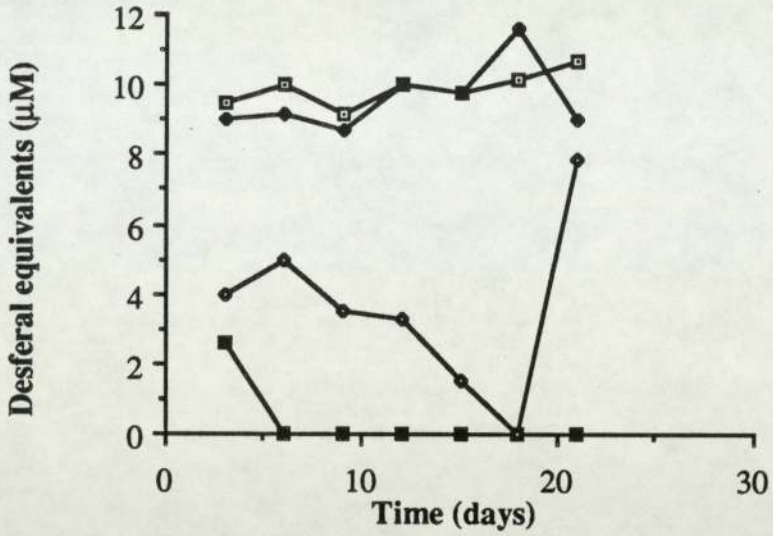


Fig.4.6A and B. Effect of temperature on siderophore production by *C.albicans* AU1. (A) Chrome azurol S was used to form a coloured iron-siderophore complex in cell-free supernatants from cultures maintained at pH 4.5 in (■) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C, X= ±0.903, Y= ±0.49; (▣) SSM+Fe at 37°C; and (◆) SSM-Fe at 37°C, X= ±1.017, Y= ±0.633. (X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times).
(B) Growth of AU1 at pH 4.5 in (■) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C; (▣) SSM+Fe at 37°C; and (◆) SSM-Fe at 37°C.

(A)



(B)

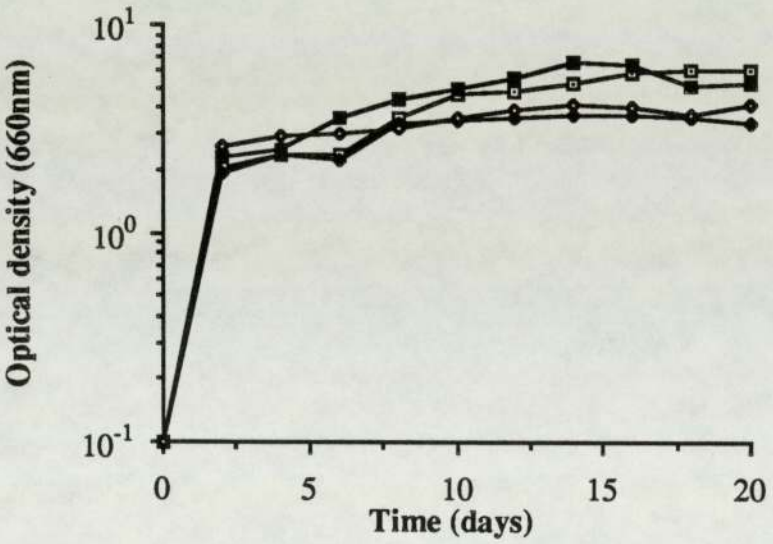


Fig.4.7A and B. Effect of temperature on siderophore production by *C.albicans* AU2. (A) Chrome azurol S was used to form a coloured iron-siderophore complex in cell-free supernatants from cultures maintained at pH 4.5 in (■) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C, X= ±0.908, Y= ±1.53; (▫) SSM+Fe at 37°C; and (◆) SSM-Fe at 37°C, X= ±0.448, Y= ±0.916. (X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times).
(B) Growth of AU2 at pH 4.5 in (▫) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C; (■) SSM +Fe at 37°C; and (◆) SSM-Fe at 37°C.

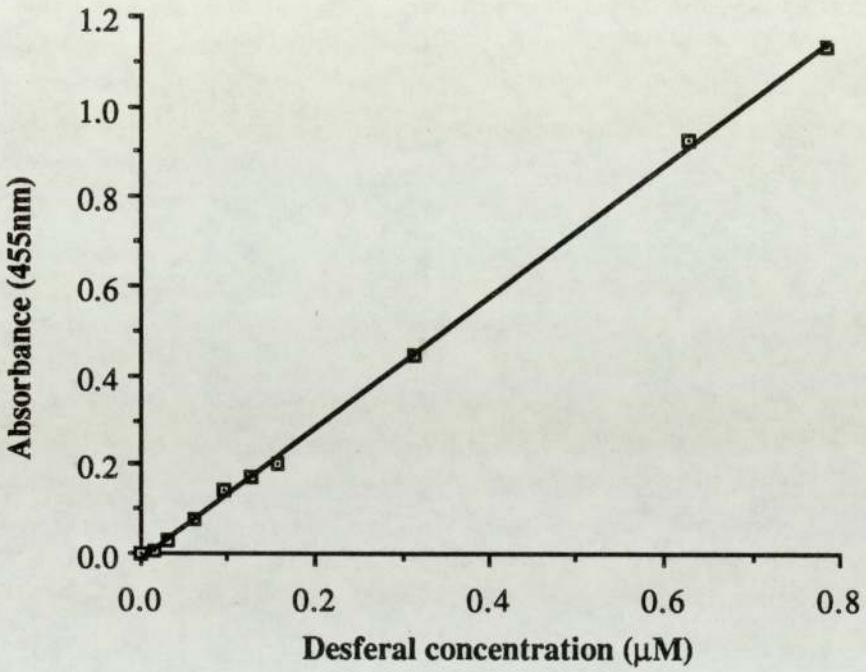


Fig.4.8. Standard curve to quantitate siderophores in terms of desferal equivalents (μM) using ferric perchlorate to form the iron-siderophore complex.

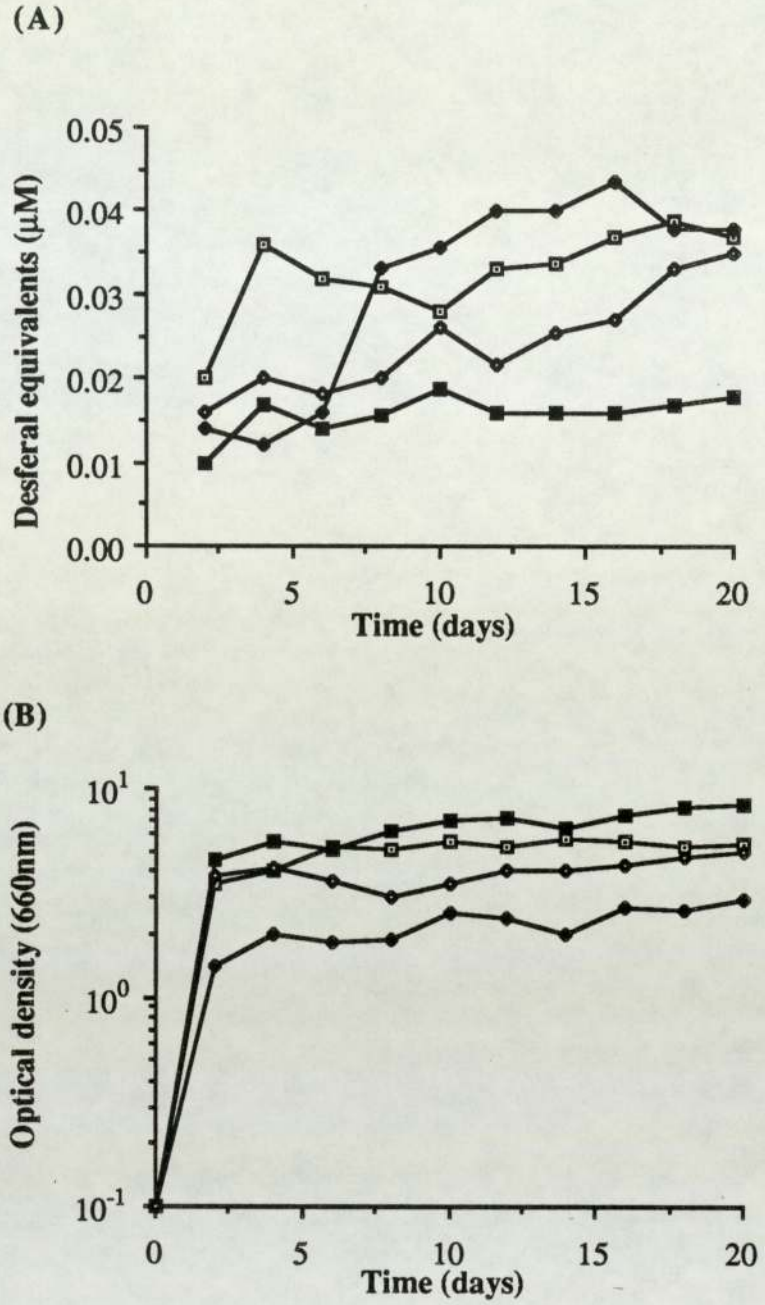


Fig.4.9A and B. Effect of temperature and pH on hydroxamate-type siderophore production by *C.albicans* AU1. (A) Ferric perchlorate was used to form a coloured iron-complex in cell-free supernatants from cultures maintained at pH 4.5 in (■) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C, X= ±0.0017, Y= ±0.0044; (▣) SSM+Fe at 37°C; (◆) SSM -Fe at 37°C, X= ±0.0039, Y= ±0.0086. (X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times).

(B) Growth of AU1 at pH 4.5 in (▣) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C; (■) SSM +Fe at 37°C; and (◆) SSM-Fe at 37°C.

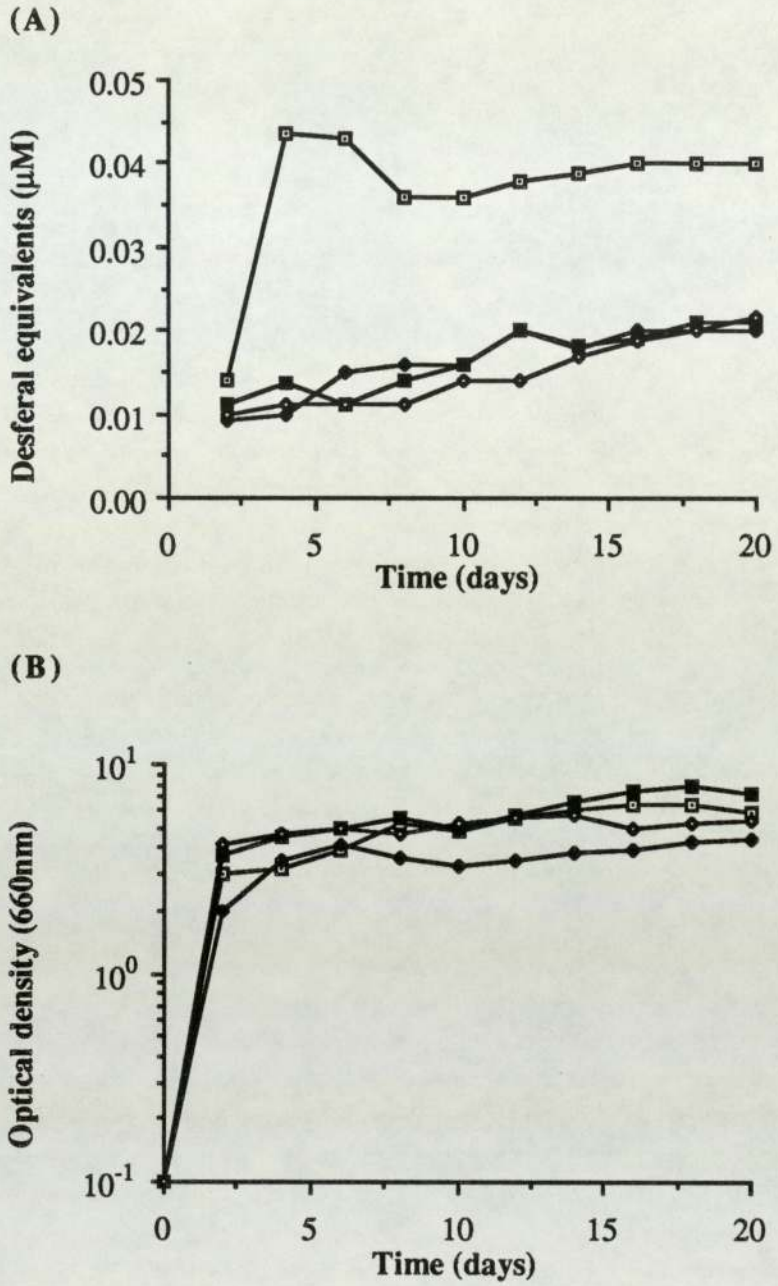


Fig.4.10A and B. Effect of temperature and pH on hydroxamate-type siderophore production by *C.albicans* AU2 (A) Ferric perchlorate was used to form a coloured iron-complex in cell-free supernatants from cultures maintained at pH 4.5 in (■) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C, X= ±0.0027, Y= ±0.0031; (□) SSM+Fe at 37°C; (◆) SSM -Fe at 37°C, X= ±0.006, Y= ±0.003. (X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times).

(B) Growth of AU2 at pH 4.5 in (□) SSM+Fe at 25°C; (◆) SSM-Fe at 25°C; (■) SSM +Fe at 37°C; and (◆) SSM-Fe at 37°C.

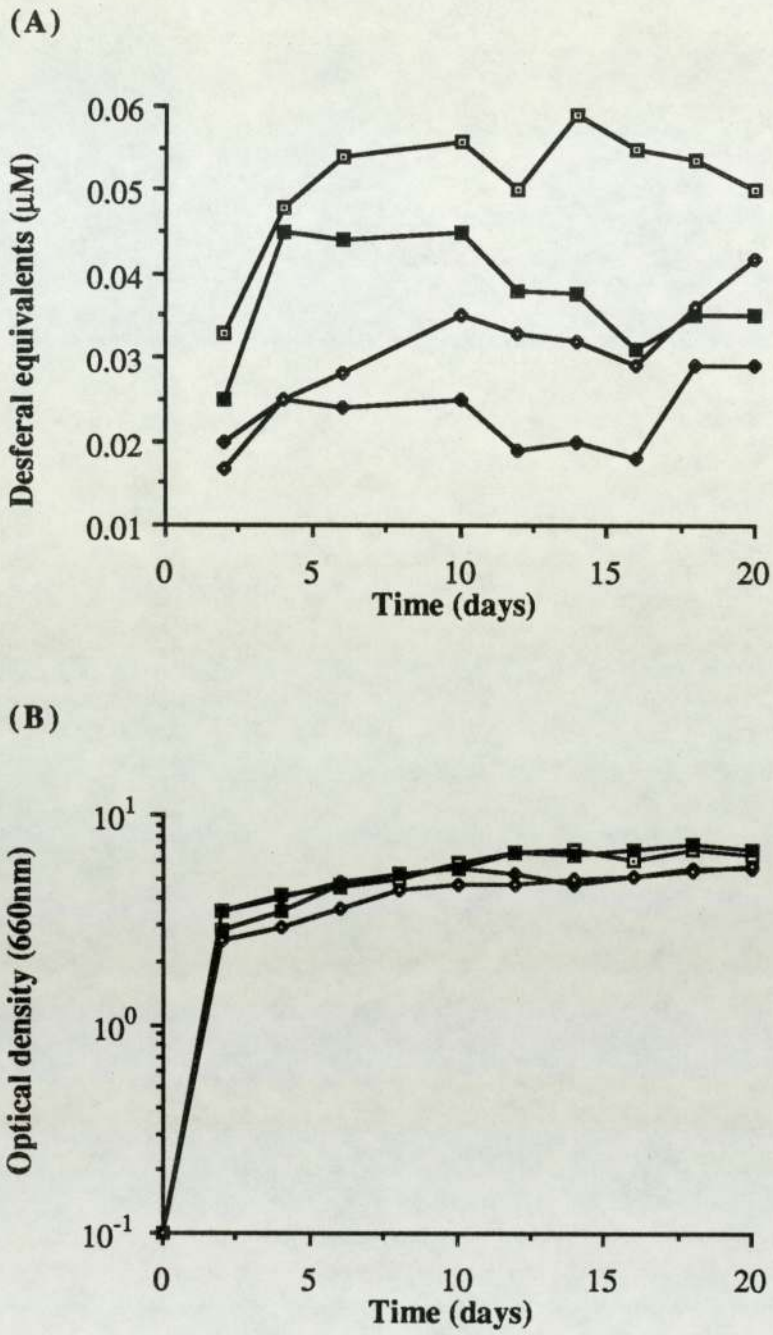


Fig.4.11A and Bffect of temperature and pH on hydroxamate-type siderophore production by *C.albicans*. (A) Ferric perchlorate was used to form a coloured iron-complex in cell-free supernatants from cultures maintained at pH 7.0 (37°C). AU1 was grown in (▫) SSM+Fe and (◆) SSM-Fe, X= ±0.0086, Y= ±0.0045. AU2 was grown in (■) SSM+Fe and (◆) SSM-Fe, X= ±0.008, Y= ±0.007. (X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times).

(B) Growth of AU1 at pH 7.0 (37 C) in (▫) SSM+Fe and (◆) SSM-Fe; and AU2 in (■)SSM+Fe and (◆) SSM-Fe.

4.3. Discussion

Microbial multiplication in the environment of host tissues is an essential feature of any infection and an ability to interfere with this process plays a major role in host defence against infection. Iron is essential for microbial growth, furthermore, it has been established that withholding of iron by host iron-binding proteins creates an iron-restricted environment which in turn induces phenotypic changes in the metabolism and surface composition of bacteria growing *in vivo* (Bullen, 1981; Griffiths *et al.*, 1983; Brown *et al.*, 1984; Brown and Williams, 1985 a and b; Griffiths, 1987a).

A large number of investigations into microbial virulence have been carried out with organisms grown *in vitro* under conditions that do not necessarily reflect microbial behaviour *in vivo*. These organisms are usually cultivated under iron-plentiful conditions in complex or simple salts media, therefore growth in media where the availability of iron is minimal may mimic more closely the host environment.

Methods employed for removal or restriction of trace metals from culture media are diverse (Waring and Werkman, 1942; Donald *et al.*, 1952; Kadurugamuwa *et al.*, 1987) and include the use of synthetic or biological iron chelators and ion-exchange resins. Results of this study demonstrated that both Chelex-100 resin and 1'10-phenanthroline were capable of removing or restricting sufficient iron from media to promote conditions of iron-deprivation.

When growth of a culture is limited by a specific nutrient, its final population is directly related to initial nutrient concentration, rate of uptake and transport into the cell (Klemperer *et al.*, 1979). In the presence of excess iron, yeast cells stopped multiplying

as a consequence of toxic secondary metabolites accumulation or limiting concentrations of oxygen, since all other nutrients were present in excess. Conversely, under iron-depleted conditions, iron became the limiting nutrient before sufficient secondary metabolites accumulated to inhibit growth, hence the final yeast concentration was less than that of cultures grown under iron-sufficient conditions.

This preliminary investigation is fundamental to subsequent studies which attempt to relate surface properties of yeast cells and their expression of antigens with growth under defined conditions. Furthermore, this form of approach has provided a comparison of relative iron requirements amongst *C.albicans* strains. Although potential differences amongst strains may have occurred through unique surface properties or uptake and metabolism pathways, the strains under study appeared to respond in a uniform manner to iron-depletion.

Many microorganisms respond to iron deprivation by secreting low MW iron-chelating agents termed siderophores which are part of the high-affinity iron transport systems (Raymond and Carrano, 1979; Neilands, 1981a and b; Griffiths, 1987a and b). Fungi produce siderophores (Neilands, 1981a and b), but studies on their secretion by pathogenic yeasts and fungi are limited. Furthermore, the literature currently available describing mechanisms of iron uptake in *C.albicans* is often contradictory and little agreement exists amongst workers on a precise role for siderophores in iron uptake. Therefore, one is led to speculate on possible mechanisms of iron acquisition by *C.albicans* with respect to the present results.

Siderophores are classified as hydroxamates or phenolates, and are detectable by chemical and biological means. Chemical detection by colorimetric assays include; Arnow's method (Arnow, 1937) for detecting phenolate-type siderophores, and Csa'ky's test (Csa'ky, 1948), or the ferric perchlorate test (Atkin and Neilands, 1968) for detecting hydroxamate-type siderophores. Preliminary investigations using Csa'ky's method were hampered by false-positive absorbance values with uninoculated SSM, therefore this method was not used. Instead, the ferric perchlorate test was used, which, although less sensitive than Csa'ky's method, is quicker to perform and is specific for hydroxamate-type siderophores.

Recently, Schwyn and Neilands (1987) described a sensitive assay for detecting both classes of siderophore, which is based on their affinity for ferric ions and is therefore independent of structure. In the colour change from blue to orange, at 630 nm, the orange-coloured iron-free dye has essentially no absorption, hence this provides a qualitative check for the presence of siderophores in supernatant solutions. Using this assay, siderophore production by AU1 was not influenced by growth conditions at 25°C. However, under the same conditions, production by AU2 was markedly affected by growth in the presence or absence of iron, and with time. Regardless of growth conditions, siderophore production by both strains was significantly greater at 37°C than at 25°C, although growth was similar at both temperatures. This observation indicates that with increasing siderophore concentrations, no concomitant increase in growth occurs.

It is apparent from present results and that of previous studies (Holzberg and Artis, 1983; Ismail *et al.*, 1985a and b) that concentrations of hydroxamate-type siderophores secreted by *C.albicans* are relatively low. Comparisons between these studies highlights the diversity of culture conditions under which *C.albicans* is grown to yield siderophores, and in the methods utilized for removal of iron from culture media. When Ismail *et al.* (1985a and b) utilized an iron chelator, 1,10-phenanthroline (OP) in their culture medium, it resulted in enhanced siderophore levels mediated by severity of iron restriction. However, some chelators may be inappropiate because they may interact with and in turn damage microbial surfaces (Schulman and Dwyer, 1964).

Several studies have demonstrated that inhibition of *C.albicans* yeast growth by OP is attributed to iron chelation (Schulman and Dwyer, 1964; Bedell and Anderson, 1985; Ismail and Bedell, 1986) and is consistent with the findings of this study which showed that inhibitory action of OP could be reversed by iron and no other metal cations. Although OP has a high affinity for ferrous ions it also binds zinc (Schulman and Dwyer, 1964) and probably other cations. Consequently, Ismail and Bedell (1986) demonstrated that the inhibitory effect of OP on *C.albicans* could be reversed by addition of zinc as well as iron. However, these workers proposed that under their growth conditions, addition of excess zinc might release iron bound by media constituents which in turn is taken up by cells. Hence, the reversal of inhibition by OP may be due to iron not zinc, and if plausible, would suggest that iron requirement for yeast growth is exceedingly low.

Biological chelators such as transferrins, have been used in several studies and are valuable because they more closely represent conditions *in vivo*. However, there are practical difficulties involved in using transferrins including a need to dialyze out metal and citrate ions before use. Despite this, Valenti *et al* (1986) failed to detect either class of siderophore in supernatants from cultures of *C.albicans* incubated in the presence of either lactoferrin or ovotransferrin for 72h. Consequently, further investigations by these, and other workers (Rolling and Lupan, 1988) have suggested that the antifungal action of these transferrins may not be due to iron chelation but involves interaction between iron-binding proteins and yeast surfaces in the absence of siderophores.

This mechanism has been proposed to explain similar events observed with other fungal and bacterial species including, *Neisseria meningitidis* (Simonson *et al.*, 1982), *Rhodotorula pilimanae* (Carrano and Raymond, 1978), and *Rhizopus oryzae* and *Trichophyton mentagrophytes* (Artis *et al.*, 1983). Simonson *et al.* (1982) showed that iron-starved meningococci were capable of removing and incorporating iron from human transferrin by a cell surface mechanism that specifically recognized transferrin rather than iron. Furthermore, there may or may not be a role for either a cell-free or surface-associated hydroxamate-type siderophore in these bacteria to transport iron within the cell after its incorporation from transferrin.

In contrast to the above studies, Ismail and Lupan (1988) proposed that the yeast-transferrin interaction may be mediated by expression of *C.albicans* surface receptors and demonstrated binding of both classes of siderophores to these receptors. Since some

clinical isolates of *C.albicans* produce phenolate-type as well as hydroxamate-type siderophores (Ismail *et al.*, 1985a), this would explain binding of both types to surface receptors, although hydroxamate-type siderophores were preferentially bound. It is not known why *C.albicans* produces both classes of siderophores.

Schwyn and Neilands (1987) reported that the stability of iron-chrome azurol S (CAS) complexes generated in their siderophore assay was unknown, and that its blue colour was subject to change at and above pH 7. This colour change was believed to be a result of iron hydroxide formation, which in turn interferes with interpretation of assay results. Given the current limitations of Schwyn and Neilands assay, the affect of physiological pH on siderophore production was examined using the ferric perchlorate test. The specificity of this test allowed detection of hydroxamate-type siderophores amongst mixtures which include phenolate-type siderophores, since complexes generated with the latter do not form in the presence of ferric perchlorate.

In this study, siderophore production by *C.albicans* was monitored at physiological temperature and pH, and at pH 4.5 to mimic conditions during colonization or infection at various sites of infection. The significance of this approach is apparent upon consideration that *C.albicans* is capable of invading most host tissues, including the vagina. Several studies have revealed that vaginal colonization and infection by yeasts is influenced by hormones, nutritional status and other physiological factors (Ryley, 1986; Galask, 1988). Vaginal pH is an important factor affecting colonization, and *Candida* is present at all pH ranges. Since the majority of *Candida* infections occur in the normal

vaginal pH range of 3.5 to 4.5 (Kaufmann, 1988), the acidity of this environment could serve to solubilize iron to a utilizable form which may in turn induce expression of low-affinity iron transport systems.

In support of this view, Brener *et al.* (1981) reported that growth of *N.meningitidis* under iron starvation at low pH (conditions simulating an inflammatory reaction) profoundly enhanced virulence for mice. Furthermore, Nilius *et al.* (1988) reported that the lack of inhibition of *Aspergillus fumigatus* by unsaturated transferrin may be due to a combination of factors including, secretion of fungal metabolites resulting in an acid pH environment thereby reducing affinity of transferrin for iron, secretion of proteases capable of digesting transferrin, and/or siderophores capable of mobilizing iron from transferrin. Therefore, one may speculate that *C.albicans* may possess several effective mechanisms to circumvent iron-withholding by host-binding proteins during infection.

Using both detection methods, *C.albicans* produced siderophores under iron-depleted, as well as under iron-sufficient conditions, a finding consistent with previous studies by Ismail and Bedell (1986) and Miller *et al.* (1988). These results may be explained by the expression of low-affinity iron transport systems in *C.albicans* operating in the presence of freely available iron. Harris *et al.* (1979) also proposed the presence of a similar system in *E.coli* which utilizes the hydroxamate-type siderophore, aerobactin, however little is known about the mechanisms involved. In contrast, Neilands (1981b) reported that neither siderophores nor membrane receptors were required for low-affinity systems, however, in the absence of an alternative mechanism, little further

progress has been gained. Consequently, it has been suggested that some of the surface atoms of insoluble ferric oxy-hydroxide polymers (FeOOH) may be unstable and loosely bound providing a source of available iron to cells. Alternatively, metal binding sites or receptors may be located within an organisms' surface allowing direct interaction between metal ions and organism.

In the absence of a convincing explanation for synthesis of siderophores by *C.albicans* in the presence of iron, it would appear relevant to focus emphasis in the direction of iron uptake and transport mechanisms of *C.albicans*. Unfortunately, although much is known about various aspects of solute transport in eukaryotic microorganisms, there is little understanding of transport systems in *C.albicans*, especially as insufficient data to describe even the kinetics and specificity of a single solute exists (Prasad, 1987). Nevertheless, a recent study by Lesuisse *et al.* (1987), describing iron uptake by the yeast *Saccharomyces cerevisiae* may provide a useful framework for a better understanding of mechanisms of iron uptake by *C.albicans*.

Lesuisse *et al.* (1987) reported that it was unlikely that surfaces of *S.cerevisiae* expressed high-affinity uptake systems for transporting ferric iron. Alternatively they found that rate of iron uptake was greatly dependent on the oxidation state, and that yeast cells were able to take up iron from several siderophores, in particular from two extremely stable complexes, ferricrocin and ferrioxamine B, with stability constants of $10^{30.4}$ and $10^{30.5}$ respectively (Raymond *et al.*, 1984). When used as an iron source, such an iron-siderophore complex could be dissociated by reduction outside the cells

before uptake, as in dicotyledonous plants (Bienfait, 1985). These workers also demonstrated that iron was physiologically reduced prior to uptake by cells, using a transplasmamembrane redox system, which was induced under iron-deficient conditions. The iron status of yeast cells appeared to control the reducing capacity of the redox system, thereby contributing an important system in regulation of iron uptake by *S.cerevisiae*. Plasma membrane-bound redox systems have been detected in several other eukaryotic organisms (Goldenberg, 1982), therefore the presence (or absence) of a similar or closely related system in *Candida* may help elucidate mechanisms of iron uptake.

5. Effect of iron-depletion on cell wall antigens of *C.albicans*

5.1. Analysis of cell wall (Cw) protein profiles of *C.albicans* grown under iron-depleted and iron-sufficient conditions

Figs. 5.1A and B show the protein profiles of walls isolated from AU1 and AU2 grown in SSM-Fe and SSM+Fe at 25°C and 37°C stained with Coomassie Brilliant Blue. All profiles were essentially similar with approximately 40 bands visualized in each profile. The molecular weights (MW) of proteins ranged from 200 to 14kd, and all profiles were characterized by four major proteins of MW 66, 47, 45 and 23/25kd. No significant qualitative differences were associated with growth temperature, the presence or absence of iron, or yeast strain, for these stained preparations.

5.2. Antibody recognition of *C.albicans* cell wall antigens

Immunoblotting is a sensitive, high resolution technique with the attributes of a quantitative solid-phase immunoassay (Towbin and Gordon, 1984) which can be used to identify specific microbial antigens or antibody species in sera of patients or other body fluids. Blots of electrophoretically separated proteins from isolates of *C.albicans* grown in SSM+Fe and SSM-Fe at 25°C and 37°C were stained with aurodye to assess qualitative transfer of proteins to Nc paper (Fig. 5.2.). These stained blots revealed similar protein profiles with no apparent qualitative differences associated with strain, presence or absence of iron, or growth temperature. Therefore, blots of AU1 shown in

Fig. 5.2. are representative of all the isolates studied and indicates satisfactory transfer of proteins to Nc paper.

5.3. Antibody recognition of wall antigens in yeasts grown at 25°C

Blots of electrophoretically separated proteins from isolates AU1 and AU2 grown in SSM+Fe and SSM-Fe are shown in Figs.5.3A and B, and 5.4A and B. Each blot was incubated with serum from a patient with systemic candidosis (patient 1) or normal human serum (NHS). The results reveal a marked heterogeneity in antibody response to Cw antigens from both isolates.

The IgG response to wall antigens of AU1 was marked by a dense, diffuse staining to high MW antigens (Fig.5.3A). IgG antibodies from both patient 1 serum and NHS reacted with a 47kd antigen in walls of yeasts grown in SSM+Fe and SSM-Fe. The same sera also reacted with a 41, 40, 35 and 25kd antigen in walls of yeasts grown in SSM-Fe (Fig.5.3A lanes 2 and 4), but these antigens were not detected in walls of cells grown in SSM+Fe (Fig.5.3A lanes 1 and 3). A 47kd antigen was the predominant antigen recognized by IgM antibodies from both sera in walls of iron-depleted and iron-sufficient cells (Fig.5.3B).

IgG antibodies similarly reacted intensely to the upper region of each blot of AU2 (Fig.5.4A). Patient 1 serum revealed a 44kd and a 35kd antigen (lane 2), whereas NHS revealed only the 35kd antigen (lane 4) in walls of iron-depleted yeasts. Neither antigen was detected in walls of yeasts grown under iron-sufficient conditions. As with AU1, a

47kd antigen was strongly detected by IgM antibodies from both sera in each blot of AU2 (Fig.5.4B). In addition, patient 1 serum revealed a 45, 41 and 14kd antigen (lane 2), whereas NHS revealed only the 14kd antigen (lane 4) in walls of cells grown in SSM-Fe. These antigens were not detected in yeasts grown in SSM+Fe.

5.4. Antibody recognition of wall antigens in yeasts grown at 37°C

Given the significance of studying physiological properties of cells under *in vitro* conditions that reflect *in vivo* environments, subsequent investigations relating to antibody recognition of wall antigens were carried out using cells cultured at physiological temperature. This work comprised an extensive study of antibody responses of patient sera against wall antigens from three clinical isolates of *C.albicans*. Blots of wall antigens from AU1, AU2 and OMC3 grown in SSM-Fe and SSM+Fe were incubated with serum from a patient with systemic candidosis (patient 1 serum) or oral candidosis (patient 2 serum), or NHS. Each blot was developed with IgG, IgM or IgA alkaline phosphatase anti-human conjugates, and resulting antigenic profiles revealed a marked heterogeneity in the IgG, IgM and IgA antibody response of patients' serum to antigens from each isolate.

5.4.1. Recognition of wall antigens by patient 1 antibodies

AU1

The IgG response was marked by a dense heterogeneous staining of the upper region of

each blot (Fig.5.5A) and negatively stained images of bands were visualized within these diffusely stained area of blots. IgG antibodies from patient 1 reacted strongly with a 47kd antigen of cells grown in SSM+Fe and SSM-Fe (Fig.5.5A lanes 1 and 2), however, NHS did not recognize this antigen (lanes 3 and 4). Both sera reacted strongly with antigens of MW 45, 41, 40, 35 and 25kd in walls of cells grown in SSM-Fe but not in SSM+Fe.

The IgM response to the high MW antigens of each blot was similar to the IgG response, however using NHS the staining was less intense (Fig.5.5B). A 47kd antigen was strongly detected by antibodies from both sera, whereas a 44kd antigen was detected by both sera in walls of cells grown in SSM-Fe but not in SSM+Fe (lanes 2 and 4). In addition, only patient serum reacted strongly with the 41, 40, 35 and 25kd antigens of cells grown under iron-depleted but not under iron-sufficient conditions (lanes 1 and 2). There was no IgA response from patient serum or NHS towards any wall proteins in AU1 (data not shown).

AU2

The IgG response was again characterized by a dense diffuse staining as described for blots of AU1. A 47kd antigen was strongly detected by patient 1 serum from cells grown in SSM+Fe and SSM-Fe (Fig.5.6A lanes 1 and 2), otherwise the profiles were similar. NHS produced a weak IgG response to the 47kd antigen, and reacted with antigens of MW 45, 40, 35 and 19kd in walls of iron-depleted cells, yet these antigens were barely detectable in iron-sufficient cells (lanes 3 and 4).

IgM antibodies reacted with several antigens in each blot, including a 40kd antigen which was strongly recognized by patient 1 serum, and a 55kd antigen which was detected by the same serum in walls of yeasts grown in SSM-Fe (Fig.5.6B lanes 1 and 2) but not in SSM+Fe. In addition, NHS detected a 43kd antigen in iron-depleted but not in iron-sufficient cells (lanes 3 and 4).

Both NHS and patient 1 serum produced a weak IgA response resulting in detection of a 47kd antigen by the latter serum in cells cultivated in SSM-Fe but not SSM+Fe (Fig.5.6C lanes 1 and 2).

OMC3

IgG antibodies from patient 1 reacted intensely to the upper portion of each blot as described previously, and detected a 47kd and a 40kd antigen (Fig.5.7A lanes 1 and 2), whereas NHS reacted weakly with the 47kd antigen in both iron-depleted and iron-sufficient cells, and failed to detect the 40 kd antigen (lanes 3 and 4) in walls of the same cells. IgM antibodies from patient 1 produced a similar response to IgG, except that the 47kd was not detected (Fig.5.7B lanes 1 and 2). In addition, NHS produced a weak IgM response to several antigens from yeasts grown in SSM-Fe (Fig.5.7B lanes 3 and 4) but not in SSM+Fe. IgA antibodies from patient 1 reacted strongly to a 81kd antigen from iron-sufficient cells but not from iron-depleted cells, whereas NHS produced a weak response to this and other antigens (Fig.5.7C lanes 1-4).

5.4.2. Recognition of wall antigens by patient 2 antibodies

AU1

Both IgG and IgM responses of serum from patient 2 were considerably weaker than serum from patient 1 (Fig.5.8.). However, unlike antibodies from patient 1, all antibody classes from patient 2 produced the typical reaction to the high MW region of each blot (Fig.5.8.). IgM and IgA antibodies reacted strongly to a 47kd antigen from iron-depleted cells (lanes 4 and 6), but weakly to the same antigen in iron-sufficient cells.

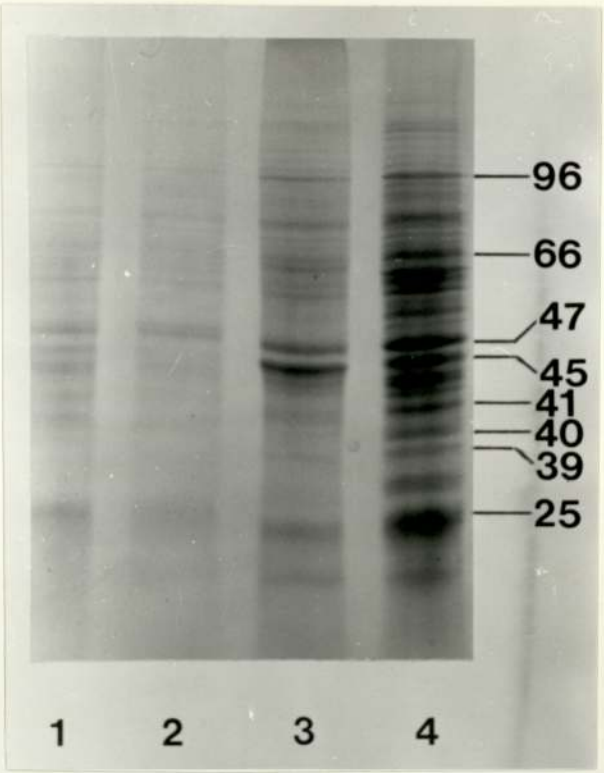
AU2

The IgG, IgM and IgA responses of serum from patient 2 were poor compared to the response of serum antibodies from patient 1 (Fig.5.9.). In addition, IgG antibodies did not produce the characteristic staining to the upper portion of each blot, and few antigens were barely detectable (lanes 1 and 2). A strong IgM response to the high MW antigens and to a 40kd antigen was observed in walls of yeasts cultivated in SSM+Fe and SSM-Fe (lanes 3 and 4), few antigens were detected by IgA antibodies (lanes 5 and 6).

OMC3

Both IgG and IgM responses of patient 2 serum resulted in typical reactions to each blot as described previously, otherwise their profiles were similar (Fig.5.10. lanes 1-4). No antigenic differences were apparent between walls of cells grown in SSM+Fe and SSM-Fe when incubated with IgA (lanes 5 and 6).

(A)



(B)

Fig.5.1A and B. Cell wall protein profiles of *C.albicans* AU1 (A) and AU2 (B). Yeasts were grown in SSM+Fe (lane 1) and in SSM-Fe (lane 2) at 25°C; and in SSM+Fe (lane 3) and in SSM-Fe (lane 4) at 37°C. Molecular weights are in kilodaltons

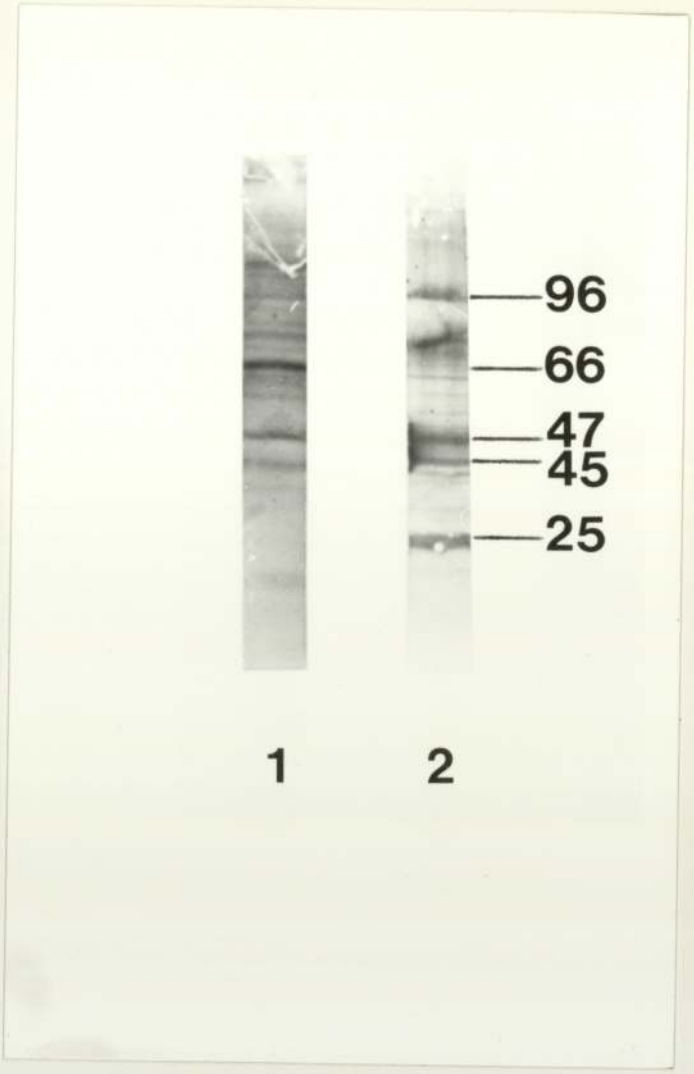
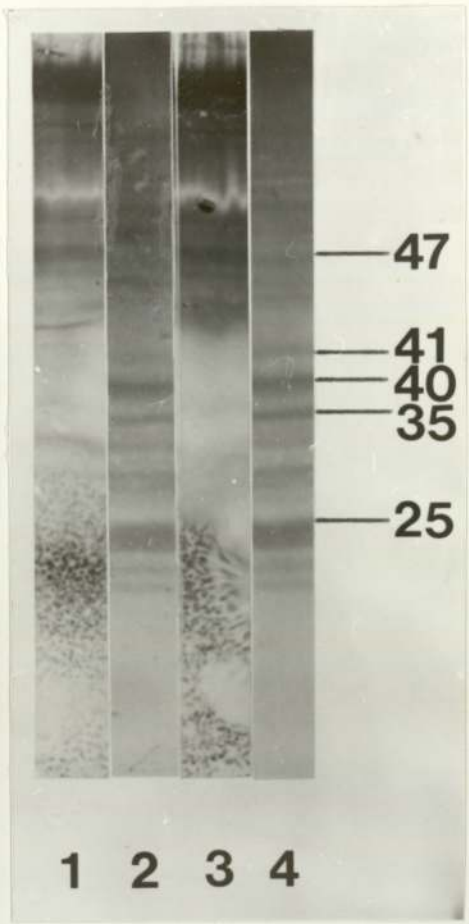


Fig.5.2. Cell wall proteins of *C.albicans* AU1 grown in SSM+Fe (lane 1) and SSM-Fe (lane 2) at 37°C. Wall proteins were separated by SDS-PAGE and electrophoretically transferred to Nc paper then stained with aurodye.

(A)



(B)

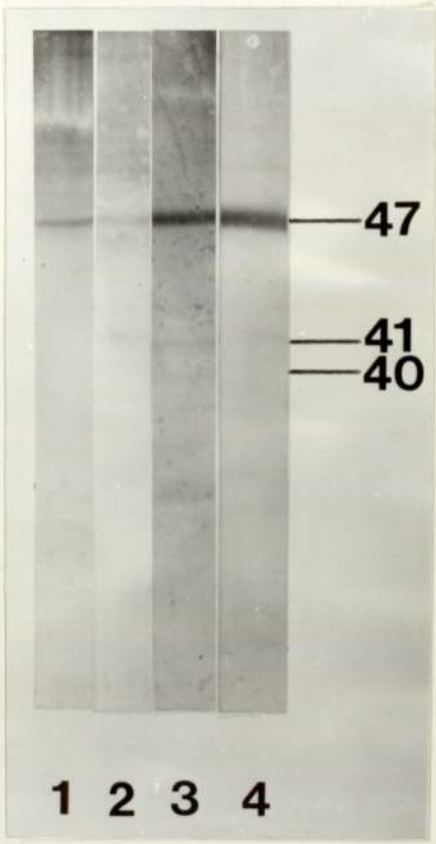


Fig.5.3A and B. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM+Fe (lanes 1 and 3) and in SSM-Fe (lanes 2 and 4) at 25°C as described in Fig.5.1A and incubated with serum from a patient with systemic candidosis (patient 1) (lanes 1 and 2) and with serum from normal individuals (lanes 3 and 4). Blots were developed with alkaline phosphatase anti-human IgG (A) or IgM (B). Molecular weights are in kilodaltons.

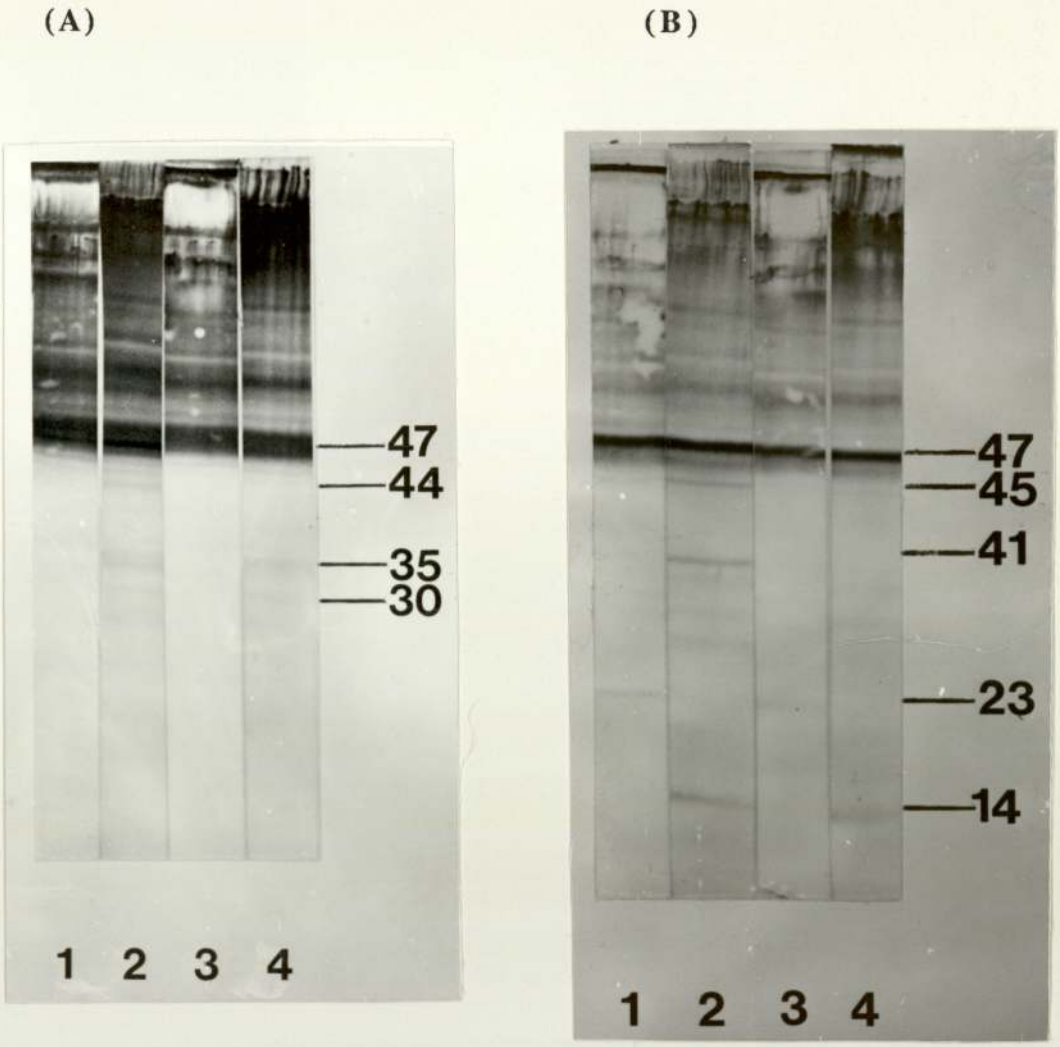


Fig.5.4A and B. Immunoblot of wall proteins from *C.albicans* AU2 grown in SSM+Fe (lanes 1 and 3) and SSM-Fe (lanes 2 and 4) at 25°C as described in Fig.5.1B and incubated with serum from patient 1 (lanes 1 and 2) and with serum from normal individuals (lanes 3 and 4). Blots were developed with alkaline phosphatase anti-human IgG (A) or IgM (B). Molecular weights are in kilodaltons.

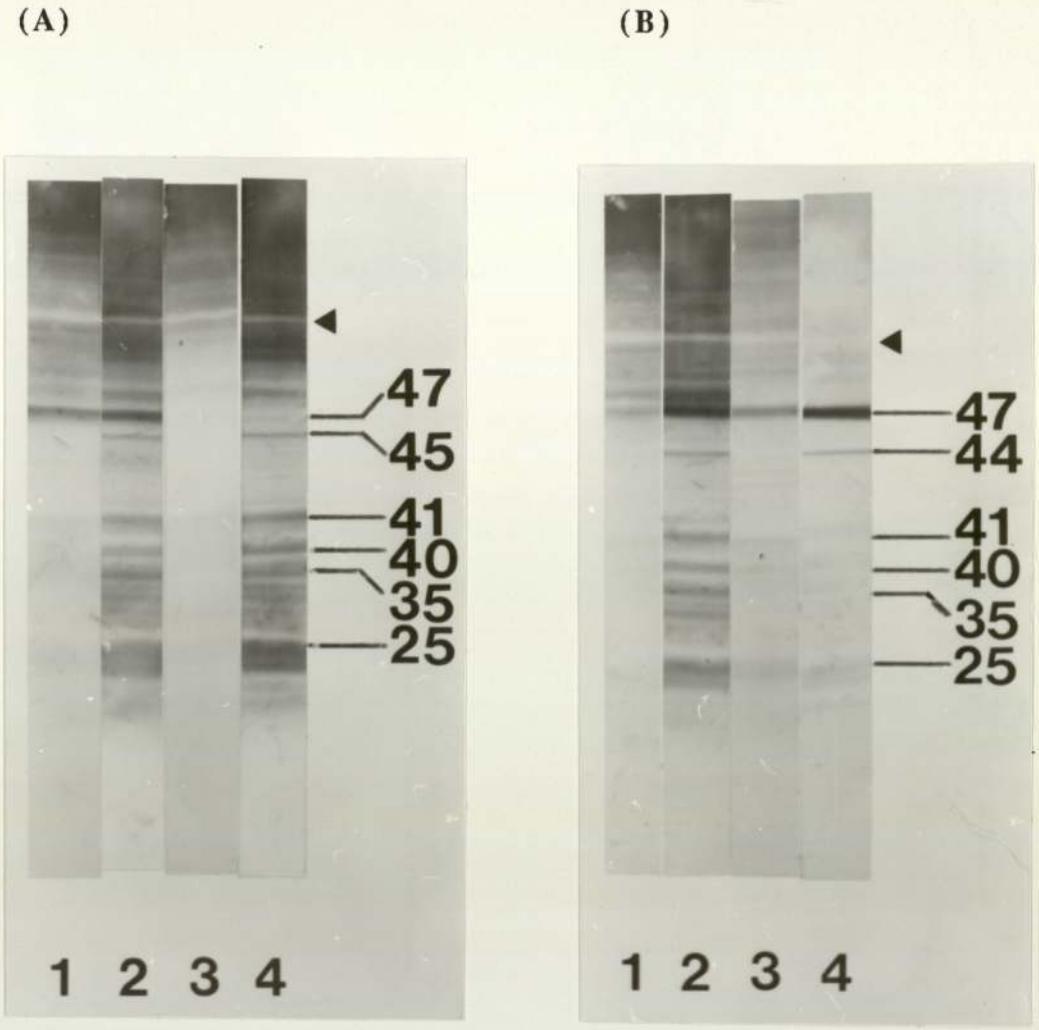


Fig.5.5A and B. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM+Fe (lanes 1 and 3) and SSM-Fe (lanes 2 and 4) at 37°C as described in Fig.5.1A and incubated with serum from patient 1 (lanes 1 and 2) and with serum from normal individuals (lanes 3 and 4). Blots were developed with alkaline phosphatase anti-human IgG (A) or IgM (B). Arrows indicate negatively stained regions of bands. Molecular weights are in kilodaltons.

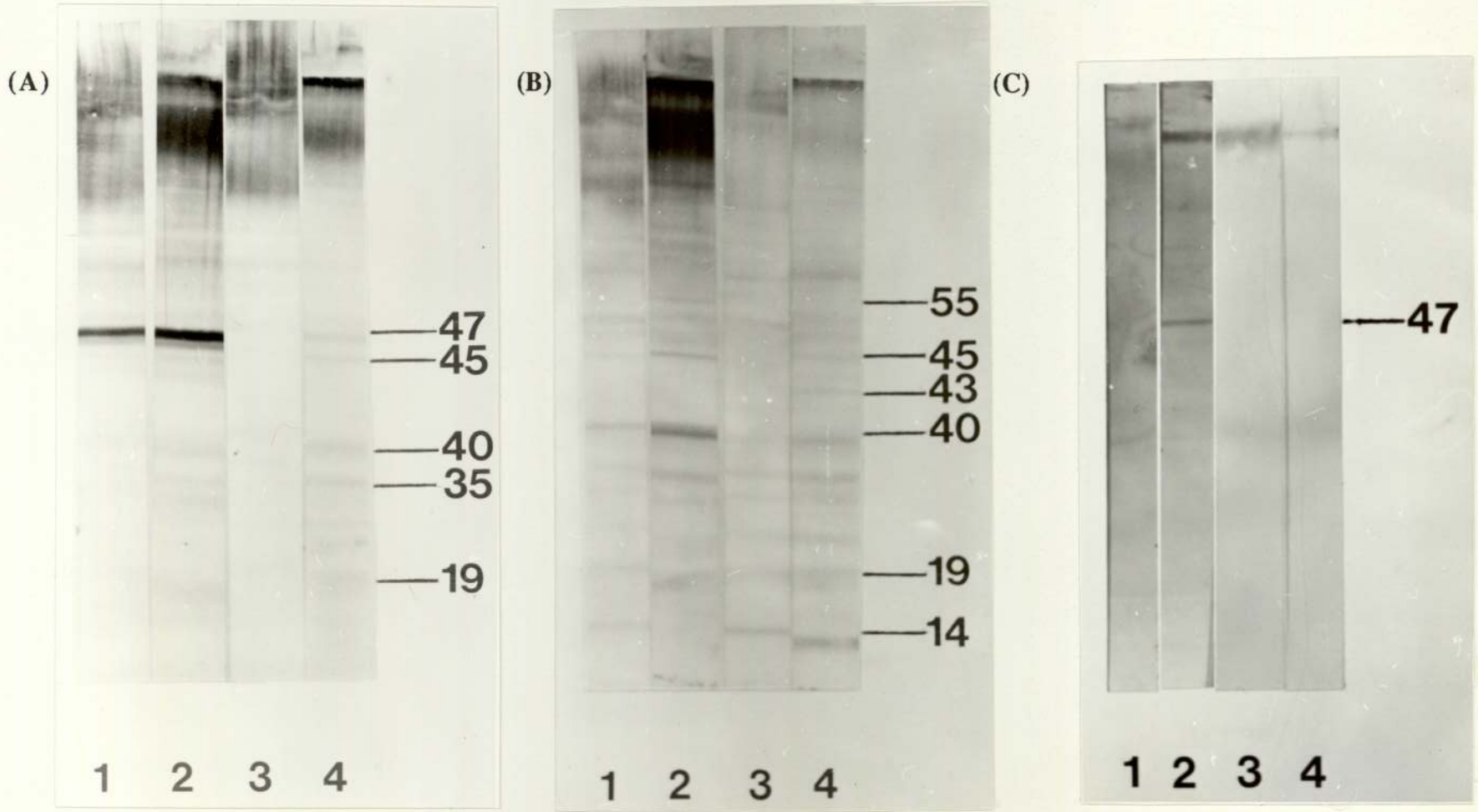


Fig.5.6.A, B and C. Immunoblot of wall proteins from *C.albicans* AU2 grown in SSM+Fe (lanes 1 and 3) and SSM-Fe (lanes 2 and 4) at 37°C as described in Fig.5.1B incubated with serum from patient 1 (lanes 1 and 2) and with serum from normal individuals (lanes 3 and 4). Blots were developed with alkaline phosphatase anti-human IgG (A), IgM (B) or IgA (C). Molecular weights are in kilodaltons.

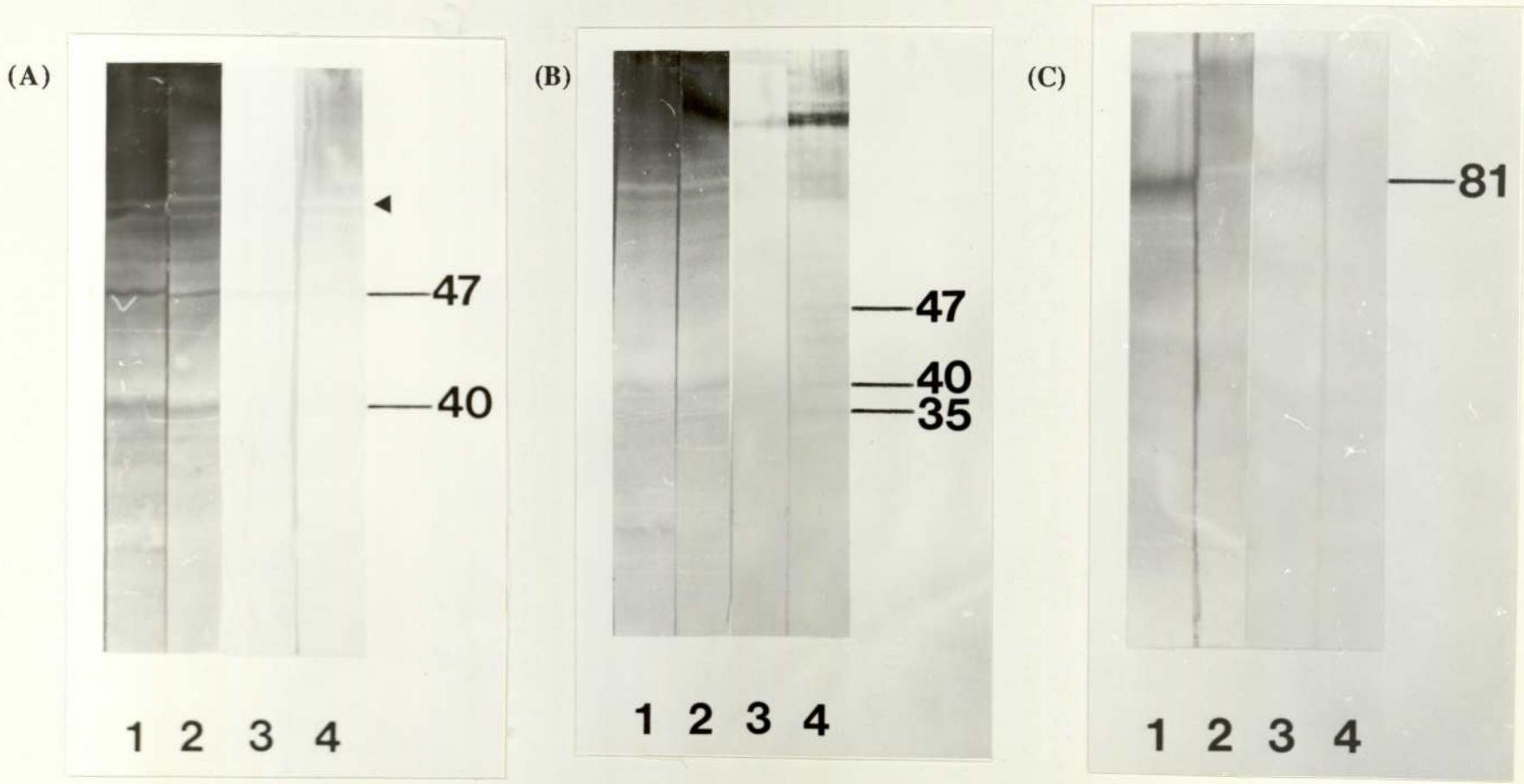


Fig.5.7A, B and C. Immunoblot of wall proteins from *C.albicans* OMC3 grown in SSM+Fe (lanes 1 and 3) and SSM-Fe (lanes 2 and 4) at 37°C and incubated with serum from patient 1 (lanes 1 and 2) and with serum from normal individuals (lanes 3 and 4). Blots were developed with alkaline phosphatase anti-human IgG (A), IgM (B) or IgA (C). Molecular weights are in kilodaltons.

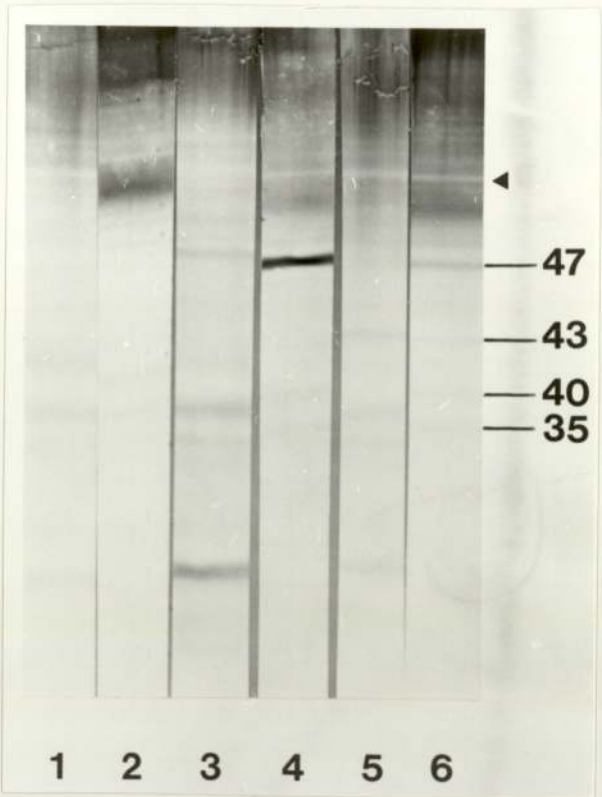


Fig.5.8. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM+Fe (lanes 1, 3 and 5) and SSM-Fe (lanes 2, 4 and 6) at 37°C as described in Fig.5.1A and incubated with serum from a patient with superficial candidosis (patient 2). Blots were developed with alkaline phosphatase anti-human IgG (lanes 1 and 2), IgM (lanes 3 and 4) or IgA (lanes 5 and 6). Molecular weights are in kilodaltons.

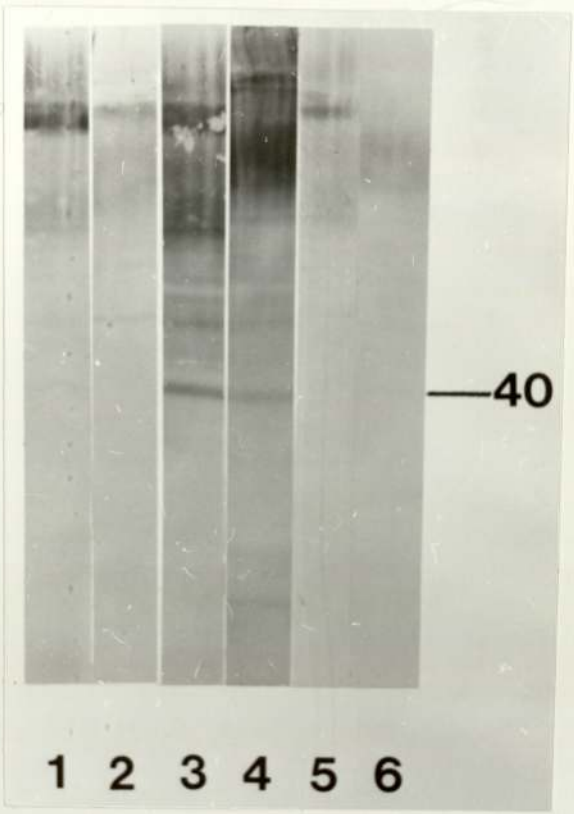


Fig.5.9. Immunoblot of wall proteins from *C.albicans* AU2 grown in SSM+Fe (lanes 1, 3 and 5) and SSM-Fe (lanes 2, 4 and 6) at 37°C as described in Fig.5.1B and incubated with serum from a patient with superficial candidosis (patient 2). Blots were developed with alkaline phosphatase anti-human IgG (lanes 1 and 2), IgM (lanes 3 and 4) or IgA (lanes 5 and 6). Molecular weights are in kilodaltons.

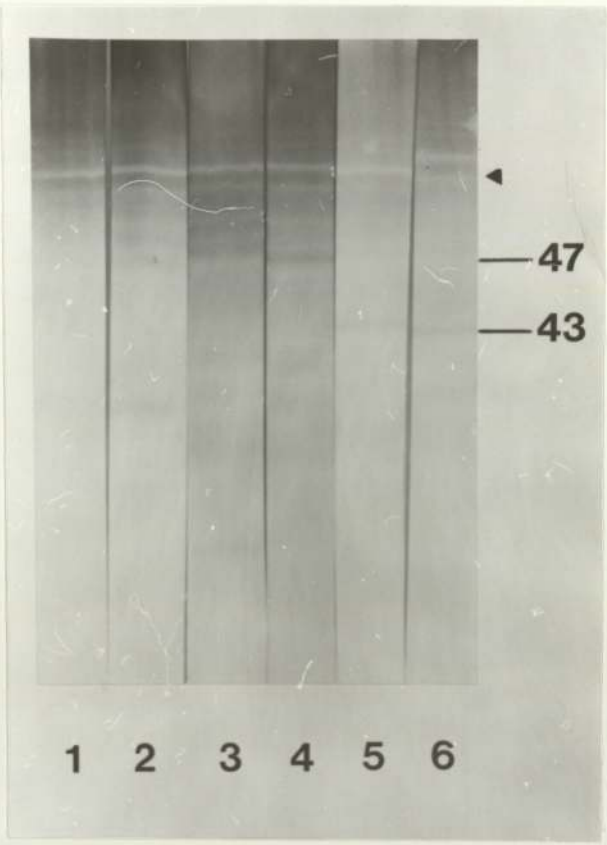


Fig.5.10. Immunoblot of wall proteins from *C.albicans* OMC3 grown in SSM+Fe (lanes 1, 3 and 5) and SSM-Fe (lanes 2, 4 and 6) at 37°C and incubated with serum from a patient with superficial candidosis (patient 2). Blots were developed with alkaline phosphatase anti-human IgG (lanes 1 and 2), IgM (lanes 3 and 4) or IgA (lanes 5 and 6). Molecular weights are in kilodaltons.

5.5. Discussion

SDS-PAGE and immunoblotting have found widespread use in numerous investigations to characterize specific antibody responses to individual components of antigenic surfaces of *C.albicans* and other microorganisms. Previous studies have shown that irrespective of growth temperature, growth phase (Chaffin and Stocco, 1983), or growth media (Gatermann *et al.*, 1986), SDS-PAGE profiles of *C.albicans* strains stained with Coomassie Blue revealed no apparent qualitative differences (Bruneau and Guinet, 1987), a finding consistent with the present results. Radiolabelling studies, which frequently provide a more sensitive determination of the quantity of cellular components, and is capable of distinguishing newly synthesized from pre-existing molecules, also failed to detect any changes in cell wall protein synthesis (Chaffin and Stocco, 1983). Hence, there is as yet no explanation for the apparent uniformity of cell wall proteins in *C.albicans* strains following growth *in vitro* under varying conditions.

The large number of wall proteins visualized by Coomassie Blue staining both in this and other studies, highlights the complex nature of *Candida* cell walls (Chaffin and Stocco, 1983), yet, little is known relating to functions of individual proteins. Apart from their general structural and enzymatic roles, wall proteins may have the potential to play a significant part in many cellular functions, including mediating adhesion to host epithelial cells (see section 1.5.1.) or forming receptor molecules for recognition by, or interaction with host immune components (Gilmore *et al.*, 1988).

Few studies on *C.albicans* have addressed the importance of nutrient deprivation

(Johnson *et al.*, 1978) and other host factors on cell wall structure and composition with respect to environmental conditions during infection in host tissues. Consequently, there is no information relating to phenotypic changes in yeast cells during growth under iron-depletion. In this study, emphasis was given to determining serum antibody responses in patients with candidosis, and to identify wall antigens of yeasts cultivated under iron-depleted and iron-sufficient conditions at physiological temperature. Since two of the three strains employed were isolated from patients with candidosis who provided serum, a more relevant assessment of individual antibody class responses to the infecting strain was obtained. In addition, comparisons could be made between strains, type of *Candida* infection and antibody response.

In one of the few studies that has considered the influence of growth conditions on antigenic profiles of *C.albicans*, Gatermann *et al.* (1986) prepared a membrane and a soluble antigen fraction of yeast cells following growth in a basic salts medium, a defined medium, two complex media, and a tissue culture based medium. Using SDS-PAGE and immunoblotting (with rabbit or human antisera) they found no significant antigenic differences between antigen preparations after growth in the five media at 37°C.

In contrast, the present study revealed gross antigenic differences in walls from yeasts of *C.albicans* which were influenced by growth environment and host antibody responses. Growth temperature affected cell wall antigen expression of *C.albicans* AU1 and AU2, as demonstrated by IgM antibodies from a patient with systemic candidosis (patient 1) reacting with more antigenic components in walls from AU1 and AU2 grown

at 37°C than at 25°C. However, the IgG response towards wall antigens from both isolates was similar regardless of temperature. Thus, it appears that for IgM antibodies at least, growth at physiological temperature gives rise to greater antibody recognition of wall antigens in some *C.albicans* isolates.

This study clearly demonstrates the heterogeneity in both quantity and specificity of antibody responses to wall antigens of *C.albicans* in patients with candidosis. Generally, antibodies from patient 1 produced a stronger response and recognized a greater number of antigens than antibodies from the patient with a superficial (oral) *Candida* infection (patient 2). This observation appears to be consistent with the demonstration of a greater antibody titre (determined by ELISA) in serum from patient 1 than from patient 2 (data not shown).

When walls from OMC3 (an isolate obtained from patient 2) were incubated with sera from patient 1 or 2, few discernible antigens were recognized. However, when walls from either AU1(a vaginal isolate) or AU2 (an isolate derived from patient 1) were incubated with the same sera, a greater number of antigens were detected. Hence, the antibody response to a systemic infection appears not only to be greater than to a superficial infection, but antibody recognition of wall antigens was also dependent on yeast strain. Furthermore, patients' antibody responses appeared to be governed by the site and severity of infection. These results may suggest that *C.albicans* isolated from different host tissue sites express antigens that reflect a particular type of infection which in turn evokes a characteristic antibody response. This view is supported by studies of

Matthews *et al.* (1984, 1987) who investigated heterogeneity in antibody responses to various antigenic determinants of *C.albicans* in 45 patients with systemic candidosis. Despite wide variation in antigens recognized by different patients, patterns of antibody responses were categorised into six groups. In their studies, the nature of infecting strains also appeared to influence patterns of antibody response.

Results of immunoblotting and ELISA (data not shown) revealed that IgA antibody titres in both patients 1 and 2 were extremely low, and a relatively poor IgA response was produced towards wall antigens from each *Candida* isolate. These results are not surprising given the predominance of non-specific local host factors against oral infections in compromised hosts. These factors include the epithelial barrier, microbial interactions, lysozyme, lactoferrin and levels of iron.

Secretory IgA (SIgA) is the predominant immunoglobulin present in saliva and is believed by many workers to be important in limiting oral infection, possibly by affecting growth (Grappel and Calderone, 1976), metabolism and adherence (Kimura and Pearsall, 1978) in *C.albicans*. In contrast, Epstein *et al.* (1982) suggested that increased titres of SIgA in mucosal secretions may be insufficient to prevent oral candidosis. However, in oral infections, antibody titres tend to reflect the degree of antigenic stimulation, i.e. it is higher in patients with greater numbers of organisms (Epstein *et al.*, 1984). Therefore, a small population of infecting organisms in the oral cavity of patient 2, could explain a relatively poor IgA, as well as a poor IgG and IgM response of this patient towards *C.albicans* isolates observed in this study.

Iron, which is an essential nutrient for both bacteria and fungi, is also an important host factor in oral infections, and appears to modulate lysozyme activity which in turn lyses *Candida* species and stimulates their phagocytosis (Epstein *et al.*, 1984). Hence, a modulating role for iron in lysozyme-lactoferrin interactions, may be significant in preventing multiplication of *C.albicans* during oral colonization and infection, since reduced levels of iron are associated with greater lysozyme activity.

In the absence of extensive literature relating to antigenic stimulation during superficial infections, one could also speculate that a poor antibody response may be a consequence of minimal antigenic stimulation by *C.albicans* of humoral immune systems during oral infections. Alternatively, the action of cellular immune mechanisms combined with local factors may be of greater importance in combating oral infection than humoral immune mechanisms.

Regardless of growth temperature, *C.albicans* expressed several wall antigens following growth under iron-depleted conditions that were either barely detectable or not expressed in walls from cells grown under iron-sufficient conditions. The magnitude and variability of antibody responses to these iron-regulated proteins were dependent on the type of *Candida* infection, antibody class and yeast strain. IgG antibodies from patient 1 or normal individuals, and IgM antibodies from patient 1 recognized several iron-regulated proteins of MW 45, 44, 41, 40, 35 and 25kd in walls of AU1, however, IgM antibodies from normal individuals only recognized a 44kd iron-regulated protein in walls of AU1 grown at 37°C. IgM (55 and 40kd) and IgA (47kd) antibodies from patient 1

detected fewer iron-regulated proteins than IgG in walls of AU2, whereas only IgM antibodies from patient 1 were capable of detecting proteins of MW 47, 40 and 35kd in walls of OMC3. IgM and IgA were the only antibodies from patient 2 which recognized a 47kd iron-regulated protein in walls of AU1, no such proteins were detected in AU2 or OMC3.

In response to iron-deprivation *C.albicans* secretes siderophores of the hydroxamate-type (Holzberg and Artis, 1983), and some clinical isolates may produce phenolate-type siderophores (Ismail *et al.*, 1985a) to acquire iron from host iron-binding proteins. Recently, Ismail and Lupan (1988) tentatively demonstrated the presence of a putative siderophore receptor in both cytoplasmic membranes and cell wall proteins of *C.albicans* yeasts that preferentially bound radiolabelled hydroxamate-type siderophores. When these results are considered along with findings of this study they provide some evidence for existence of iron-regulated proteins in cell walls of *C.albicans*. The precise role of these proteins in iron uptake remains to be determined, however, one function may involve siderophore-mediated iron transport.

Griffiths *et al.* (1985a and b) observed that the relative abundance of IRMPs expressed in different pathogenic *E.coli* strains under the same iron-restricted growth conditions, varied considerably, and that pathogenic strains produced more IRMPs than a non-pathogenic laboratory strain. In addition, expression of IRMPs in pathogenic *E.coli* isolates varied with the site of infection. Similarly, in this study, expression of iron-regulated proteins in *C.albicans* varied between strains, and it appeared that strains AU1

and AU2 expressed more wall proteins that were immunogenic than OMC3. These results provide additional evidence for antigenic variation between *C.albicans* strains grown under uniform conditions, however, further studies including more clinical isolates and laboratory strains of *C.albicans* could help elucidate the extent of variability in expression of iron-regulated proteins in this fungus.

Since it has been shown that IRMPs are expressed by Gram-negative organisms *in vivo* during infection (Griffiths *et al.*, 1983; Brown *et al.*, 1984; Shand *et al.*, 1985), the possibility that receptors for ferric siderophores could be important protective antigens is likely to receive increasing attention. Unfortunately, an attempt to isolate *C.albicans* directly and without subculture from kidneys of a rabbit model of systemic candidosis was unsuccessful, hence it is not known whether *C.albicans* expresses iron-regulated proteins *in vivo*. Despite this, the diverse antibody response of normal individuals to these antigens suggests that there may be naturally occurring antibodies which react with iron-regulated proteins of some *C.albicans* strains and may afford a protective role. In support of this view, naturally occurring IgG and IgA antibodies that react with IRMPs of *E.coli*, including the siderophore receptors, have previously been found in normal healthy individuals (Griffiths, 1987b).

The problems associated with diagnosis of systemic candidosis has been extensively investigated (Matthews *et al.*, 1984; Gatermann *et al.*, 1986; Manning-Zweerink *et al.*, 1986), and has prompted the use of sensitive immunological techniques, such as immunoblotting, to examine sera for anti-*C.albicans* antibody and *Candida* antigens that

may be associated with systemic infection. So far few *Candida* antigens have emerged as potential serodiagnostic markers, however, there is increasing interest in a 47kd antigen.

In this study, IgG antibodies from patient 1 reacted consistently and frequently strongly with a 47kd antigen in walls of AU1 and AU2. Similarly, when Matthews *et al.* (1984, 1987) analysed antibody responses of patients with systemic candidosis, they found that 73% produced antibody to a 47kd antigen in cytoplasmic extracts. This has been described as an immunodominant antigen of *C.albicans* (Matthews *et al.*, 1984), and antigens with similar molecular weights (Greenfield and Jones, 1981; Strockbine *et al.*, 1984a and b; Au-Young *et al.*, 1985; Bruneau and Guinet, 1987) may be identical or closely related since apparent differences in molecular weight may arise through variations in antigen preparation and antibody detection.

This antigen appears to be characteristic of systemic candidosis because antibodies to it are rare in patients with superficial *Candida* infections and normal healthy individuals (Matthews *et al.*, 1984, 1987; Au-Young *et al.*, 1985; Neale *et al.*, 1987). In agreement with this view, the 47kd antigen was seldom detected by antibodies from the patient with a superficial infection or normal individuals in this study. Furthermore, Matthews *et al.* (1984) found that antibody to a 47kd antigen was produced by all hospital patients who survived, whereas those who died had either a poor or absent response to this antigen. The immunodominance of this antigen and its shedding during infection, makes it an obvious candidate for developing a new generation of serodiagnostic tests for systemic candidosis. Indeed, Matthews and Burnie (1988)

described a test based on detection of the circulating 47kd antigen, and found its sensitivity and specificity for disseminated *C.albicans* infections increased. This enhanced sensitivity was important in detecting cases of systemic candidosis early in the course of infection.

In addition to the 47kd antigen, other antigens have been recognized as potential candidates for diagnosis of systemic candidosis. Gatermann *et al.* (1986) observed an IgM response to an 80kd antigen from a soluble antigen fraction of *C.albicans*, whereas 40% of patients with systemic candidosis produced either an IgG or IgM response to a 60kd antigen in a cytoplasmic extract of *C.albicans* (Matthews *et al.*, 1984).

Typically, in the studies described above, each *C.albicans* isolate was cultivated under iron-plentiful conditions, thus limiting expression of antigens of potential value for diagnosis of systemic candidosis. The present results suggest that future investigations to identify and isolate antigens that may be of diagnostic value, would benefit from growing cells under iron-depleted conditions. Since iron-depleted yeast cells expressed a greater complement of wall antigens than iron-sufficient cells, these antigens may not only reflect growth during infection, but could present useful targets for developing monoclonal antibodies, therefore increasing specificity of diagnostic tests. Furthermore, some of these proteins may be responsible for nutrient uptake by the cell during growth *in vivo*, therefore characterization of these components would provide a basis for a better understanding not only of host recognition of *C.albicans*, but also of cell wall structure and its role in other functions.

6. Tentative characterization and cellular location of *C.albicans* antigens

6.1. Detection of glycosylated cell wall components

Given the increasing importance of characterizing *C.albicans* antigenic components to elucidate their role during infection, the following study describes a partial chemical analysis of cell wall antigens isolated from yeasts grown under iron-depleted and iron-sufficient conditions. The high affinity of lectins for specific sugar residues or sequences has been widely used to characterize microbial surfaces and isolated components of cells (Pistole, 1981). In particular, concanavalin A (Con A) has been shown to react with α-D-mannopyranosyl residues found in mannans (mannoproteins) of *C.albicans* yeast cells (**Pistole,** 1981; Miragall *et al.*, 1988). Since mannoproteins are major constituents of *C.albicans* cell walls, Con A provides a valuable tool for detecting such components.

Blots of electrophoretically separated wall proteins from three isolates of *C.albicans* grown in SSM+Fe and SSM-Fe at 25°C and at 37°C revealed no apparent antigenic differences when stained with Con A. Therefore, isolate AU2 was chosen as representative of these isolates (Fig.6.1.). Lectin blotting revealed a marked heterogeneity in the antigenic patterns of each blot, in particular, upper regions of each blot, except those of cells grown in SSM+Fe at 25°C (lane 1), were characterized by an intense diffuse staining. Con A also reacted strongly with major antigens of MW 47, 41, 35, 30 and 19kd in walls of cells grown in SSM-Fe at 25°C (lane 2) and 37°C (lane 4). However, few of these antigens were detectable in walls of yeasts cultivated under iron-sufficient conditions (lanes 1 and 3).

6.2. Recognition of *C.albicans* antigens by yeast-absorbed human serum

Serum from a patient with systemic candidosis (patient 1) was absorbed with yeasts from the patients' own isolate (AU2) grown in SSM-Fe, as described in section 3.8.2., and blots of electrophoretically separated proteins from AU1, AU2 and OMC3 were incubated with this absorbed serum (Fig.6.2.). All blots were developed with an alkaline phosphatase-conjugated anti-human IgG. Absorbing serum with yeast cells removes antibodies responsible for a dense heterogeneous staining of the upper region of blots as described previously, and consequently reveals discrete bands.

The major antigen recognized in all isolates had a MW of 47kd, however, the IgG response was somewhat weaker to this antigen in yeasts grown in SSM+Fe (Fig.6.2.). A 45kd and a 35kd antigen were detected by serum in walls from AU1 grown in SSM-Fe (lane 2) but not SSM+Fe (lane 1). Similarly, a 45kd and a 19kd were recognized only in walls from AU2 grown in SSM-Fe (lane 4), whereas there was no apparent distinction between blots of walls from OMC3 grown in SSM+Fe and SSM-Fe (lane 5 and 6).

6.3. Immunocytochemical detection of surface antigens of *C.albicans*

This investigation attempts to determine whether the presence or absence of iron influences surface antigen distribution on *C.albicans* yeast cells. Expression of yeast cell surface antigens was studied by reacting stationary phase yeasts grown under iron-

depleted and iron-sufficient conditions with serum from a patient with systemic candidosis. These cells were then incubated with protein A conjugated with colloidal gold and sections were cut, mounted, stained, and visualized using transmission electron microscopy. Yeast cells revealed a uniform distribution of single gold particles located on the outermost part of a cell wall fibrillar layer (Fig.6.3. and 6.4.). In negative controls no evidence of non-specific binding of gold was apparent (Fig.6.5.). These results indicate that antigens located on yeast surfaces are accessible to IgG antibodies.

Modification of cell preparation and fixing procedures was attempted, including sectioning of yeast cells prior to incubation with patient sera, to determine whether growth conditions affect intracellular antigen expression. However, this procedure proved unsuccessful, probably due in part to inadequate fixation of yeast cells.

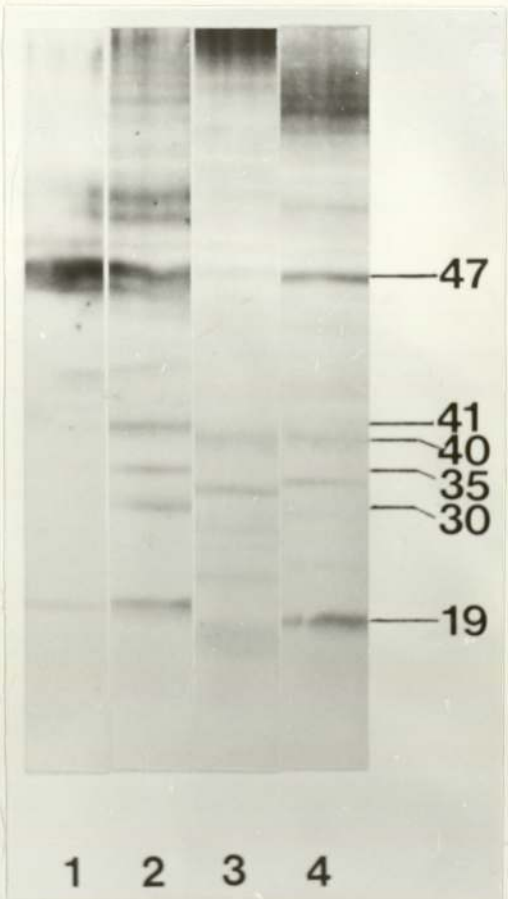


Fig.6.1. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM+Fe (lane 1) and SSM-Fe (lane 2) at 25°C; and in SSM+Fe (lane 3) and SSM-Fe (lane 4) at 37°C. Blots were stained concanavalin A-peroxidase. Molecular weights are in kilodaltons.

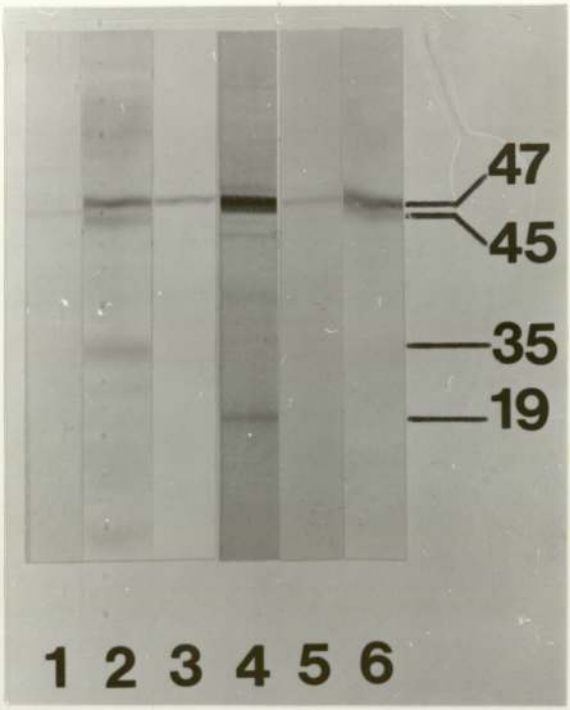


Fig.6.2. Immunoblot of wall proteins from *C.albicans* AU1 (lanes 1 and 2), AU2 (lanes 3 and 4) and OMC3 (lanes 5 and 6) grown in SSM+Fe (lanes 1, 3 and 5) and SSM-Fe (lanes 2, 4 and 6) at 37°C. Blots were incubated with yeast-absorbed serum from patient 1 and developed with alkaline phosphatase anti-human IgG. Molecular weights are in kilodaltons.

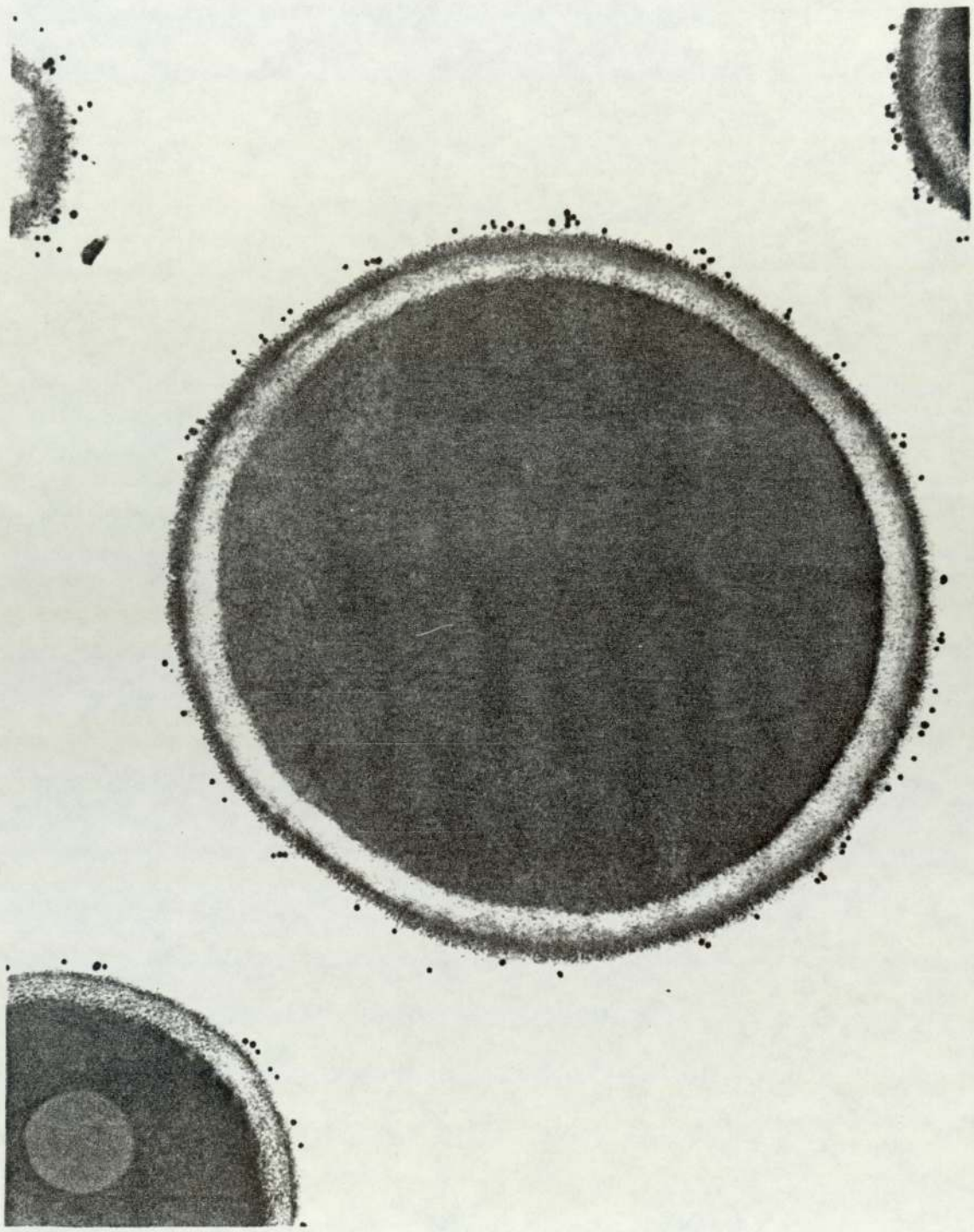


Fig.6.3. Expression of surface antigens on yeasts of Candida albicans AU1. Antigens detected with colloidal gold-conjugated secondary antibodies were distributed on the outer fibrillar layer. Cells were grown in SSM+Fe. Bar=0.5μM x 23000

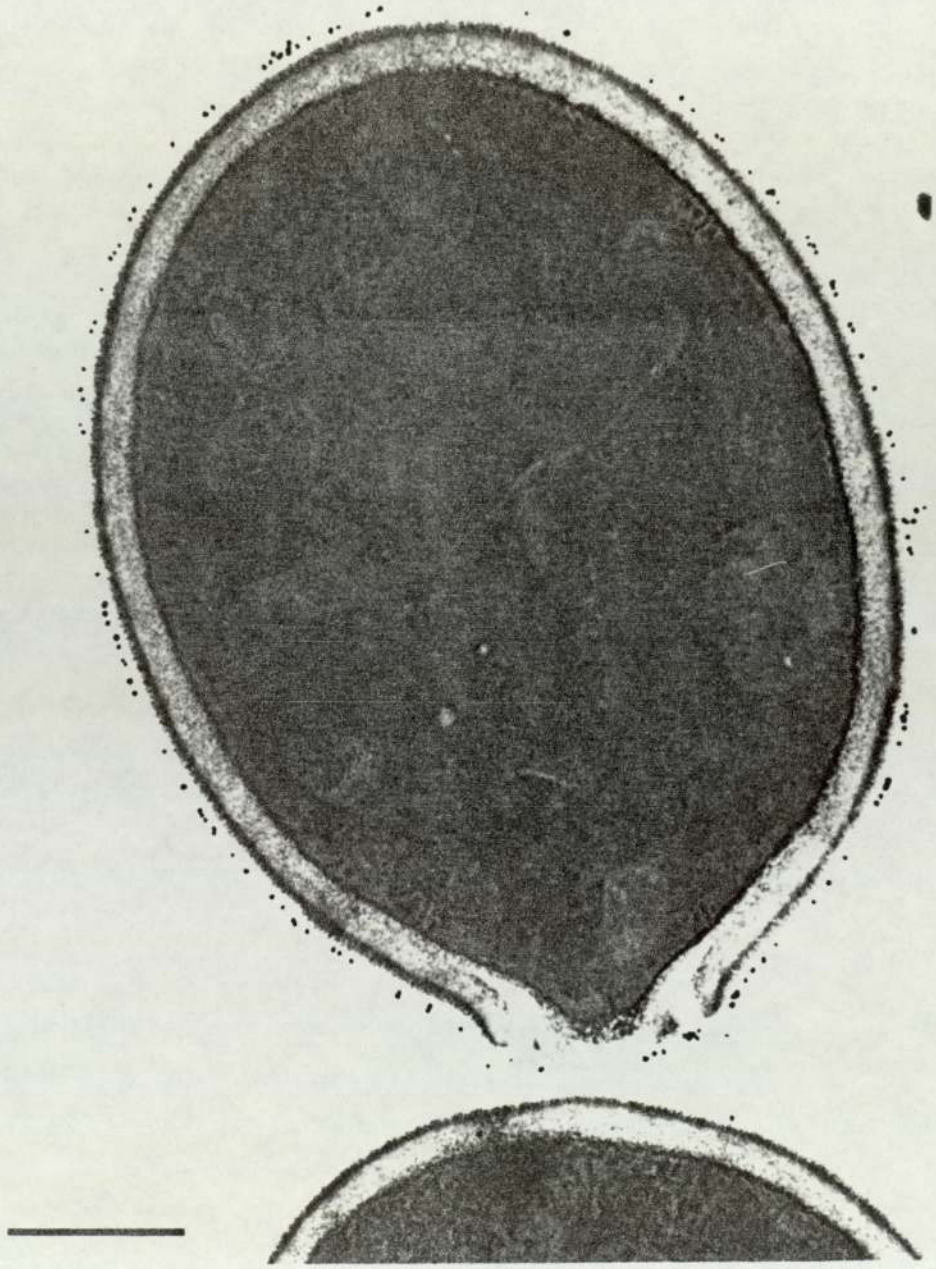


Fig.6.4. Expression of surface antigens on yeasts of Candida albicans AU1. Antigens were detected as described in fig.6.3. and distributed on the outer fibrillar layer. Cells were grown in SSM-Fe. Bar=0.5μM x 18000

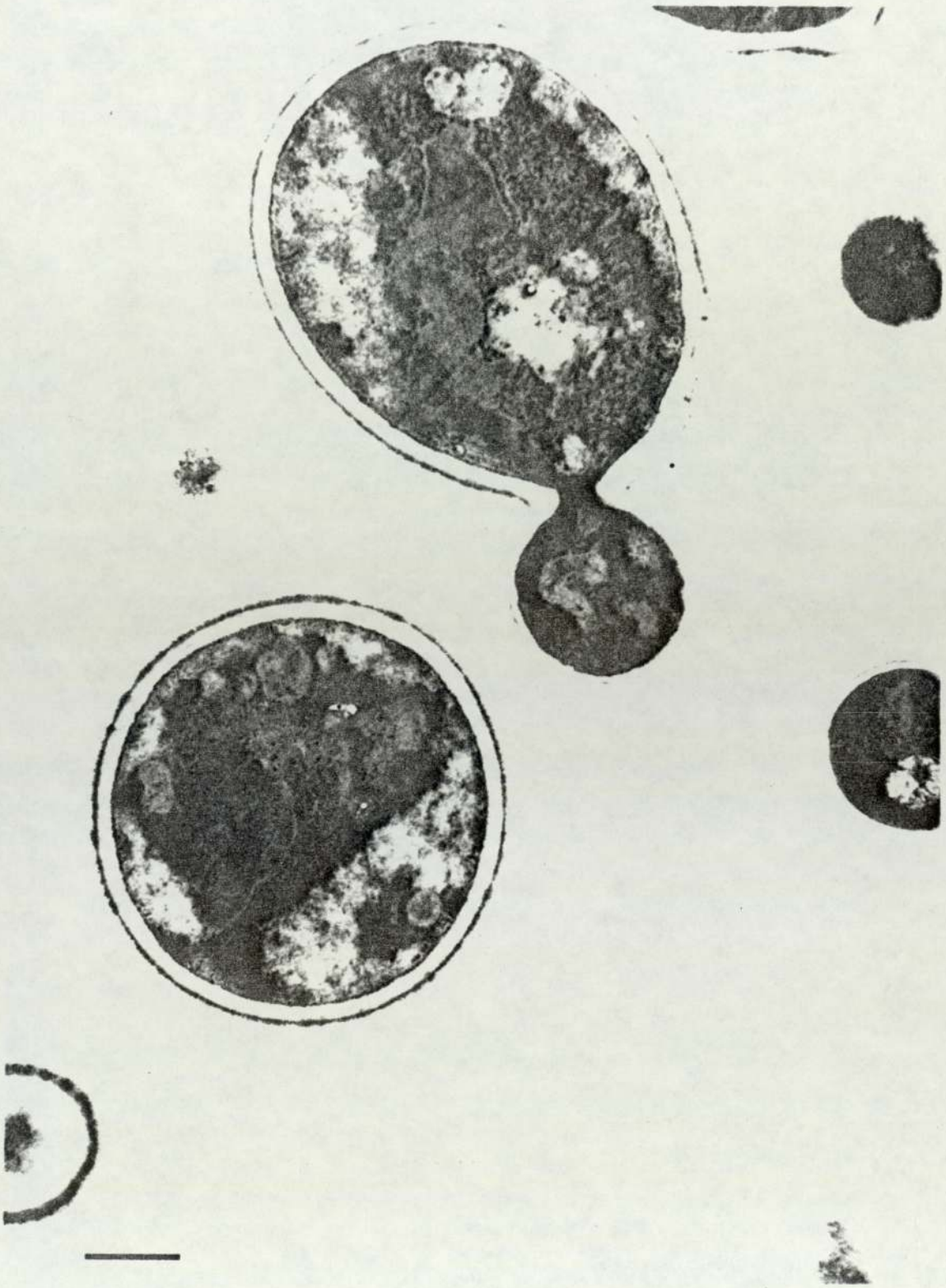


Fig.6.5. Control for non-specific gold-labelling of yeasts in the absence of primary antibody. Bar = 0.5 μM x 9800

6.4. Discussion

Definition of surface constituents and their antigenic determinants is important to understanding host-*C.albicans* interactions. These components are involved in evoking host immune responses as well as other cellular functions, however, their surface epitopes remain poorly defined. Given the lack of literature relating to characterization of surface antigenic components of *C.albicans* cultivated under conditions that may reflect growth during infection *in vivo*, the present investigation describes the ultrastructural and cellular location, and partial chemical analysis of some of these antigens following growth under iron-depleted and iron-sufficient conditions.

By using concanavalin A (Con A), a lectin with a high affinity for mannose polymers, results of cytochemical, ultrastructural, and agglutination studies have shown that mannose-containing constituents (mannoproteins) are present on *C.albicans* surfaces (Cassone *et al.*, 1978; Tronchin *et al.*, 1981, 1988; Linehan *et al.*, 1988; Miragall *et al.*, 1988). These results have been supported by studies of Elorza *et al.* (1985) who detected over 40 different bands from a cell wall mannoprotein extract using SDS-PAGE. To confirm a mannoprotein nature of these solubilized molecules, Con A-Sepharose was added to the extract and over 85% of mannoproteins were retained by Con A. Furthermore, virtually all molecules were released by addition of α-methyl-D-mannoside (a mannan inhibitor), and mannoprotein profiles before and after Con A treatment were identical, suggesting that treatment did not discriminate between mannoproteins.

Using Con A, this study revealed the presence of numerous mannoproteins in cell walls of *C.albicans*. Virtually all antigens stained with Con A were also recognized by

serum from patients with systemic or superficial candidosis, or normal individuals as described in section 5.4.1. However, several antigens detected with human sera reacted poorly or not at all with Con A, including some iron-regulated proteins, a 47kd antigen in walls from cells cultivated in SSM+Fe at 37°C, and the high MW antigens in walls from cells grown in SSM+Fe at 25°C.

These findings may be explained by levels of mannose residues in these mannoproteins which are too low to be detected by Con A, suggesting that these antigens could be predominantly protein in nature. Alternatively, growth in the presence of excess iron may alter mannose composition in mannoprotein molecules resulting in a loss or masking of sites accessible to binding by Con A. This latter view is consistent with that of Chaffin *et al.* (1988) and Sundstrom *et al.* (1988) who proposed that surface antigen heterogeneity may partly be a result of variability in surface polysaccharides. Regulation may involve mechanisms of synthesis and loss by degradation or cell wall reorganization, which is in turn influenced by growth conditions (Brawner and Cutler, 1984). Therefore, the antigenic nature of *C.albicans* cell walls appears to consist of a complex array of proteins and mannoproteins whose chemical composition could be influenced by growth under iron-depleted and iron-sufficient conditions.

The glycoprotein nature of *C.albicans* surfaces may be important in mediating adhesion to host tissues (see section 1.5.1.), and considerable evidence suggests that *C.albicans* adherence is mediated by mannoproteins, but it is not known whether polysaccharide or protein portions of mannoprotein molecules are involved in adhesion

processes. Studies by Critchley and Douglas (1987) revealed that predominant interactions between yeasts and buccal epithelial cells involved the protein portion of mannoprotein molecules. Such a mechanism would be analogous to that found in many Gram-negative bacteria where adhesion to mucosal surfaces is mediated by proteinaceous, carbohydrate-binding adhesins. Consequently, Critchley and Douglas (1987) suggested that protein moieties of mannoprotein molecules may be more important than polysaccharide moieties in mediating yeast attachment to epithelial cells.

Furthermore, a potential role for glycoproteins in pathogenicity has been reported by Gilmore *et al.* (1988) who showed that receptor-like proteins on candidal surfaces bound the complement fragment, iC3b non-covalently, thus inhibiting opsonization and preventing effective phagocytosis of organisms. Mycelial transformation and enhanced glucose concentrations augmented expression of iC3b receptors on *Candida* surfaces and increased resistance of *C.albicans* to phagocytosis.

To determine the cellular location of *C.albicans* antigens, electrophoretically separated proteins were incubated with yeast-absorbed serum from a patient with systemic candidosis. Absorbing serum with yeast cells removes antibodies responsible for the dense, diffuse staining of high MW components, implying that these heterogeneous antigens are located on yeast cell surfaces. Since surface antigens of *C.albicans* are predominantly mannoprotein in nature, these high MW antigens are likely to be the same because it stained intensely with Con A, and was not visualized in gels stained with Coomassie Blue, thus reflecting its low proportion of protein. Similar

observations have been reported by other investigators (Manning-Zweerink *et al.*, 1986; Ponton and Jones, 1987; Sundstrom *et al.*, 1987).

It was also apparent that absorbed serum detected fewer antigens than unabsorbed serum, indicating that the majority of antigens are located on yeast surfaces. However, several antigens of MW 47, 45, 35 and 19kd were present in cell walls but not on yeast surfaces as shown by their ability to react with both absorbed and unabsorbed sera. Similarly, Ponton and Jones (1986) detected antigens in wall extracts from yeasts in the range of 22 to 50kd when stained with yeast-absorbed serum, and suggested that these antigens could be expressed preferentially in yeast cell walls at locations other than its outer surface.

Isolates AU1 and AU2 cultivated under iron-depleted conditions expressed antigens that were either barely detectable or not expressed in walls from cells grown under iron-sufficient conditions when detected with absorbed serum. Hence, some of these iron-regulated proteins or mannoproteins (as determined by Con A binding) appear to be located within cell walls, whereas others are located on yeast surfaces.

The location of IgG antibody binding sites on yeast surfaces was determined by reacting ultrathin sections of yeast cells with serum from a patient with systemic candidosis and staining the preparations with a secondary antibody conjugated with colloidal gold. Antigenic determinants which reacted with IgG antibodies were distributed uniformly over yeast cell surfaces. These antigens were associated with an outer fibrillar layer of cell walls which have also been observed by other workers (Poulain *et al.*,

1985; Brawner and Cutler, 1986b, 1987; Tokunaga *et al.*, 1986).

Surface antigens which appear to be accessible to antibodies *in vitro*, probably comprise many of the antigens located on yeast surfaces as demonstrated by using absorbed and unabsorbed sera, and are also likely to be mannoprotein and protein in nature. Absorbed and unabsorbed serum revealed the presence of iron-regulated proteins on yeast surfaces using immunoblotting, yet immunoelectron microscopy showed no apparent differences in antigen expression between cells cultivated under iron-depleted and iron-sufficient conditions. This discrepancy is likely to reflect a difference in sensitivity between techniques. Alternatively, given the denaturing aspect of immunoblotting, it is possible that some epitopes recognized by antibodies may not be expressed on surface proteins or mannoproteins in their native configuration in cell walls. Conversely, some epitopes in the outermost fibrillar layer may easily be lost or altered from their native state due to chemical treatment in preparation for electron microscopy.

Expression of candidal surface antigens and their antigenic determinants *in vitro* does not necessarily reflect expression *in vivo* (Brawner and Cutler, 1987). Using a similar technique to the present study, Brawner and Cutler (1987) examined the intracellular and cell surface expression of two antigenic determinants of *C.albicans* which specifically react with monoclonal antibodies. Differences between *in vitro* and *in vivo* antigen expression (in cells recovered from infected mice) were observed during early stages of growth. Antigen expression *in vivo* appeared to be concentrated in deeper cell wall layers, however, both *in vivo* and *in vitro* grown cells possessed antigen associated

with an outer fibrillar layer. In addition, expression of antigens in yeasts growing in kidney cells differed from cells isolated from animals inoculated intraperitoneally. Therefore, it appears that host factors, and site of infection influence surface antigen expression in *C.albicans*. These factors should be considered when designing such experiments and interpretating *in vitro* antigen expression. Furthermore, the results of Brawner and Cutler (1987) reveals that monitoring of intracellular antigen expression could help identify cytoplasmic organelles which are sites for assembling antigens.

With increasing emergence of monoclonal antibody (MAb) probes for yeast surface antigens, it would be valuable to isolate MAbs directed against polysaccharide and protein moieties of iron-regulated protein or mannoprotein molecules on *C.albicans* surfaces to gain a better understanding of their significance and regulation during infection.

7. IgG response to *C.albicans* in immunized rabbits

7.1. Sequential rabbit IgG response to *C.albicans* using immunoblotting

Immunoblotting was used to monitor the IgG response in four rabbits immunized biweekly with *C.albicans* AU1 over a period of 8 weeks. Cell walls of isolate AU1, prepared from cells cultivated in SSM-Fe and SSM+Fe, were separated by SDS-PAGE, transferred to Nc paper then incubated with rabbit serum and blots were developed with alkaline phosphatase-conjugated goat anti-rabbit IgG. Figs.7.1. to 7.4. show blots of wall proteins from iron-depleted yeasts detected with sequential sera from each rabbit. Lane 9 in each figure represents the IgG response of a high titre reference antiserum (Dakopatts, DAKO Ltd.) raised against an aqueous extract of homogenized *C.albicans*.

No antigens were detected by pre-immune serum, or serum collected after three weeks of immunization (Figs.7.1. to 7.4. lanes 1-3), but after week 4, each rabbit serum initiated antigen recognition. Sera from rabbits 1 (Fig.7.1. lane 4) and 3 (Fig.7.3. lane 4) produced a weak IgG response towards antigens of MW 28 and 35kd respectively, whereas sera from rabbits 2 (Fig.7.2. lane 4) and 4 (Fig.7.4. lane 4) produced a similar response to a 70kd antigen.

From week 5 onwards, each rabbit gave rise to a response of increasing intensity to numerous wall antigens, however, different major antigens were recognized by each rabbit serum. After week 5, serum from rabbit 1 produced a weak response to major antigens of MW 106-94, 70, 66, 47, 40, 35 and 20kd (Fig.7.1. lane 5). Thereafter, the

response to these predominant antigens, in addition to a 44 and 43kd antigen, intensified greatly (lanes 6-8) whereas the response to the 28kd antigen diminished. IgG antibodies from rabbit 3 reacted strongly with a 106-94, 36 and 35kd antigen and weakly with a 44kd antigen after week 5 (Fig.7.3. lane 5), however, the response to these antigens, as well as a 70kd antigen increased with time (lanes 6-8). The predominant antigen recognized by serum from rabbits 2 (Fig.7.2. lane 5) and 4 (Fig.7.4. lane 5) after week 5 was of MW 70kd, but weaker responses by both rabbit sera to antigens of MW 44, 43, 40, 36, 35 and 20kd were also observed. As described for other rabbits the response to these antigens continued to increase with time.

Blots of wall proteins from cells grown under iron-sufficient conditions incubated with sequential sera from four rabbits revealed essentially similar antigenic profiles, therefore blots obtained following incubation with serum from rabbit 1 were chosen as representative of all blots (Fig.7.5.). No antigens were detected by pre-immune serum, but, in contrast to blots of walls from iron-depleted yeasts, a weak IgG response was produced to a 44kd antigen after only 2 weeks of immunization (lane 2). In addition, after week 3, a weak response emerged to a 70kd antigen (lane 3). The response to these two antigens increased with time, however, the intensity of reaction was strongest towards the 70kd antigen.

7.2. Sequential rabbit IgG response to *C.albicans* using ELISA

Enzyme-linked immunosorbent assay (ELISA) was used in parallel to immunoblotting to

detect and quantitate the anti-*C.albicans* AU1 IgG response in rabbits immunized as described in section 3.6.3. Whole cells of AU1 prepared from iron-depleted and iron-sufficient cultures were used as solid phase antigens in ELISA microtitre plates, and the results using serum taken sequentially from four rabbits is shown in Table 7.1.

Antibody titres did not rise above the pre-immune value until 3 weeks after immunization of rabbits 1 and 2, and 5 weeks after immunization of rabbits 3 and 4. Thereafter, a marked increase in antibody titre was observed in each animal. Serum titres in rabbits 1 and 3 were greater when measured with solid phase antigens prepared from cells cultivated in SSM+Fe than from SSM-Fe. Conversely, titres in rabbits 2 and 4 were higher when detected with antigens prepared from SSM-Fe than from SSM+Fe.

Table 7.1. Comparison of IgG antibody titres* in four rabbits immunized with *C.albicans* AU1.

No. of weeks post-immunization	Rabbit 1		2		3		4	
	Solid phase antigen†							
	Fe+	Fe-	Fe+	Fe-	Fe+	Fe-	Fe+	Fe-
Preimmune	<20	20	<30	40	<40	<60	<40	<60
1	<20	20	<30	40	<40	<60	<40	<60
2	20	50	<30	40	<40	<60	<40	<60
3	50	80	<30	550	<40	<60	<40	<60
4	52	120	37	700	<40	<60	40	60
5	90	130	30	970	40	60	100	100
6	260	190	32	1300	240	200	140	300
7	650	320	37	2000	520	300	200	800

* Titres are expressed as the reciprocal of the highest serum dilution that resulted in an absorbance at least twice the mean of negative controls.

† Serum was tested against whole cell antigens of AU1 prepared from cells grown in SSM+Fe and SSM-Fe.

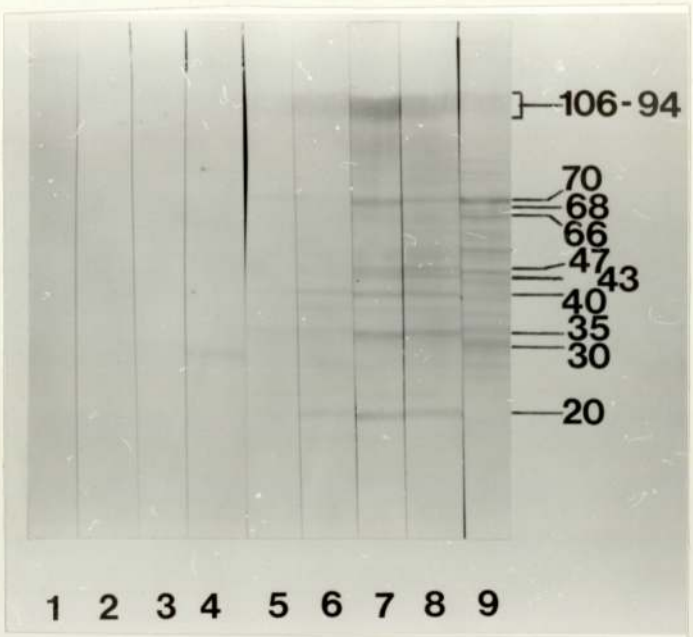


Fig.7.1. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM-Fe at 37°C and incubated with sera taken sequentially from rabbit 1 immunized with the same isolate. Lane 1 represents preimmune serum; lanes 2-8 represent the number of weeks of subcutaneous injections, lane 9 represents a commercially obtained high antibody titre serum raised against a homogeneous extract of *C.albicans*. The IgG response was visualized with alkaline phosphatase anti-rabbit IgG. Molecular weights are in kilodaltons.

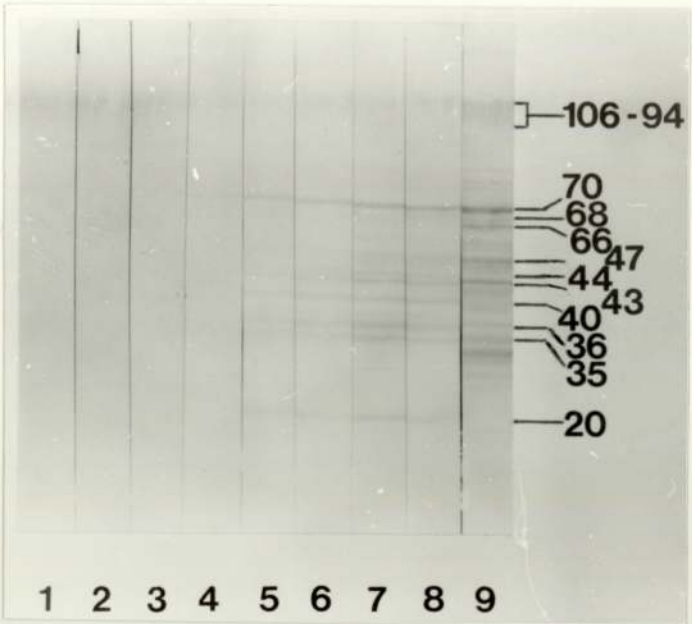


Fig.7.2. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM-Fe at 37°C and incubated with sera taken sequentially from rabbit 2 immunized with the same isolate. Lane 1 represents preimmune serum; lanes 2-8 represent the number of weeks of subcutaneous injections, lane 9 represents a commercially obtained high antibody titre serum raised against a homogeneous extract of *C.albicans*. The IgG response was visualized with alkaline phosphatase anti-rabbit IgG. Molecular weights are in kilodaltons.

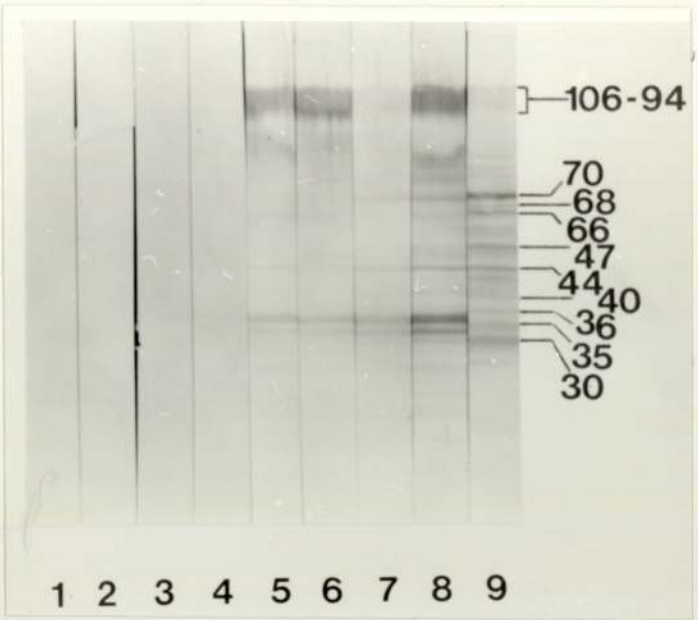


Fig.7.3. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM-Fe at 37°C and incubated with sera taken sequentially from rabbit 3 immunized with the same isolate. Lane 1 represents preimmune serum; lanes 2-8 represent the number of weeks of subcutaneous injections, lane 9 represents a commercially obtained high antibody titre serum raised against a homogeneous extract of *C.albicans*. The IgG response was visualized with alkaline phosphatase anti-rabbit IgG. Molecular weights are in kilodaltons.

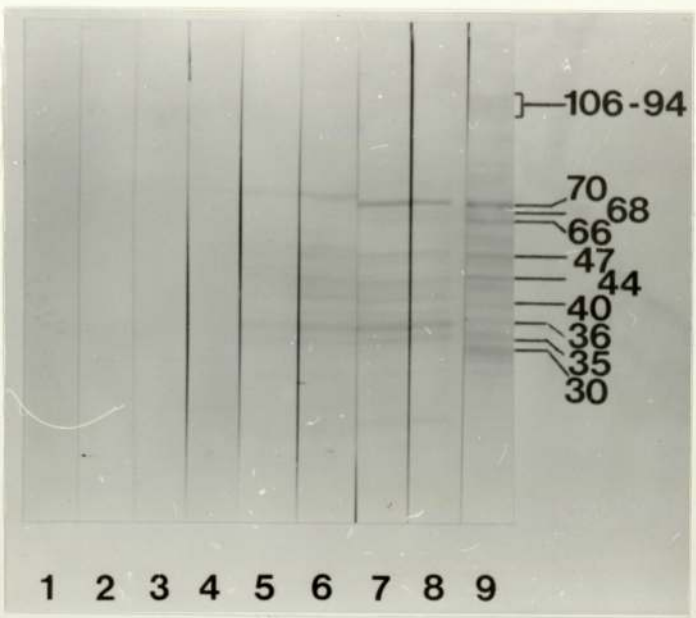


Fig.7.4. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM-Fe at 37°C and incubated with sera taken sequentially from rabbit 4 immunized with the same isolate. Lane 1 represents preimmune serum; lanes 2-8 represent the number of weeks of subcutaneous injections, lane 9 represents a commercially obtained high antibody titre serum raised against a homogeneous extract of *C.albicans*. The IgG response was visualized with alkaline phosphatase anti-rabbit IgG. Molecular weights are in kilodaltons.

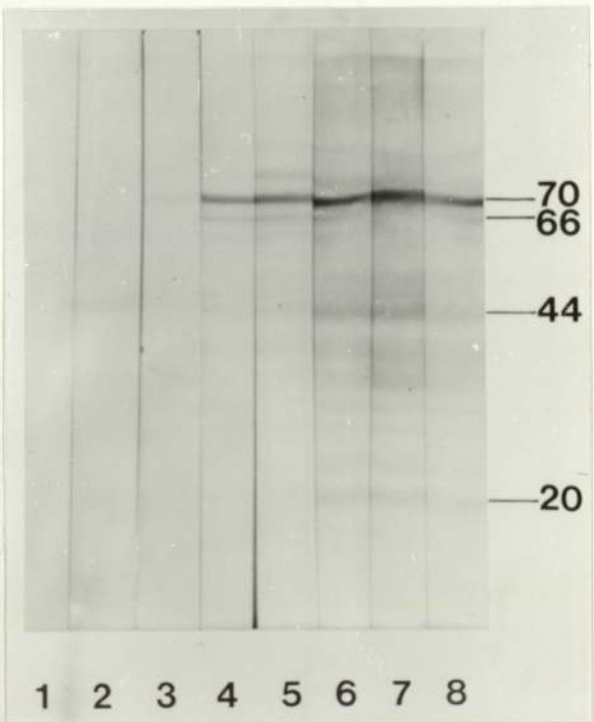


Fig.7.5. Immunoblot of wall proteins from *C.albicans* AU1 grown in SSM+Fe at 37°C and incubated with sera taken sequentially from rabbit 1 immunized with the same isolate. Lane 1 represents preimmune serum; lanes 2-8 represent the number of weeks of subcutaneous injections. The IgG response was visualized with alkaline phosphatase anti-rabbit IgG. Molecular weights are in kilodaltons.

7.3. Discussion

C.albicans possesses numerous antigens located within cell walls and cytoplasm, and is also associated with extracellular metabolites or products. These antigens include mannans (or mannoproteins) and proteins, and have been shown to elicit immune responses in normal individuals, patients with candidosis and experimental animals (Axelsen, 1973; Ellsworth *et al.*, 1977; Lehmann and Reiss, 1980a). Given that the primary site of contact between host and fungus occurs at cell surfaces, purification of cell wall antigens by several investigators has revealed that mannans (or mannoproteins) are the major antigens against which antibodies are directed. However, most antigenic preparations of *C.albicans* for antibody detection have been obtained by disintegration of yeast cells *in vitro* (Mackenzie, 1983; Richardson and Warnock, 1983), resulting in a mixture of antigenic components. Furthermore, the assumption that these preparations are biochemically and antigenically comparable to those expressed *in vivo*, remains to be substantiated.

In this study emphasis was directed towards the antibody response against yeast surface antigens of *C.albicans*. Some reports have suggested that heat killing of yeast cells is not recommended because some clinically significant antigens appear to be heat labile (Huppert, 1983). Therefore, formalin-fixed whole cells were used in this study to achieve effective killing and to minimize denaturing of yeast antigens. Furthermore, whole cells were used as opposed to whole cell or cytoplasmic extracts, so that immune responses will be directed predominantly towards surface antigens. Since microbial surfaces are known to alter their biochemical and antigenic characteristics in response to

changes in environmental conditions, in particular nutrient limitation, it is important to simulate such conditions when attempting to increase the relevance of an *in vitro* test or assay (Brown and Williams, 1985a and b; Gilbert *et al.*, 1987). Hence, *C.albicans* yeast cells were cultivated under iron-depleted conditions *in vitro* prior to immunization of rabbits in an attempt to mimic the metabolic and surface properties of cells likely to be expressed *in vivo*. Furthermore, whole cell antigens prepared from yeasts grown in iron-depleted and iron-sufficient medium were used as antigens to measure anti-*C.albicans* IgG antibodies during immunization.

Enzyme-linked immunosorbent assay (ELISA) was used in this study to provide a sensitive and quantitative method (Kostiala and Kostiala, 1981; Mackenzie, 1983; Richardson and Warnock, 1983; Fujita *et al.*, 1986) for detection of anti-*C.albicans* AU1 IgG antibodies in immunized rabbits. Advantages of ELISA over existing immunoassays include the use of objective endpoints, and automation of assay procedures allows rapid testing of numerous samples. In addition, it is easy to perform and reagents employed have a long shelf life which confers a marked economic advantage over other assays e.g. radioimmunoassays. Furthermore, by amending the basic procedure, individual antibody classes can be measured.

Antibody titres did not exceed pre-immune values until 3 weeks (rabbits 1 and 2) and 5 weeks (rabbits 3 and 4) after immunization. Beyond this period, the IgG response increased profoundly for all rabbits, however, when iron-sufficient cells were used to measure antibodies of rabbit 2, the response remained constant throughout the

immunization period. The reason for this observation is not clear, but it is apparent that variations in the IgG response of rabbit 2 towards antigens prepared from iron-depleted and iron-sufficient yeast cells exists. In support of these findings, yeast cells are known to vary in their capacity to elicit antibodies in experimental animals and even different strains produce different responses for any given serological test (Mackenzie, 1983). Therefore variations in antibody response may be attributed to the antigenic heterogeneity of *C.albicans* (Poulain *et al.*, 1985) which in turn is influenced by growth phase and environmental conditions (Brawner and Cutler, 1984, 1986a and b; Brown and Williams, 1985a), as well as variations in individual host responses.

Immunoblotting with serum taken sequentially from rabbits during immunization, in addition to affirming ELISA data, helped to elucidate which surface antigens were recognized by IgG antibodies, and at what stage during immunization antibodies emerged. Blots of wall proteins from cells cultivated under iron-depleted conditions revealed that a 28kd and a 35kd antigen were the first antigens detected by sera from rabbits 1 and 3 respectively after 4 weeks of immunization, whereas a 70kd antigen was the first antigen recognized by sera from rabbits 2 and 4 after the same period.

Antibodies from rabbits 1 and 3 also reacted strongly to a high MW antigen (106-94kd) after 5 weeks of immunization, but serum from rabbits 2 and 4 did not recognize this antigen. Generally, blots incubated with serum from rabbits 1 and 3 produced slightly different antigenic profiles than blots incubated with serum from rabbits 2 and 4. As time progressed, each rabbit developed antibodies to the full complement of wall

proteins, and several major antigens were recognized by each serum.

Blots of wall proteins from iron-sufficient cells revealed a different antigenic profile to blots of iron-depleted cells. Antigenic patterns of blots incubated with serum from rabbit 1 was typical of the IgG response in serum from rabbits 2, 3 and 4. Antigenic bands were somewhat diffuse rather than discrete, and the profile was characterized by recognition of a 44kd antigen as early as 2 weeks of immunization, and a strong IgG response to a 70kd antigen after 3 weeks of immunization.

Variations in antigen recognition appear to reflect different antibody responses amongst rabbits, and the physiological state of cells used to prepare antigens was shown to be an important factor when interpretating results. These results correlated well with the increasing antibody titres demonstrated by ELISA. A notable exception being the low antibody titre observed in rabbit 2 against cells prepared from iron-sufficient cultures. Not surprisingly, blots incubated with serum from rabbit 2 detected many antigens in walls from iron-sufficient yeasts (data not shown, similar to Fig.7.5.) thus demonstrating that immunoblotting provides greater sensitivity in detecting antibody responses to antigens compared to ELISA. Given the complexity of antigenic profiles described in this study, it remains to be determined whether similar profiles are obtained with serum from rabbits undergoing an acute or chronic candidal infection, since the animals will be subjected to a greater physiological stress than those immunized with formalin-fixed whole cells.

One advantage of multicomponent antigen preparations of fungi is that they tend to

increase the chances of detecting immunological host responses. However, many preparations are produced in different laboratories with variations occurring amongst strains, growth medium, culture conditions, source of antigen and methods of antigen preparation. At present antigens are prepared from cells cultured under iron-plentiful conditions, with little consideration of environmental conditions faced by organisms during infection in host tissues. The results of this study suggests that antibody recognition of antigens specific to cell wall surfaces of iron-depleted cells may provide a valuable basis for a reproducible and specific method for detection of *C.albicans* during infection.

In view of the current emphasis on understanding the complex mechanisms by which host cells recognize and respond to microbial antigens, there is a need for greater refinement of antigens (Huppert, 1983; Haun *et al.*, 1987). These refined antigens could serve as a means of discriminating between antibody responses during colonization and infection of *C.albicans*. Purification of antigens by monoclonal antibodies could give rise to unique antigenic markers, such as iron-regulated proteins, that would increase the specificity of antibody detection by ELISA and immunoblotting. Further investigations could also help to determine whether such antigens afford protection to the host.

This study focussed primarily on the IgG response of immunized rabbits largely because these are predominant antibodies produced against experimental *C.albicans* infections (Lehmann and Reiss, 1980b; Mackenzie, 1983). In addition, IgG antibodies possess a longer half-life in serum of 23 days compared to 5.1 days of IgM.

Furthermore, the appropiate affinity-purified alkaline phosphatase anti-rabbit IgM or IgA conjugate was not commercially available at the time of this investigation. Nevertheless, an extensive study of both IgG, IgM or IgA responses to cell wall antigens during the course of a *C.albicans* infection would be valuable.

The present study provides a useful framework in which future studies may be designed to monitor antibody responses in human patients with candidosis to isolates derived from the same patients and cultivated under conditions which reflect growth during infection, such as iron restriction.

8. Influence of sub-inhibitory concentrations of antifungal antibiotics on growth and surface antigens of iron-depleted yeast cells of *C.albicans*

8.1. Influence of growth conditions on MICs of antifungal antibiotics

Several reports have demonstrated that numerous factors may have profound affects on results of antifungal susceptibility tests (Johnson *et al.*, 1978; Doern *et al.*, 1986; Galgiani, 1987) including, yeast strain, inoculum size, incubation conditions and composition of test media. These factors, as well as the pharmacological and pharmacokinetic properties of a given antifungal, are also responsible for large variations in MIC values between different laboratories (Galgiani, 1987). This current lack of reproducibility in antifungal susceptibility testing warrants further investigation to elucidate which variables influence results, and whether they are associated with the physiological state of yeasts cultivated under laboratory conditions that reflect *in vivo* environments.

This study examines the affect of inoculum size, pH, length of incubation, and, presence and absence of iron on MICs of Amp B, nystatin and fluconazole against *C.albicans* AU1. In one study ketoconazole was also included for comparison but was not part of any further investigations. The approach taken in this study allows a comparison of MIC values for each drug tested against yeasts grown under varying conditions. MIC data obtained can then form a basis for preparing sub-inhibitory concentrations, which subsequently is used to study their effects on growth and surface

antigens of *C.albicans* under iron-depleted and iron-sufficient conditions.

The final concentration of dimethyl sulphoxide (DMSO) present in the test media (<0.0000078%) appeared not to influence results, a finding in agreement with several studies that have also employed DMSO to dissolve and store polyene and azole antifungals (Nugent and Couchot, 1986; Odds *et al.*, 1986; Schaude *et al.*, 1987). Determination of MIC values after incubation for 24h and 48h was identical, hence MICs evaluated after incubation for 48h were chosen to ensure complete inhibition of growth. All inocula used throughout this study were previously grown under iron-depleted conditions.

Regardless of inoculum size, growth in SSM+Fe or SSM-Fe did not affect MICs of all drugs tested (Table 8.1.). Increasing inocula from 10^3 to 10^5 yeast cells/ml did not alter the MICs of Amp B or nystatin, however, the MIC of fluconazole was at least 25-fold higher with an inocula of 10^5 than with 10^3 yeast cells/ml. Given the extent of this increase in MIC, all subsequent studies were performed with an inocula adjusted to 10^3 yeast cells/ml.

Irrespective of pH, growth in SSM+Fe or SSM-Fe did not affect the MICs of Amp B, nystatin or fluconazole (Table 8.2.). In marked contrast, MICs of ketoconazole were 2-fold and 4-fold higher for cells grown in untreated SSM and SSM+Fe respectively, than for cells grown in SSM-Fe. When pH was adjusted to 7.0 it resulted in 3-fold and at least 2-fold higher MICs for Amp B and fluconazole respectively, but failed to alter the MIC of nystatin. Similarly, no affect of pH on the MIC of ketoconazole was observed for

cells grown in SSM+Fe or SSM-Fe, however, raising pH, increased the MIC from 0.92μg/ml to 1.84μg/ml for cells grown in untreated SSM (Table 8.2).

8.2. Influence of sub-inhibitory concentrations of antifungals on growth of *C.albicans*

MICs of Amp B, nystatin and fluconazole in SSM (pH 4.5), as determined in section 8.1, were 0.25, 8.0 and 4.0μg/ml respectively, and presence or absence of iron did not affect MICs of these drugs against AU1. Three sub-inhibitory concentrations of each antifungal were chosen for further investigations based on criteria described in section 3.4.2. The effect these antifungal concentrations on growth characteristics of AU1 cultivated in untreated SSM, SSM+Fe and SSM-Fe is shown in figs.8.1 to 8.3A, B and C.

Regardless of presence or absence of iron, growth in 0.05 or 0.083μg/ml Amp B was marked by a prolonged lag phase and a slightly lower growth rate than control cultures (Fig.8.1.). However, growth in SSM-Fe and in the presence of 0.083μg/ml Amp B was characterized by an early onset of stationary phase (Fig.8.1A). A similar pattern of growth was observed when cells were grown in the presence of 1.6μg/ml nystatin, particularly in SSM-Fe (Fig.8.2A), yet, growth was profoundly inhibited by 2.66μg/ml nystatin under all test media (Fig.8.2A, B and C).

All three concentrations of fluconazole produced similar effects on growth characteristics of cells cultured in untreated SSM or SSM+Fe (Fig.8.3B and C), but in

SSM-Fe and in the presence of 0.8 or 1.33μg/ml fluconazole (Fig.8.3A), growth was characterized by early onset of stationary phase and a marked inhibition.

A concentration equivalent to 1/15th MIC of each antifungal (0.016μg/ml Amp B, 0.53μg/ml nystatin, and 0.266μg/ml fluconazole) had little effect on growth kinetics of AU1, and resulted in curves essentially similar to control cultures. Furthermore, when samples of cultures were removed during logarithmic and stationary phase growth, no morphological changes were observed using light microscopy, but this does not preclude the occurrence of ultrastructural changes in yeast cells.

8.3. Influence of sub-inhibitory concentrations of antifungals on surface antigens of *C.albicans*

The effects of three sub-inhibitory concentrations of Amp B, nystatin and fluconazole on protein profiles of iron-depleted and iron-sufficient cells of *C.albicans* AU1 was investigated by SDS-PAGE and immunoblotting. Whole cells from cultures harvested during logarithmic and stationary phase growth at 37°C, were electrophoretically separated and stained with Coomassie Brilliant Blue. Amp B or fluconazole did not reveal any qualitative differences in protein profiles of AU1 (data not shown).

8.3.1. *Analysis of C.albicans surface proteins by SDS-PAGE.*

Figs.8.4A and B, and 8.5A and B illustrate protein profiles of cells grown in SSM+Fe and SSM-Fe, in the presence and absence of nystatin. Visualization of the

profile of 12h cells (Fig.8.4A. lane 6) grown in SSM+Fe and 0.53μg/ml nystatin was relatively poor and probably a result of insufficient loading on the gel, but despite this, all profiles were similar. In the absence of drug there was a reduction in intensity of a 68, 47 and 23kd protein, and conversely, an increase in intensity of a 45Kd protein was observed (Figs.8.4. lanes 1-4). Growth in 1.6 or 2.66μg/ml nystatin resulted in a reduction in intensity of bands, in particular, a 23Kd protein was barely detectable compared to control profiles (Figs.8.4A and 8.4B), however regardless of growth phase, major proteins of MW 96, 66, 64, 47, and 45 remained visible throughout.

Profiles of cells grown under iron-depletion and in the absence or presence of 0.53μg/ml nystatin were virtually identical to corresponding profiles of whole cells prepared from iron-sufficient cultures (Figs.8.4A and 8.5A). Growth in 1.6μg/ml nystatin resulted in an overall reduction in intensity of bands during early stages of growth, with several bands becoming virtually undetectable compared to control profiles (Figs.8.5A and 8.5B), yet this intensity increased gradually with time to reveal a complete protein profile (Fig.8.4B lanes 1-4). Growth in 2.66μg/ml revealed an analogous pattern, but profiles did not reveal a full complement of protein bands until after 25h growth (Fig.8.5B. lanes 5-8).

8.3.2. *Antibody recognition of surface antigens*

Proteins electrophoretically separated as shown in Figs.8.4A and B, and 8.5A and B were transferred onto Nc paper and incubated with serum from a patient with vaginal

candidosis. Growth in the presence and absence of Amp B or fluconazole did not reveal any qualitative differences in their antigenic profiles (data not shown). Figs.8.6A and B, and 8.7A and B represent blots of cells grown in SSM+Fe and SSM-Fe, in the presence and absence of nystatin. The antigenic profile of 8h cells grown in SSM+Fe and in 0.53μg/ml nystatin was faint and may reflect insufficient sample loading onto the gel (Fig.8.6A lane 5).

The IgG response to antigens from cells cultivated in SSM+Fe, in the presence and absence of drug was similar (Fig.8.6A and B) and marked by a dense diffuse staining to the upper portion of each blot. IgG antibodies also reacted strongly with antigens of MW 66, 64, 47 and 45Kd, however this response profoundly diminished with time (Fig.8.6.A lanes 4 and 8). A 66Kd antigen was the only major antigen recognized by serum in cells grown in 1.6 or 2.66μg/ml nystatin (Fig.8.6B).

Antigenic profiles of cells grown under iron-depleted conditions in 0.53μg/ml nystatin or in the absence of drug were virtually identical to corresponding profiles of cells grown in SSM+Fe (Figs.8.6A and 8.7A), however, it is acknowledged that profiles of cells harvested after 16h and 25h were relatively poor (Fig.8.7A lanes 4 and 5). IgG antibodies reacted with a 66Kd antigen in cells harvested after all time periods in 1.6μg/ml nystatin (Fig.8.7B lanes 1-4). In addition, as cells aged, a strong response developed towards a diffuse antigen in the upper region of each blot, and conversely, a weak response was observed towards antigens of MW 64, 47, 45 and 23Kd (Fig.8.7B lanes 1-4). Following growth in 2.66μg/ml nystatin, only the 66Kd antigen was

recognized in cells harvested after 8, 12 and 16h, however, by 25h a strong response developed towards a diffuse antigen as described above, in addition to the 66Kd antigen (Fig.8.7B lanes 5-8).

Table 8.1. Effect of inoculum size and iron on mean* MIC values (μg/ml) of three antifungal agents against yeast cells of *C.albicans* AU1.

Antifungal agent	Test medium (pH 4.5)	Inoculum size (CFU/ml)	
		10^3	10^5
Amphotericin B	SSM-Fe	0.25	0.25
	Untreated SSM	0.25	0.25
	SSM+Fe	0.25	0.25
Nystatin	SSM-Fe	8.0	8.0
	Untreated SSM	8.0	8.0
	SSM+Fe	8.0	8.0
Fluconazole	SSM-Fe	4.0	≥100[a]
	Untreated SSM	4.0	≥100[a]
	SSM+Fe	4.0	≥100[a]

[a] maximum tube dilution used; * mean of duplicate replicates for six experiments

Table 8.2. Effect of pH and iron on mean* MIC values (μg/ml) of three antifungal agents against yeast cells of *C.albicans* AU1.†

Antifungal agent	Test medium	pH of test medium	
		4.5	7.5
Amphotericin B	SSM-Fe	0.25	2.0
	Untreated SSM	0.25	2.0
	SSM+Fe	0.25	2.0
Nystatin	SSM-Fe	8.0	8.0
	Untreated SSM	8.0	8.0
	SSM+Fe	8.0	8.0
Fluconazole	SSM-Fe	4.0	≥8.0[a]
	Untreated SSM	4.0	≥8.0[a]
	SSM+Fe	4.0	≥8.0[a]
Ketoconazole	SSM-Fe	0.23	0.23
	Untreated SSM	0.92	1.84
	SSM+Fe	3.68	3.68

† inoculum size 10^3 CFU/ml; [a] maximum tube dilution used; * mean of duplicate replicates for six experiments.

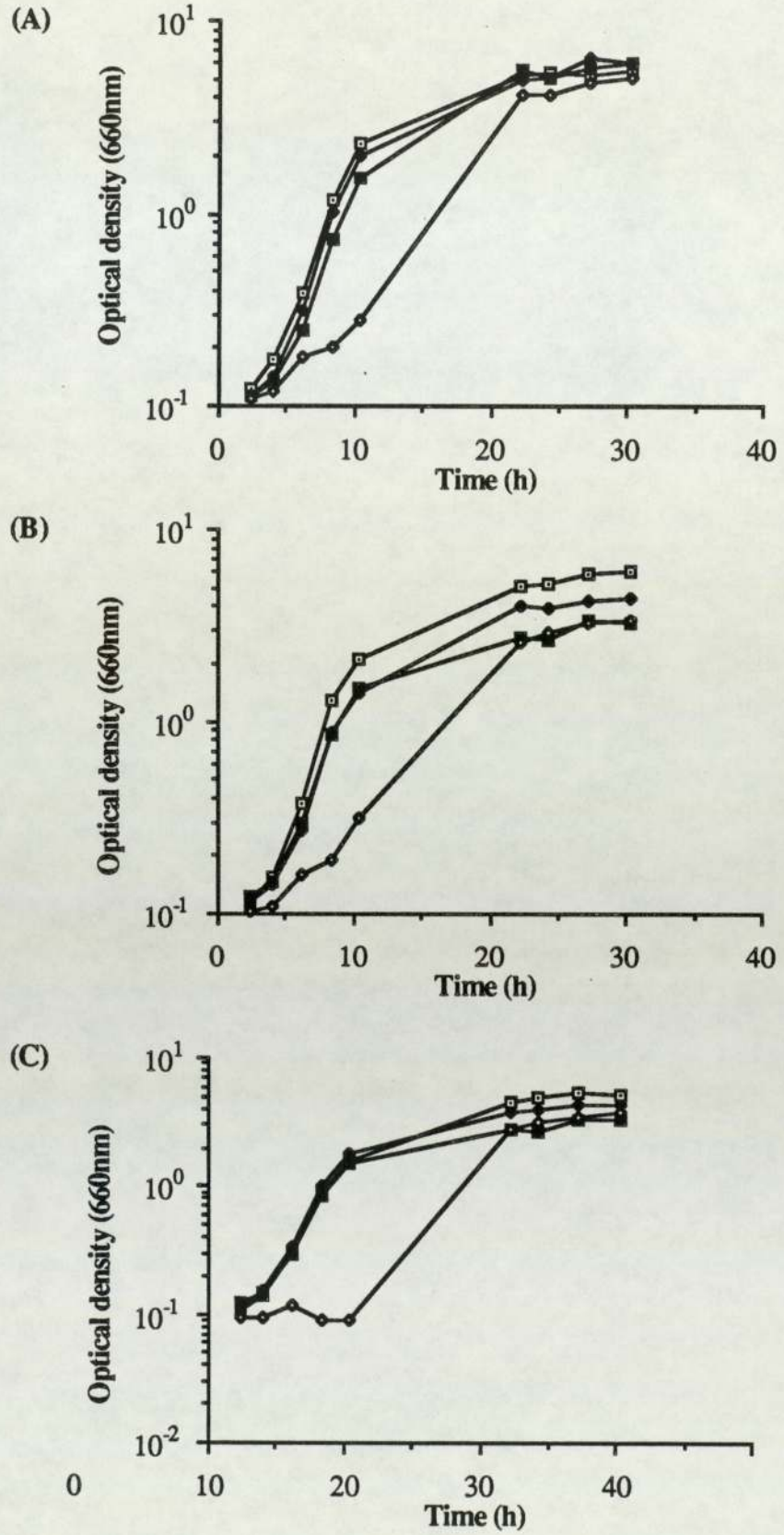


Fig.8.1A, B and C. Effect of sub-inhibitory concentrations of amphotericin B on growth of *C.albicans* AU1 in (A) SSM+Fe, (B) untreated SSM, and (C) SSM -Fe, and in (▫) no drug; (◆) 0.016µg/ml; (■) 0.05µg/ml and (◆) 0.083µg/ml Amp B.

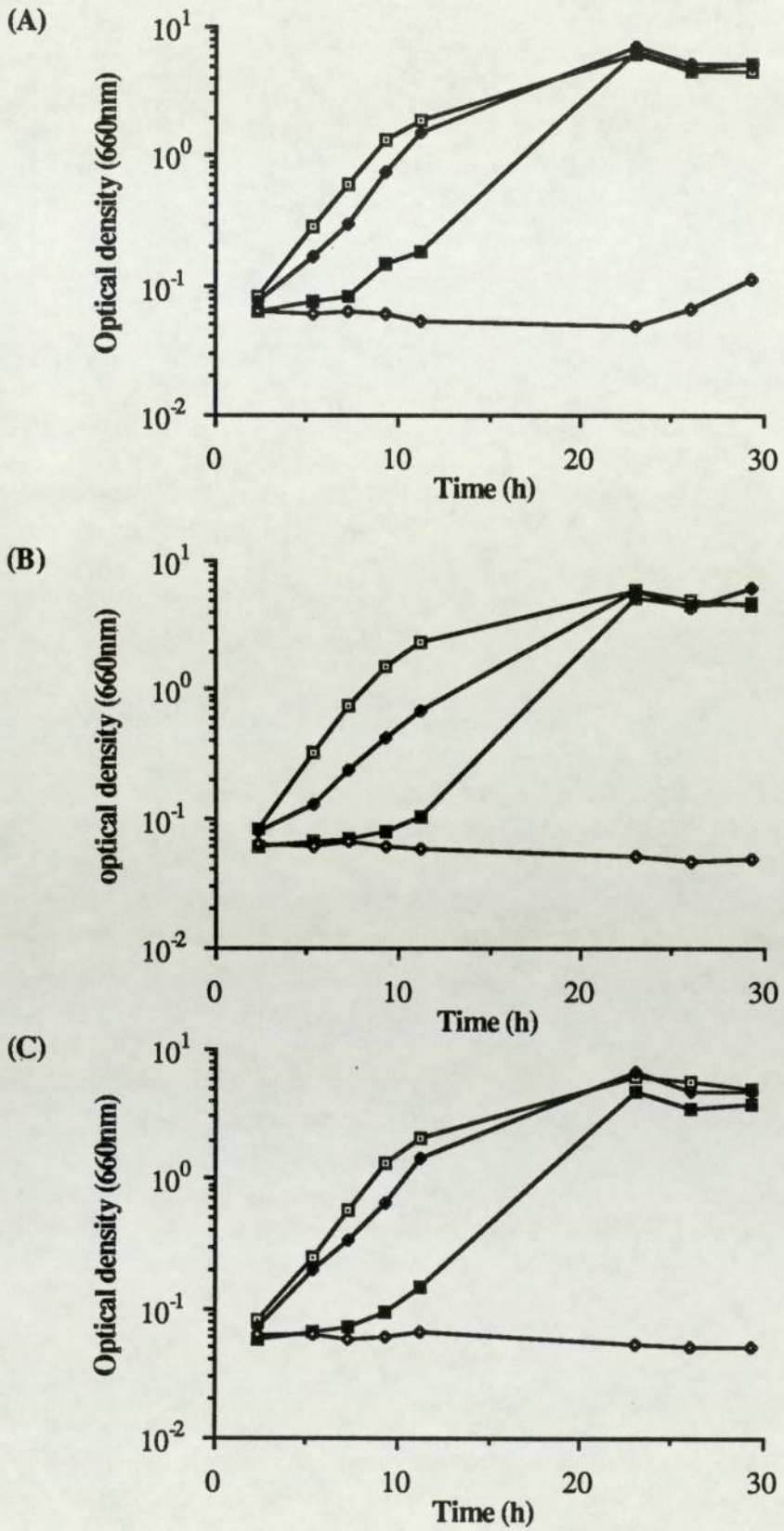


Fig.8.2A, B and C. Effect of sub-inhibitory concentrations of nystatin on growth of *C.albicans* AU1 in (A) SSM+Fe, (B) untreated SSM, and (C) SSM -Fe, and in (▫) no drug; (◆) 0.053µg/ml; (■) 1.6µg/ml and (◆) 2.66µg/ml nystatin.

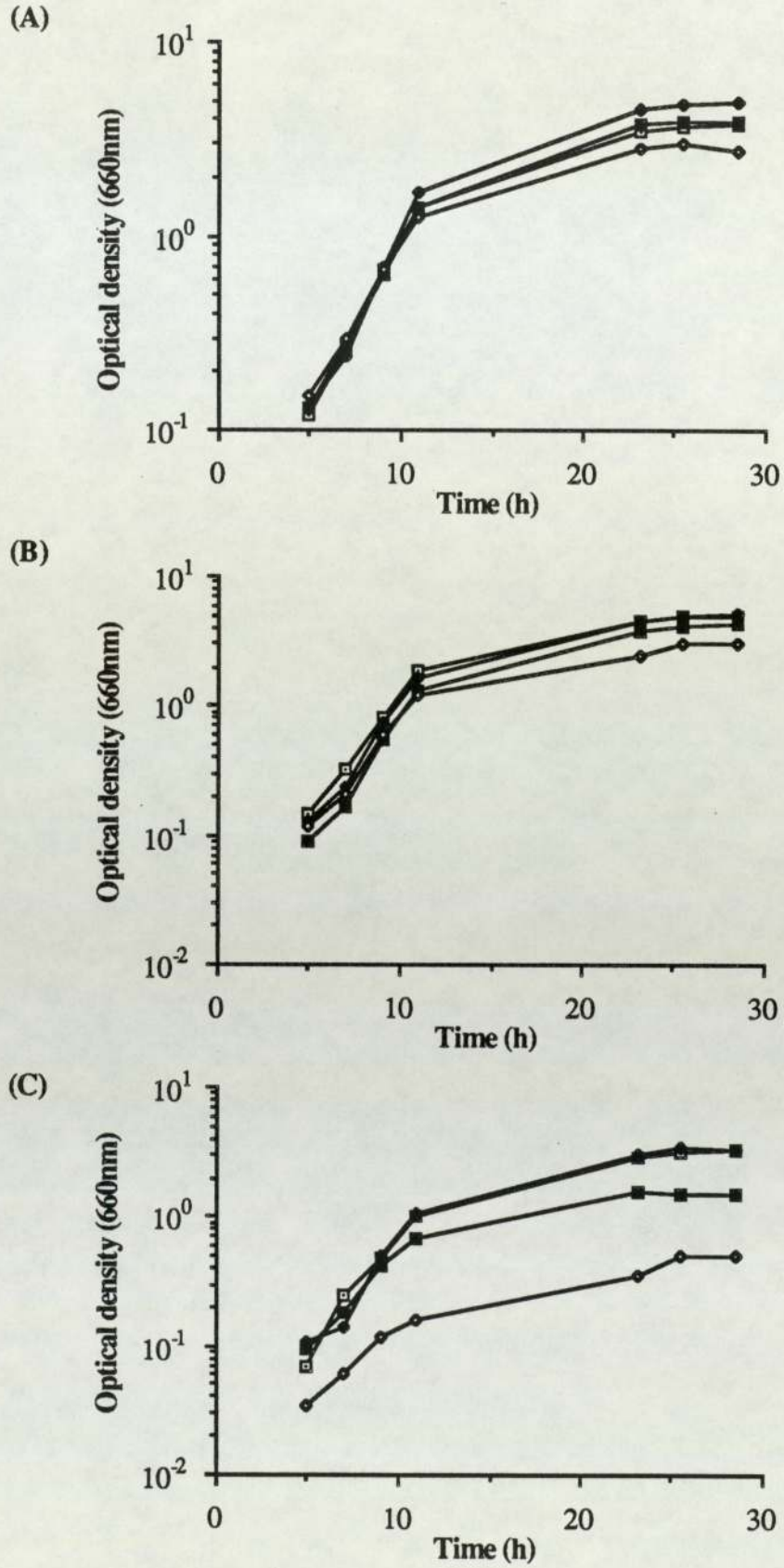
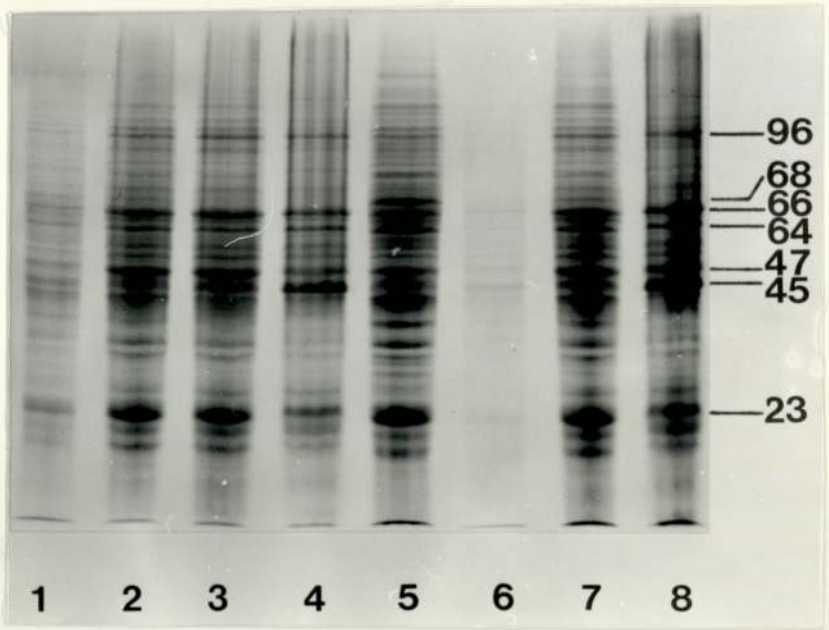


Fig.8.3A, B and C. Effect of sub-inhibitory concentrations of fluconazole on growth of *C.albicans* AU1 in (A) SSM+Fe, (B) untreated SSM, and (C) SSM-Fe and in (□) no drug; (◆) 0.266µg/ml; (■) 0.8µg/ml and (●) 1.33µg/ml fluconazole

(A)



(B)

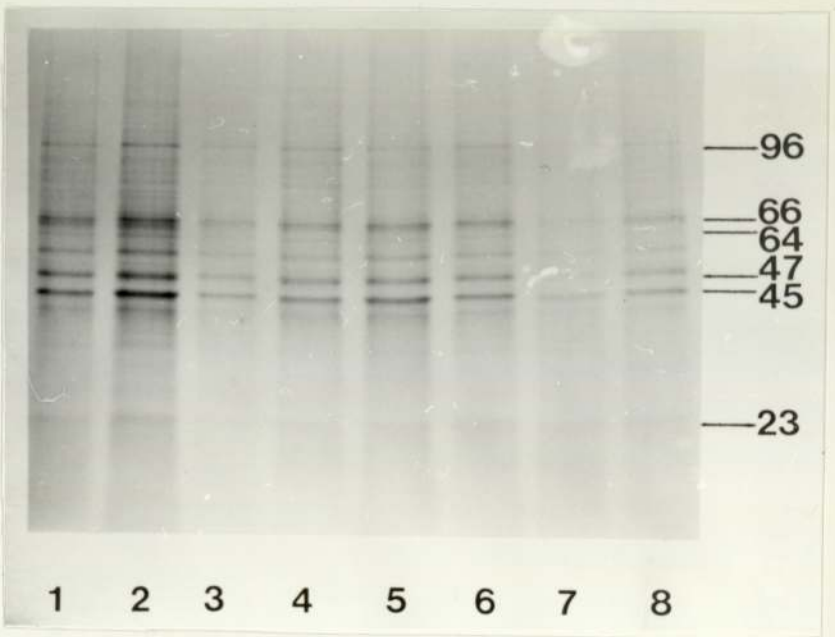
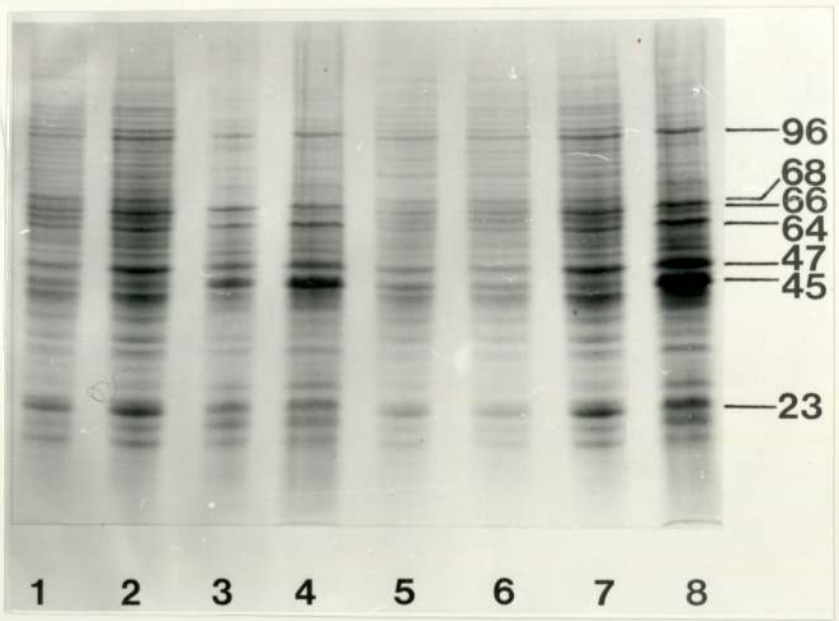


Fig.8.4A and B. SDS-PAGE of whole cell surface proteins of *C.albicans* AU1 grown in SSM+Fe, in the presence and absence of nystatin, and stained with Coomassie Blue. (A) Whole cells were prepared from cultures grown in the absence of drug (lanes 1-4) and in 0.53μg/ml nystatin (lanes 5-8). (B) Whole cells were prepared from cultures grown in 1.6μg/ml (lanes 1-4) and 2.66μg/ml nystatin (lanes 5-8). Cells were harvested after 8h (lanes 1 and 5), 12h (lanes 2 and 6), 16h (lanes 3 and 7) and 25h (lanes 4 and 8). Molecular weights are in kilodaltons.

(A)



(B)

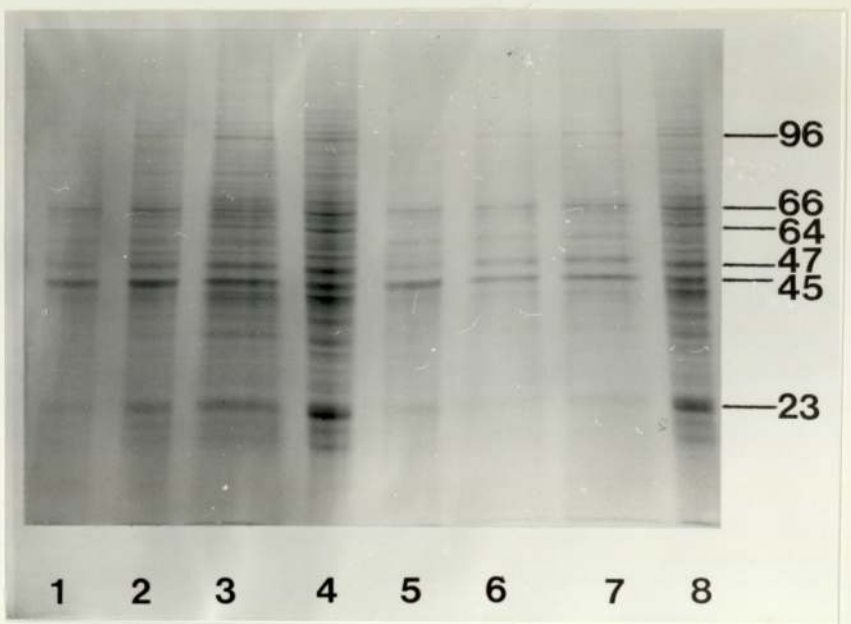


Fig.8.5A and B. SDS-PAGE of whole cell surface proteins of *C.albicans* AU1 grown in SSM-Fe, and in the presence and absence of nystatin, and stained with Coomassie Blue. (A) Whole cells were prepared from cultures grown in the absence of drug (lanes 1-4) and in 0.53µg/ml nystatin (lanes 5-8). (B) Whole cells were prepared from cultures grown in 1.6µg/ml (lanes 1-4) and 2.66µg/ml nystatin (lanes 5-8). Cells were harvested after 8h (lanes 1 and 5), 12h (lanes 2 and 6), 16h (lanes 3 and 7) and 25h (lanes 4 and 8). Molecular weights are in kilodaltons.

(A)

(B)

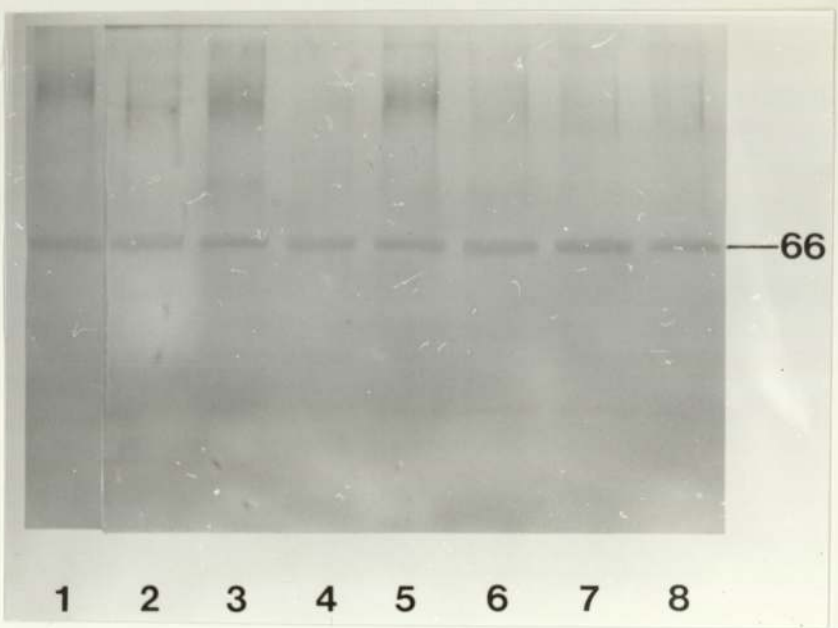
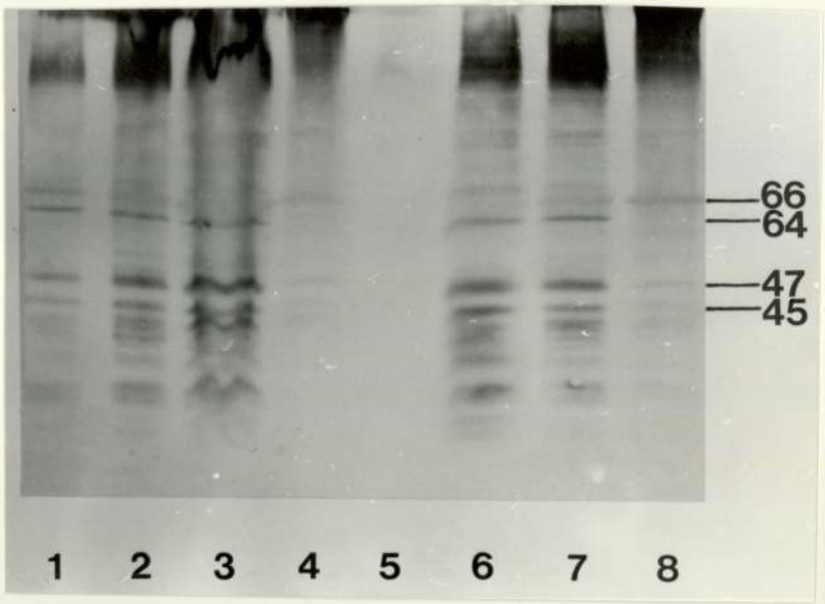


Fig.8.6A and B. Immunoblot of cell wall proteins of *C.albicans* AU1 grown in SSM+Fe as described in Fig.8.4A and B. Proteins were electrophoretically transferred to Nc paper and incubated with serum from a patient with vaginal candidosis, then developed with alkaline phosphatase anti-human IgG. Molecular weights are in kilodaltons.

(A)



(B)

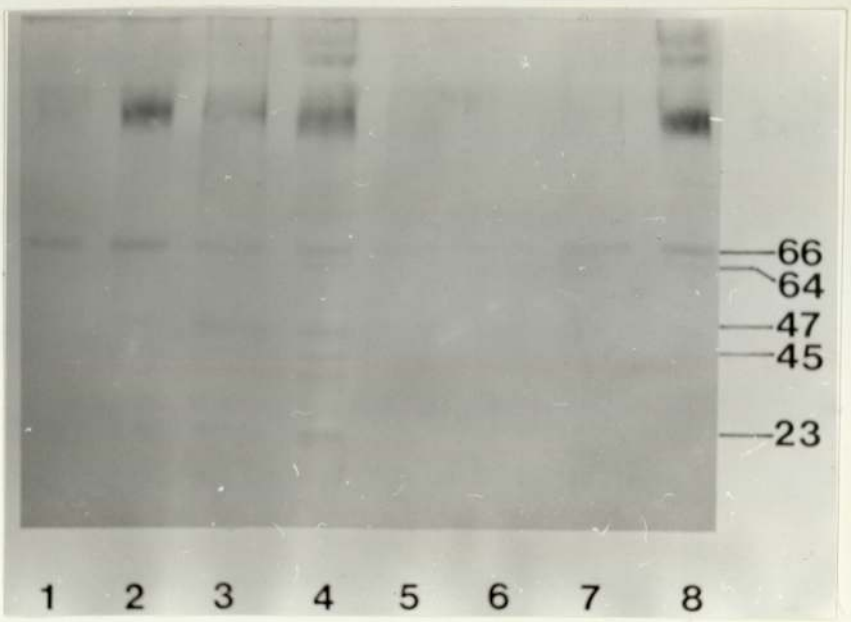


Fig.8.7A and B. Immunoblot of cell wall proteins of *C.albicans* AU1 grown in SSM-Fe as described in Fig.8.5A and B. Proteins were electrophoretically transferred to Nc paper and incubated with serum from a patient with vaginal candidosis, then developed with alkaline phosphatase anti-human IgG. Molecular weights are in kilodaltons.

8.4. Discussion

Several factors have been shown to influence results of antifungal susceptibility tests (Johnson *et al.*, 1978; Doern *et al.*, 1986; Galgiani, 1987) and contribute profoundly to a lack of reproducibility between different laboratories performing these tests. Consequently, conclusions relating to the antifungal activity of a given agent against a specific pathogen remains controversial (Galgiani, 1987).

It is apparent from previous reports relating to effects of antifungals on *C.albicans* that little attention has been paid to the importance of physiological characteristics of yeast cells grown under conditions that may reflect *in vivo* environments (Johnson *et al.*, 1978). Given that microorganisms are likely to encounter iron-restricted environments during growth within host tissues and fluids, many studies could benefit by including cells grown under analogous conditions *in vitro*.

This study describes the affect of some environmental factors, including iron, on MICs of two widely used polyene antifungals (Amp B and nystatin), and a relatively novel azole compound (fluconazole) against *C.albicans* AU1 yeast cells. Determination of MIC values allows the preparation of sub-inhibitory concentrations for each drug. This approach is fundamental to subsequent studies which attempt to investigate the affect of these concentrations on growth and surface proteins of *C.albicans* cultivated under iron-depleted and iron-sufficient conditions.

Increasing inoculum size increases the MIC of azole compounds (Galgiani and Stevens, 1976; Plempel *et al.*, 1987), a finding consistent with the results of this study.

Therefore inoculum size determines whether a given yeast strain will be classified as highly sensitive, moderately sensitive, or resistant to an azole antifungal. Currently, an inoculum size of 10^3-10^4 organisms/ml is considered to be clinically relevant (Plempel *et al.*, 1987), however, realistically this is an arbitrary number, since *in vivo, Candida* is present in various morphological forms. In the case of mycelium formation, the influence of inoculum size on MIC values is completely obscure, since microbial counts or weights of hyphae are difficult to determine.

The activity of several antifungal agents in various defined and complex media against *Candida* is affected by pH (Hamilton-Miller, 1973b; Johnson *et al.*, 1978; Minagawa *et al.*, 1983; Calhoun and Galgiani, 1984; Rogers and Galgiani, 1986). In the present study, increasing pH, led to an increase in MICs of Amp B and fluconazole. The transition from yeast to hyphal phase of growth can be regulated *in vitro* by pH (Mitchell and Soll, 1979; Buffo *et al.*, 1984). Generally, adjusting pH to above 6.7 stimulates mycelium formation in an appropiate supportive medium. However, in SSM the majority of cells remain as yeast cells after pH is adjusted to 7.0, hence, increasing pH to physiological values associated with potential hosts, could alter metabolic function and membrane integrity of a particular isolate resulting in resistance to antifungal action. This could help explain the affect of pH on MICs of Amp B and fluconazole. In contrast, Rogers and Galgiani (1986) observed a 2- to 4-fold decrease in MIC for fluconazole when pH was increased from 3.0 to 7.4. These workers employed growth media which supports mycelial growth, and since mycelia are more sensitive than yeasts to azole

antifungals, this could explain the observed fall in MIC values.

Media constituents may antagonize drug activity, particularly those found in some complex media (Johnson *et al.*, 1978; Calhoun and Galgiani, 1984; Doern *et al.*, 1986). In addition, subjective determination of MIC end-points is confounded by a tendency for azole antifungals to cause a gradual diminution of growth in the presence of increasing drug concentrations rather than having a sharp, well-defined end-point, an observation noted in this study. In response to these problems, some workers have described improved methods for determining MICs of azoles against *C.albicans* which give sharp, reproducible end-points in complex media (Odds *et al.*, 1986; Gordon *et al.*, 1988).

Odds *et al.* (1986) have tested combinations of azole antifungals with antibacterial agents for synergistic effects of potential clinical value. Their studies revealed that antibacterials which bind to 80S ribosomes, thereby blocking protein synthesis, tend to show synergy with imidazole and triazole antifungals, giving low MICs against *C.albicans* with sharp, precise end-points in complex media. The presence of these antibiotics reduced MICs, on average by 50- to 250-fold and low MICs were in close agreement with MICs obtained in a defined, tissue culture-based medium lacking added antibiotics. However, widespread acceptance of this method remains restricted because variations in MICs between tests based on different media and between microdilution plate assays using identical media indicates that even antifungal-antibiotic effects are affected by other performance variables.

Gilbert *et al.* (1987) reported that a significant but overlooked reason for lack of

reproducibility in antimicrobial susceptibility testing is the plasticity of microbial cell envelopes. Changes in bacterial cell components tend to be associated with changes in growth rate and/or nutrient-limitation which in turn influences susceptibility to antibiotics (Brown, 1977; Gilbert and Brown, 1980; Brown and Williams, 1985a and b). These effects may be mediated through changes in envelope permeability towards a drug active at a distant point from the target site or through alterations in expression of that target (Gilbert and Brown, 1978; Gilbert *et al.*, 1987).

Using chemostat cultures of *C.albicans*, Johnson *et al.* (1978) demonstrated that growth rate, nutrient-limitation, temperature and pH influenced sensitivity to Amp B and nystatin, as monitored by release of potassium ions. Given that susceptibility of *C.albicans* will vary according to the nature of a growth-limiting nutrient, then it is important to simulate such conditions when attempting to increase the significance of an *in vitro* test.

In this study growth in the presence or absence of iron did not affect MICs of Amp B, nystatin or fluconazole against AU1, therefore extensive investigations are necessary to determine whether these results reflect a similar phenomenon amongst other isolates. Despite these results, MIC values for ketoconazole were enhanced following growth in SSM containing high levels of iron, implying that the presence of available iron has a profound affect on sensitivity of AU1 to ketoconazole activity. These findings gain further significance when compared with data from previous investigations. Studies by Minagawa *et al.* (1983) demonstrated that acidic medium antagonized ketoconazole

activity, and that their range of MIC values were broad, suggesting that various *C.albicans* isolates differed markedly in sensitivity. However, at physiological pH, MIC values for the same isolates were low and essentially similar. With the exception of growth in untreated SSM, pH did not influence MICs of ketoconazole in this study. Therefore variations in iron concentration of growth media may be more important in influencing interlaboratory reproducibility of MIC data for ketoconazole than pH. However, a combined action of both factors could also have a profound affect on MICs.

Despite lack of interlaboratory agreement , there are some convincing reports of *in vitro* data correlating with treatment of experimental infections in animals (Stiller *et al.*, 1983; O'Day *et al.*, 1987) Although similar correlations with treatment in human infections are not available to any appreciable extent, establishing correlations between *in vitro* and human clinical studies will be critical to the validity of *in vitro* testing. Generally, MIC data for polyene antifungals are known to reflect their activity *in vivo* (Proctor and Mackenzie, 1980), and values obtained in this study are in agreement with other workers (Johnson *et al.*, 1978; Drouhet and Dupont, 1987). Given that the *in vitro* problems discussed above tend to be associated with testing of azole activity, *in vivo* animal models of infection have been shown to provide a better means of assessing relative azole antifungal activity (Richardson *et al.*, 1985; Rogers and Galgiani, 1986; Galgiani, 1987; Troke, 1987).

Growth characteristics of AU1 were unaffected in the presence of fluconazole under iron-sufficient conditions, but under iron-depleted conditions growth was inhibited and a

decrease in cell density occurred which was proportional to drug concentration. Similarly, Gull *et al.* (1987) observed that growth in a tissue culture based medium was reduced *in vitro* following treatment with fluconazole at concentrations as low as 0.16μg/ml (1/5th MIC).

Using cytochemical staining and electron microscopy, these same workers also observed that nuclear division was interrupted and large, often multinucleate cells with irregularly thickened walls were evident. These walls contained membranous vesicles, and cytoplasm contained increased amounts of lipid. Furthermore, enhanced fluconazole concentrations and time of exposure accelerated rate and degree of these changes without inducing any additional effects. Gull *et al.* (1987) also found that when oestrogenized mice were inoculated intravaginally with *C.albicans* and treated with fluconazole, fungal cells from vaginal tissues revealed similar morphological changes to those seen *in vitro*.

Fluconazole is a relatively new bis-triazole antifungal with some excellent pharmacological properties, and is regarded as a possible alternative to currently available antifungal treatments. At present there is little information available relating to factors influencing its *in vitro* activity against *C.albicans*. However, the present *in vitro* data suggest that investigators who include iron-deprivation in their studies may contribute to a better understanding of fluconazoles' excellent efficacy *in vivo* (Richardson *et al.*, 1985; Rogers and Galgiani, 1986; Troke, 1987).

Growth of AU1 in the presence of 0.083μg/ml Amp B or 1.6μg/ml nystatin was characterized by a prolonged lag phase, and in the presence of 2.66μg/ml nystatin by a

pronounced inhibition. Investigations by Gale *et al.* (1975) and Johnson *et al.* (1978) revealed that logarithmic phase cultures of *C.albicans* grown in either complex or defined medium were extremely susceptible both to Amp B- and to nystatin-induced release of potassium ions. However, as cultures aged, cells were associated with increased resistance to effects of both polyenes. Furthermore, resistance was greater in cultures grown in a simple salts medium than in complex medium, a phenomenon consistent with the present data. In this study, as cells aged, their recovery from polyene-inhibition was gradual and dose-dependent, but growth was eventually restored to a similar rate to control cultures. However, in the presence of 2.66μg/ml nystatin recovery from inhibition of growth was not apparent.

An important characteristic of microorganisms is their ability to alter their phenotype in response to changes in environment (Brown and Williams, 1985b). *C.albicans* is no exception, and environmentally induced changes in yeast cell surfaces may affect either composition of target components or drug penetration through cell walls. An increase in antibiotic resistance is believed to be associated with changes in cell wall structure or composition since protoplasts prepared from drug-resistant stationary phase cells are as sensitive to polyene action as logarithmic phase organisms (Kerridge *et al.*, 1976; Johnson *et al.*, 1978). There are also changes in ultrastructure, with walls of stationary phase cells losing the typical layered appearance of logarithmic phase cells and becoming progressively thicker (Cassone *et al.*, 1979), in addition, walls become tightly cross-linked as cultures age (Gale *et al.*, 1975).

Yeast cell walls contain considerable amounts of polyene-binding material, and polyenes must first penetrate the cell wall matrix containing lipids, carbohydrates and proteins to reach plasma membrane target sites. Chemical analysis of *C.albicans* walls have shown that changes in lipid composition occur after cells enter stationary phase, but no correlation has been established between lipid content and development of phenotypic drug resistance (Gale *et al.*, 1975). Similarly, development of phenotypic resistance of polyenes has not been associated with synthesis of new proteins.

Both (1→3)- and (1→6)-β-glucans are major structural components of *C.albicans* cell walls, and their synthesis and deposition is influenced not only by membrane-bound glucan synthases but also by endogenous β-glucanases within walls. Evidence has accumulated to suggest that (1→3)-β-glucan polymers function as a barrier to penetration of polyenes through cell walls. Changes in wall structures mediated by an imbalance between synthetic and degradative pathways of β-glucan metabolism can have a profound effect on sensitivity of *C.albicans* to polyene antibiotics.

β-glucan chains function by increasing their length to form a tightly cross-linked network within cell walls, thus impeding antibiotic passage. Any factor that decreases β-glucanase activity enhances resistance, and those that increase enzyme activity reduces drug resistance (Notario *et al.*, 1982). Therefore, since cell wall lipids, polysaccharides and proteins have been identified as possible glucanase-binding sites, they could also be involved in regulating enzyme activity, so that any changes in structure or composition of these components will subsequently affect drug sensitivity.

Al-Bassam *et al.* (1985) have further demonstrated the importance of cell walls in polyene sensitivity. These workers showed that sub-inhibitory doses of Amp B induced profound cell wall modifications in *C.albicans* by immuofluorescent techniques and these changes were attributed to a reduction in wall mannan components. In addition, regardless of growth phase, patient serum antibodies reacted weakly with walls altered by Amp B compared to controls, indicating that polyene antifungals may affect both chemical and antigenic structures of cell walls considerably. Alternatively, since yeast membranes are one of many sites of cell wall biosynthesis, wall modifications may be a result of drug-induced plasma membrane damage and impairment of membrane-bound enzyme systems following polyene action.

In contrast to Al-Bassam *et al.* (1985), this study failed to show any significant changes in surface proteins of *C.albicans* AU1 treated with sub-inhibitory concentrations of Amp B using SDS-PAGE and immunoblotting. This difference in results between studies may be explained by variations in analytical immunological techniques, growth conditions, physiological state of yeast cells, yeast strain, MIC values and subsequent sub-inhibitory doses employed.

Although Al-Bassam *et al.* (1985) employed an immunofluorescent method which apparently does not denature surface components of whole cells during preparation, unlike the denaturing properties of SDS used in this study, it was also clear that their MIC values were 5-fold higher than those reported here. Consequently, their higher sub-inhibitory concentrations were more likely to induce profound wall changes. In addition,

differences between results are likely to be influenced by some of the factors mentioned above which include, yeast strain, pH and media composition.

In contrast to the results associated with Amp B, sub-inhibitory doses of nystatin induced profound alterations in surface proteins. These changes were markedly influenced by growth phase and growth in the presence and absence of iron. Serum from a patient with vaginal candidosis only reacted with a 66kd antigen in walls from cells cultivated under iron-sufficient conditions in 1.6μg/ml or 2.66μg/ml nystatin. However under iron-depleted conditions several antigens were recognized in the presence of 1.6μg/ml nystatin by the same serum, whereas a diffuse high MW antigen and a 66kd antigen were recognized in the presence of 2.66μg/ml as cells aged.

An interesting question is whether the present findings relating to phenotypic changes *in vitro* have any clinical significance? There have been a few reports of vaginal candidosis where eradication of *C.albicans* by Amp B proves difficult despite its *in vitro* sensitivity to polyenes. Amp B reacts non-specifically with host tissue and serum components and has a toxicity 20-40 times its therapeutic level. Given that in any infection, cell populations will be heterogenous with growing and non-growing organisms present, administration of Amp B, at levels attainable in blood (1-3μg/ml) (Kerridge, 1986), would be expected to mediate clearing of growing cells from host tissues, but stationary phase organisms would be resistant and survive the presence of antibiotic to provide a reservoir of infection.

The role of iron in this context may have significant implications. Excess iron could

provide a protective effect, not by preventing antibiotic interaction with plasma membranes, but by maintaining an equilibrium between internal and external cell iron concentrations necessary for an organisms' survival. A similar effect has been described by Liras and Lampen (1974) and Kerridge *et al.* (1976) when K^+ and Mg^{2+} ions were added at concentrations of 85mM and 45mM respectively, to cultures of *S.cerevisiae* and *C.albicans*. With *C.albicans*, protection occurred only when polyenes were present at MIC levels. Therefore a protective effect by ferrous ions of *C.albicans* could help to explain the apparent recovery of yeast growth after prolonged inhibition by 2.66μg/ml nystatin in iron-sufficient media.

A mechanism(s) of drug action resulting in surface changes described above remains to be determined, but one may speculate that nystatin appears to induce either a loss of surface proteins or possibly to modulate surface epitopes by masking or altering antigenic sites which otherwise would be accessible for antibody recognition. Growth under iron-depletion in the presence of 1.6 and 2.66μg/ml nystatin resulted in expression of surface antigens that were either barely detectable or not expressed under iron-sufficient conditions following incubation with patient serum. These findings may suggest that recognition of iron-regulated proteins by patient serum antibodies in combination with effects of sub-inhibitory doses of nystatin on surface proteins could render yeast cells more susceptible to opsonization and phagocytosis. Conversely, during iron overload environments *in vivo*, *C.albicans* could proliferate with minimal stimulation of humoral immune responses and resist effects of polyene action.

Considerable phenotypic variations in the sensitivity of *C.albicans* to imidazole-induced release of K^+ ions also exist (Kerridge, 1986), and by analogy with polyenes, this resistance develops during stationary phase growth and is associated with changes in cell walls. A similar phenomenon has also been observed by Beggs (1984) who found that fungicidal, but not fungistatic effects of miconazole and ketoconazole were affected by growth phase, with resistance developing during stationary phase. Apart from ultrastructural studies on the effects of fluconazole on *C.albicans*, no known study has previously investigated effects of sub-inhibitory doses of this triazole antifungal on surface proteins of *C.albicans*. Thus, although the present results suggest that such concentrations of fluconazole do not alter surface protein expression *in vitro*, further investigations are necessary.

Only IgG antibodies were examined in this study, yet further investigations which include IgM and IgA would be valuable to elucidate their role in antigen recognition on surfaces of yeasts grown in the presence of sub-inhibitory concentrations of antifungals. For future work, it would be interesting to compare antigenic profiles of *C.albicans* incubated with sera from a range of patients with various forms of candidosis, before and during antifungal therapy. Such a study could shed some light on the affects of antifungals on *Candida* surface antigens during infection, and also provide a better understanding of the role of specific antibody responses during therapy.

9. Influence of iron deprivation on adherence mechanisms of *C.albicans*

9.1. Influence of iron restriction on agglutination of *C.albicans* by concanavalin A sepharose beads.

Concanavalin A (Con A) is a lectin which reacts specifically with mannopyranosyl residues in *C.albicans* yeast cells, therefore use of this lectin provides a means of assessing yeast agglutination amongst *C.albicans* isolates and factors influencing this phenomenon. Figs.9.1A and B illustrate agglutination and non-agglutination of yeasts in the absence and presence of a yeast mannan inhibitor, α-methyl-D-mannoside. Agglutination of yeasts by concanavalin A (Con A) resulted in a rough granular precipitate evenly dispersed around the bottom of microtitre wells (Fig.9.1A), whereas inhibition of agglutination was observed as a small compact pellet of yeasts at the bottom of wells, with an overlaying layer of smooth transparent beads (Fig.9.1B). Three isolates of *C.albicans* (AU1, C55 and C406) were grown to logarithmic and stationary phase in iron-sufficient media (SSM+Fe) and media supplemented with an iron chelator, 1'10-phenanthroline (OP) (SSM+OP). Yeast cells subsequently radiolabelled as described in section 3.6.2., were mixed with Con A beads, and yeasts bound to beads quantified by radioactive measurements.

Growth in the presence or absence of iron did not affect agglutination of any isolate. Similarly, growth phase did not influence agglutination of AU1 nor C406 during growth in SSM+Fe or SSM+OP, but significantly affected agglutination of C55 in SSM+Fe

($P<0.005$) (Fig.9.2.). There was no apparent trend in agglutination of *C.albicans* isolates, however, comparisons between isolates demonstrated that growth phase influenced agglutination of C406 and C55 ($P<0.01$), and C55 and AU1 ($P<0.05$) during growth in iron-sufficient but not in iron-depleted conditions. Furthermore stationary phase yeasts of all isolates appeared to be agglutinated to a lesser degree than logarithmic phase cells.

9.2. Influence of iron restriction on adherence of *C.albicans in vivo*

Isolates AU1, C55 and C406 grown to stationary phase in SSM+Fe and SSM+OP *in vitro* were harvested and inoculated into the vagina of oestrogenized hamsters. All animals were lavaged 48h after the last of three consecutive inoculations and quantification of vaginal *Candida* infection performed on lavage fluid as described in section 3.6.3.

Results of colonization *in vivo* of each isolate is shown in Fig.9.3. Growth *in vitro* in the presence and absence of iron did not influence the ability of each isolate to cause experimental vaginitis *in vivo*. When strains were ranked based on their ability to cause vaginal infection in hamsters (C55 > AU1 > C406), the order appeared to correlate with their growth characteristics *in vitro* i.e. C55 and AU1 obtained higher growth rates and achieved greater cell densities than C406.

9.3. Influence of iron-depletion on the cell surface hydrophobicity of *C.albicans*

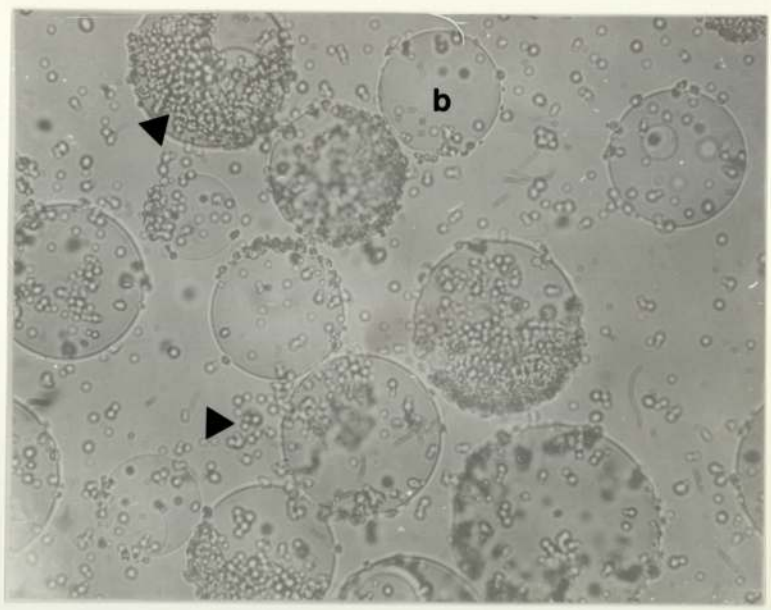
The relative hydrophobic affinity of *C.albicans* AU1, AU2 and OMC3 was determined by the adherence to a water-hydrocarbon interface assay. In this assay, emulsions of hydrocarbon were formed during vortexing, in effect increasing the hydrocarbon interfacial area available for adherence. Yeast cells entering the hydrocarbon phase were measured spectrophotometrically at 660 nm and percentage changes in absorbance calculated with respect to absorbance values of original aqueous yeast suspensions.

All isolates tested were hydrophilic regardless of growth conditions (Fig.9.4A, B and C), i.e. few yeast cells entered the hydrocarbon phase. Although no clear trends in hydrophobicity between *Candida* strains or growth conditions were observed in this study, a number of significant differences were demonstrated. Hydrophobicity of AU1 and OMC3 was significantly enhanced ($P<0.001$ and $P<0.001$ respectively) during growth in SSM-Fe compared to growth in SSM+Fe (Fig.9.4A and C), in contrast, presence or absence of iron did not influence hydrophobicity of AU2, but logarithmic phase cells were more hydrophobic than stationary phase cells ($P<0.025$) (Fig.9.4). In addition, AU2 was more hydrophobic than AU1 and OMC3 during growth in SSM+Fe ($P<0.001$ and $P<0.001$ respectively) and as a result of growth phase ($P<0.025$ and $P<0.01$ respectively).

9.3.1. *Influence of iron-depletion and sub-inhibitory concentrations of antifungals on the cell surface hydrophobicity of C.albicans*

Stationary phase yeast cells of AU1 were chosen to continue further studies on affects of sub-inhibitory concentrations of three antifungal antibiotics on surface hydrophobicity of *C.albicans* as previous investigations have employed this strain extensively (Fig.9.5). Hydrophobicity of AU1 was significantly reduced following growth in the presence of nystatin (1.6 μg/ml) or Amp B (0.05 μg/ml) under iron-sufficient conditions ($P<0.025$ and $P<0.005$ respectively), however, there was little change after growth in SSM-Fe. Furthermore, fluconazole (0.8 μg/ml) significantly enhanced hydrophobicity of AU1 ($P<0.001$) regardless of growth conditions.

(A)



(B)

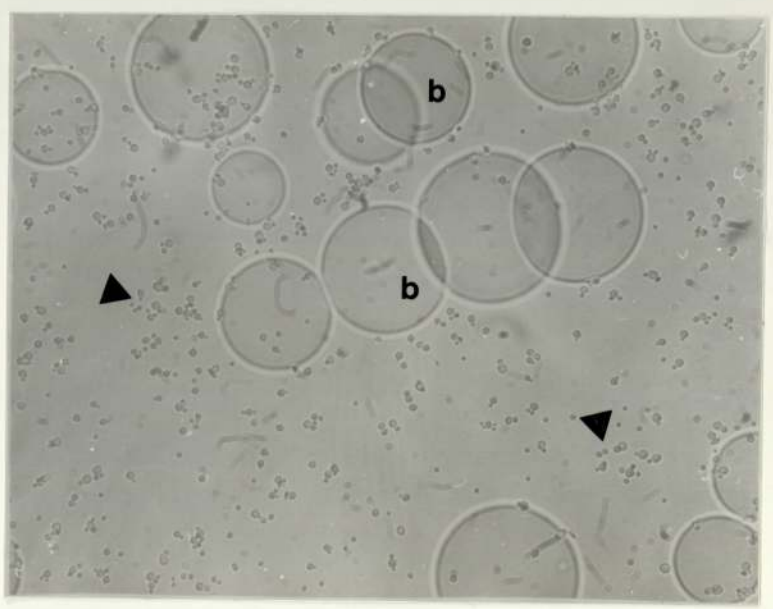


Fig.9.1A and B. Agglutination of yeast cells of *C.albicans* AU1 by concanavalin A coated sepharose beads in the (A) absence and (B) presence of a yeast mannan inhibitor, α-methyl-D-mannoside. Arrowheads indicate yeast cells. b=beads. Magnification x40.

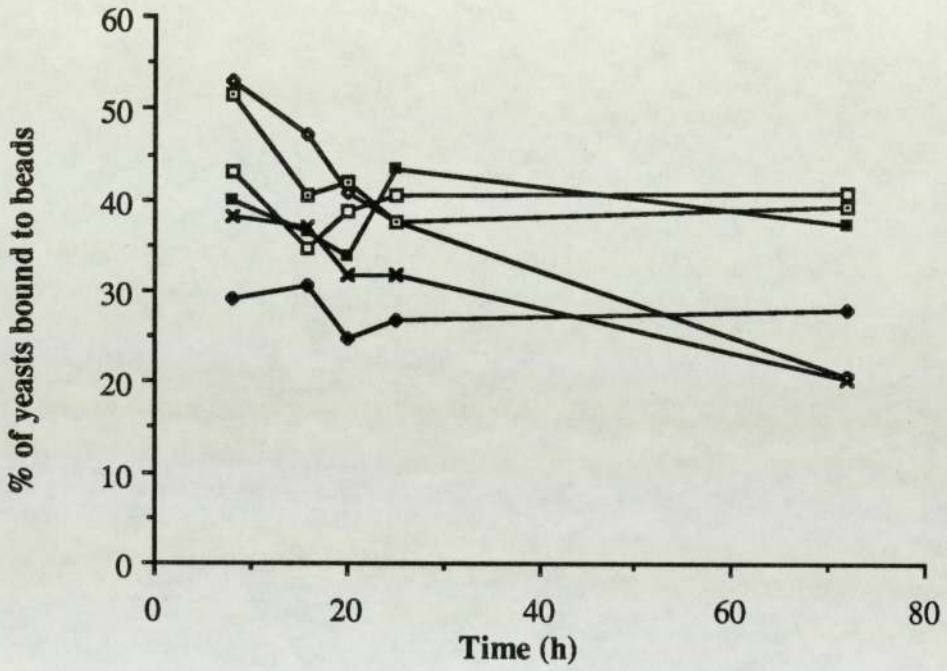


Fig.9.2. Effect of iron-restriction and growth phase on agglutination of *C.albicans* by concanavalin A sepharose beads. Yeasts bound to beads were enumerated by radiolabelling with ^{14}C-glucose. Isolate AU1 grown in (▪) SSM+Fe and (▫) SSM +OP, X= ±4.56, Y= ±3.8; C55 grown in (◆) SSM+Fe and (◈) SSM+OP, X= ±15.18, Y= ±6.8; C406 grown in (✖) SSM+Fe and (◆) SSM+OP, X= ±8.76, Y= ±2.75. (X= 95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times).

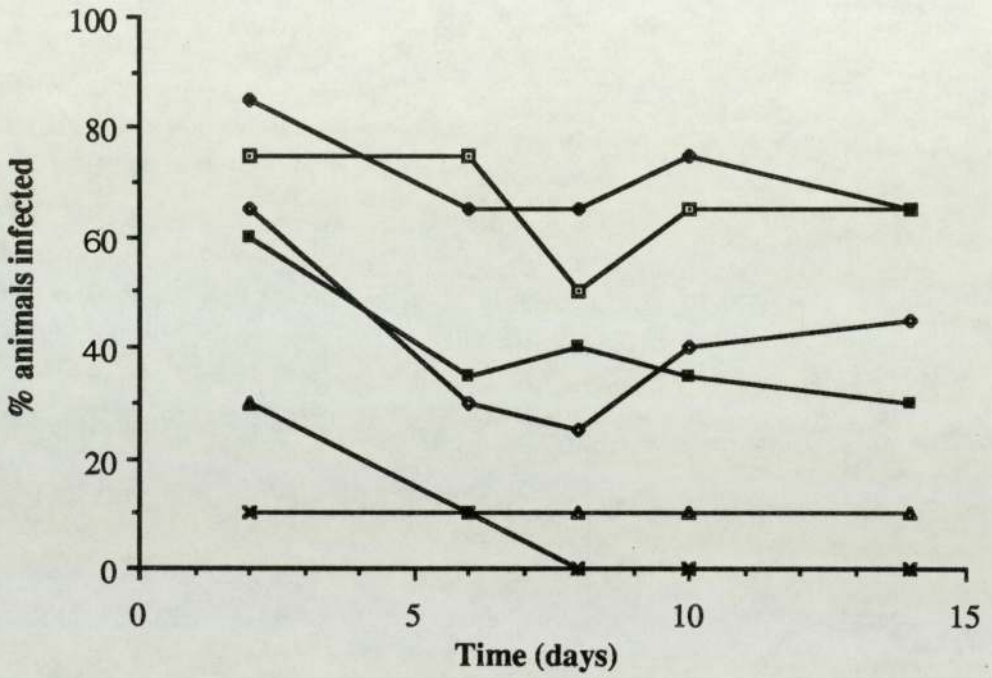


Fig.9.3. Effect of iron restriction *in vitro* on the ability of *C.albicans* to induce experimental vaginitis in hamsters. Comparison of isolates AU1 grown in (■) SSM+Fe and (◆) SSM+OP, X= ±14.55, Y= ±19.3; C55 grown in (▫)SSM +Fe and (◆) SSM+OP, X= ±12.7, Y= ±11.1; and C406 grown in (▲) SSM+Fe and (✖) SSM+OP, X= ±11.1, Y= ±6.799. All isolates were harvested during stationary phase growth prior to inoculation into animals. (X= 95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same time or different times).

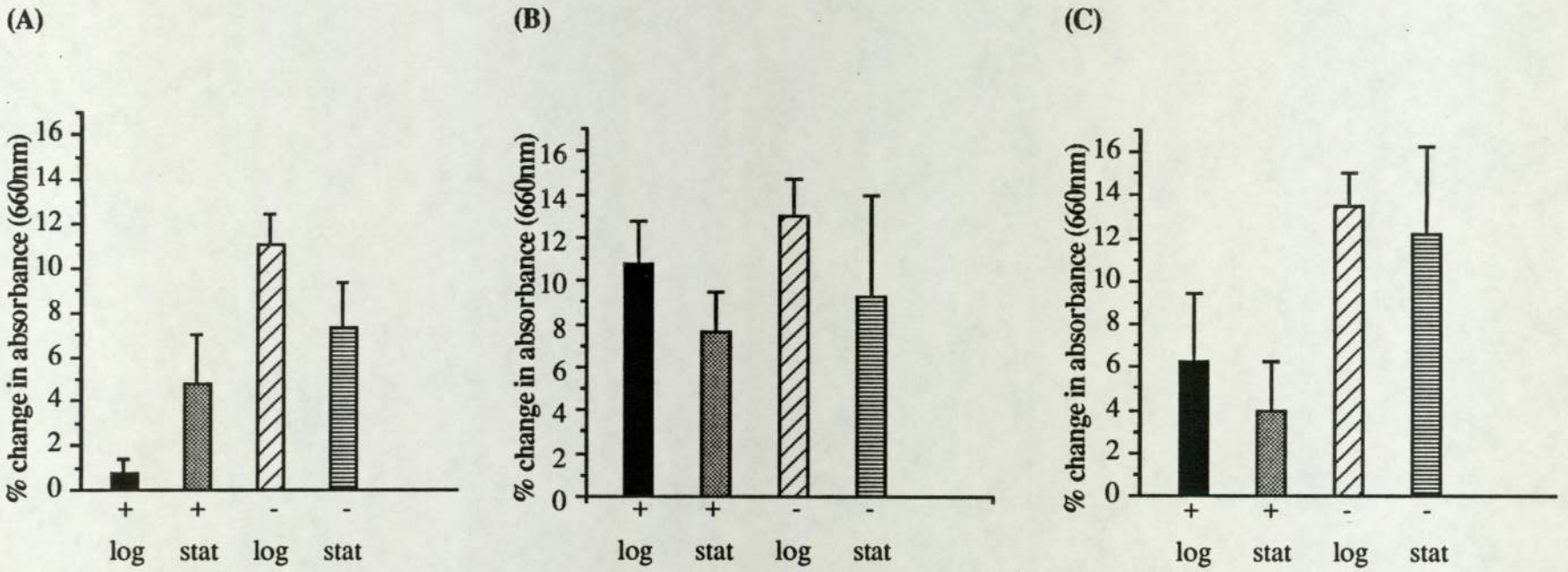


Fig.9.4A, B and C. Influence of iron and growth phase on the cell surface hydrophobicity of *C.albicans* AU1 (A), AU2 (B) and OMC3 (C). Yeasts were harvested during logarithmic (log) phase in (+) SSM+Fe and (-) SSM-Fe; and during stationary (stat) phase in (+) SSM+Fe and (-) SSM-Fe, and adherence to toluene measured. Results represent means ±95% confidence limits of six replicates.

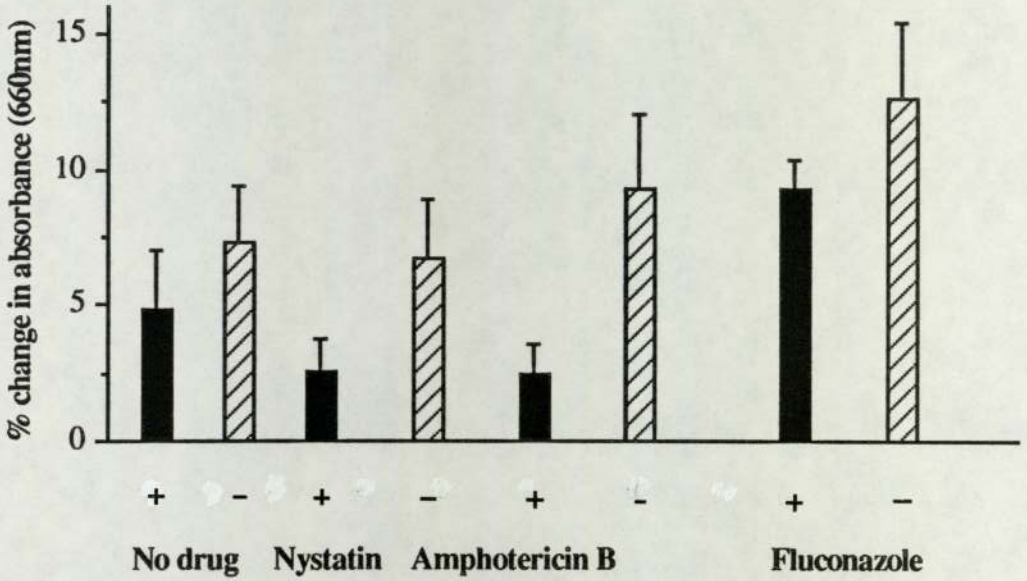


Fig.9.5. Influence of iron and a subinhibitory concentration of three antifungals on the cell surface hydrophobicity of *C.albicans* AU1. Cells were harvested during stationary phase growth in (+) SSM+Fe and (-) SSM-Fe in the absence of drug or in the presence of 1.6µg/ml nystatin, 0.05µg/ml Amp B or 0.8µg/ml fluconazole. Results represent the means ±95% confidence limits of six replicates.

9.4. Discussion

Most *Candida* infections follow a similar sequence of events initiated by adherence of yeast cells to host tissues which is succeeded by germ tube and mycelium formation, and subsequent invasion of epithelial cells. Hence, adhesion of *C.albicans* to various epithelial cells is considered to be an important event in colonization and pathogenesis of candidosis (Rotrosen *et al.*, 1986; Kennedy, 1987). Numerous *in vitro* and *in vivo* models have been developed to study mechanisms of adherence of *C.albicans* to mucosal and acrylic surfaces associated with prosthetic devices. These models have led many workers to conclude that various yeast surface components mediate adhesion to epithelial cells. However, characterization of candidal adhesins or ligands which promote attachment remains to be elucidated.

It has been suggested by several workers, that mannose-containing moieties (mannoproteins) are likely to be candidal adhesins (Maisch and Calderone, 1981; McCourtie and Douglas, 1981, 1985; Sandin and Rogers, 1982; Lee and King, 1983; Sandin, 1987), which have been shown to be associated with an outer fibrillar layer on yeast surfaces (Tronchin *et al.*, 1984). In contrast, studies by Segal *et al.*, (1982, 1988) and Lehrer *et al.*, (1983, 1988) have indicated that chitin, which is located within innermost cell wall layers, may serve as adhesive components.

Consistent with this view are recent findings of Sandin (1987) who demonstrated that adhesion of *C.albicans* to buccal epithelial cells was inhibited by concanavalin A (Con A), whereas after mannan extraction of yeast cells, pretreatment with Con A failed to

reduce the residual adhesion. Furthermore, using slide agglutination assays with Con A, extracted cells retained a significant degree of agglutination compared to strongly agglutinated unextracted cells, suggesting that *C.albicans* is capable of retaining the ability to adhere to epithelial cells even after removal of surface mannans.

In addition to supporting the expression of an adhesin beneath the outer surface layers, Kennedy *et al.* (1987) and Sandin (1987) suggest that there may be several mechanisms of adhesion, whereas other workers, who have failed to establish a definitive role for an adhesin, propose that *Candida* adhesion may not be mediated by a specific adhesin-receptor interaction alone, but that non-specific mechanisms may be involved (Reinhart *et al.*, 1985; Hazen *et al.*, 1986; Klotz and Penn, 1987).

These discrepancies are likely to reflect variations in experimental conditions used by different investigators including, differences in *Candida* strain, growth medium, variation in growth conditions, and methods for isolation and preparation of test cells (Rotrosen *et al.*, 1986; Kennedy, 1987; Kennedy and Sandin, 1988). However, while all of these factors may influence adhesion, growth conditions are probably the most important, since factors mediating adhesion are directly dependent on growth conditions (Kennedy and Sandin, 1988).

Surface composition and structure of *C.albicans* has been shown to vary with the nutritional composition of growth media (Douglas *et al.*, 1981; M^{c}Courtie and Douglas, 1981, 1985). In particular, concentration and type of carbohydrate, type, batch and manufacturer of medium, whether growth was in a liquid or on solid a medium and

whether a synthetic or undefined medium was used, have all been shown to influence adhesion.

In their extensive review of adherence of *Candida* species to host tissues and plastic surfaces, Rotrosen *et al.* (1986) recommended that the field of candidal adhesion would benefit from a systematic identification of growth conditions that influence adhesion and from interlaboratory standardization of assay techniques. Furthermore, they proposed the use of simple screening techniques that are analogous to bacterial haemagglutination assays.

Con A has been widely used as a valuable tool for detecting specific carbohydrate residues or sequences on microbial surfaces because it reacts specifically with mannan (mannoprotein) residues in *C.albicans* yeast cells. Utilizing sepharose beads coated with Con A, an assay was developed for determining yeast agglutination, whereby beads were mixed with yeasts harvested during different growth phases and cultivated under iron-restricted and iron-sufficient conditions. Visual observation of agglutination was substantiated by enumerating the number of cells attached by radioactive measurements. This assay fulfills some of the requirements outlined above by Rotrosen *et al.* (1986), Kennedy (1987) and Kennedy and Sandin (1988), in particular, the performance of yeast agglutination in microtitre wells allows screening of large numbers of different isolates or the same isolate cultivated under various conditions.

This study has demonstrated that growth phase rather than growth in the presence or absence of iron significantly influenced yeast agglutination by Con A, possibly by

modulating expression of mannose-containing moieties on yeast surfaces. The few reports supporting the view that stationary phase cells show greater adherence to epithelial cells than logarithmic phase cells (King *et al.*, 1980; McCourtie and Douglas, 1981), are matched by reports to the contrary (Segal *et al.*, 1982). Hence it is difficult to draw any firm conclusions relating to its significance based on these results. The present data revealed that stationary phase cells were agglutinated to a lesser degree than logarithmic phase cells, a characteristic clearly more evident with yeast cells of strain C55 than with any other strain. These results suggest that expression of mannose-containing moieties decreases with time, and that extent of reduction appears to be governed by yeast strain.

Using monoclonal antibodies (MAbs) directed against different soluble cell wall components, several workers have attempted to identify and study regulation of surface antigen expression in *C.albicans*. However, at present it is not known whether any surface antigenic determinants identified by MAbs are involved in, or mediate adhesion of *C.albicans* to host tissues. Most of these studies have demonstrated a greater expression of antigenic determinants on logarithmic phase cells than on stationary phase cells grown *in vitro*, and furthermore antigen expression *in vivo* has also been shown to be prominent during early stages of growth (Hopwood *et al.*, 1986; Brawner and Cutler, 1987).

The mannose composition of yeast cell walls is reported to remain fairly constant during logarithmic and stationary phase growth (Sullivan *et al.*, 1983). This view is

supported by Chaffin *et al.* (1988) and Sundstrom *et al.* (1988) who proposed that since antigenic determinants are believed to be part of a carbohydrate component of mannoproteins, only a particular surface epitope and not the overall polysaccharide structure would be subject to variable expression.

It has also been proposed that regulation of antigen expression could involve masking or shedding of some antigens into the environment during growth. Shedding of antigenic material during growth has been supported by evidence from several investigators (Tronchin *et al.*, 1984; Brawner and Cutler, 1986; Chaffin *et al.*, 1988; Sundstrom *et al.*, 1988), and could explain a reduction in yeast agglutination with time observed in this study. An examination of culture supernatants for agglutination would have confirmed this.

A recent investigation by Tronchin *et al.* (1988) attests to the rapid progress being made to isolate and characterize molecules believed to be involved in adhesion of *C.albicans*. Using electron microscopy and Con A-gold labelling, these workers demonstrated that germ tubes adhering to plastic develop an additional outermost fibrillar layer containing mannoproteins, and through analysis by SDS-PAGE, proteins of MW 60, 68, 200kd and a high MW component (>200kd) were detected in germ tube cell wall layers and on plastic surfaces. Although the 60 and 68kd components were identified by Coomassie Brilliant Blue, immunoblotting and Con A-peroxidase labelling, the high MW components were detected only after autoradiography, a more sensitive technique. Therefore these workers suggested that cell wall adhesins are retained on plastic surfaces

and are involved in enhanced adherence of germ tubes to plastic.

Many previous studies on yeast adherence have attempted to demonstrate the importance of a specific interaction between a surface ligand or adhesin and an epithelial cell receptor, yet there is currently little convincing evidence for the presence of a universal adhesin for *C.albicans*. This approach may have ignored the complexity of adhesion mechanisms in an attempt to demonstrate a single mechanism for adhesion, as has been the case for many bacterial studies (Beachey, 1981). The ubiquitous nature of *Candida* attachment to numerous tissues and inert surfaces has prompted several investigators to suggest that multiple mechanisms may contribute to *C.albicans* adhesion (Reinhart *et al.*, 1985; Kennedy *et al.*, 1987; Klotz and Penn, 1987).

Klotz and Penn (1987) suggested that *C.albicans* yeasts appear to possess the ability to adhere to intestinal epithelia by several mechanisms, including adhesin-receptor interactions, nonspecific hydrophobic and ionic bonding, and coadhesion. Since hydrophobic interactions are generally considered to play an important role in adhesion of bacteria to host cell surfaces (Peres *et al.*, 1977; Beachey, 1981; Miörner *et al.*, 1983), the adherence of three *C.albicans* isolates to a hydrocarbon interface was investigated in this study.

All yeast isolates were hydrophilic after growth in the presence or absence of iron at 37°C, a finding consistent with that of Klotz *et al.* (1985), Hazen *et al.* (1986) Minagi *et al.* (1986) and Kennedy and Sandin (1988). Subsequent studies by Hazen and Hazen (1988) demonstrated that isolates grown at 37°C became hydrophobic within 1h

incubation, but this appeared to be transient because after 6h growth, levels of hydrophobicity diminished.

In this study, growth phase influenced hydrophobicity of *C.albicans* yeasts, and this varied within and between isolates. There are few comparable studies available relating to effects of different growth phases of *C.albicans* yeast on surface hydrophobicity, but recent reports by Kennedy *et al.* (1988) have indicated that under identical growth conditions, specific and nonspecific adhesion mechanisms of *C.albicans* can be significantly influenced by the phenotypic state of an organism (Kennedy *et al.*, 1988). Furthermore, results of this and other studies (Hazen *et al.*, 1986; Hazen and Hazen, 1988) appear to suggest that cell surface hydrophobicity of *C.albicans* is strain dependent. Growth under iron-depletion also increased hydrophobicity of *C.albicans* AU1 and OMC3, but the significance of these findings requires further investigation especially given that growth *in vivo* may be under iron-restriction and that mechanisms of pathogenicity of *C.albicans* are poorly understood.

Although no direct correlation has been demonstrated between surface hydrophobicity and adhesion of *C.albicans* to epithelial cells (Kennedy and Sandin, 1988), hydrophobic properties may be an important factor in promoting yeast coadhesion, thereby, contributing to the total number of yeasts attached to epithelial cells by indirect adhesion. Aggregation of yeasts could concentrate extracellular secretions at adjacent areas of host tissue, which may damage host cells or cause increased adherence of yeasts (Klotz and Penn, 1987). Therefore, variations in hydrophobicity may influence adhesion which

could in turn affect colonization and infection.

Alternative roles for cell surface hydrophobicity during growth of *C.albicans in vivo* remain undefined. However, Zajic and Seffens (1983) believe that cell surface hydrophobicity may function as a biosurfactant, whereby cell walls could assist solubilization of hydrocarbon aggregates and other insoluble compounds therefore providing a source of nutrients for organisms. Consequently, it has been postulated that an organism displaying surface hydrophobicity would be better adapted to nutritionally stringent conditions (Hazen and Hazen, 1988). Recent studies with *Candida* suggest that hydrophobic yeast cells may be more likely to cause disease than hydrophilic yeast cells. Evidence in support of this view originates from survival experiments in which mice died faster when challenged with hydrophobic yeast cells than with hydrophilic yeast cells (Hazen and Hazen, 1988).

In their review on adherence of *C.albicans*, Rotrosen *et al.* (1986) expressed the need for studies relating to affects of antifungal drugs on adherence mechanisms in *C.albicans*. Recently, Vuddhakul *et al.* (1988) described the affects of polyene and azole antifungals on adherence of *C.albicans* to dacron fibre and reported that a possible therapeutic target of antifungal agents may be interference with candidal adhesion mechanisms. Macura (1988) also found that Amp B, nystatin and ketoconazole significantly inhibited adherence of *C.albicans* to human buccal epithelial cells and that sub-inhibitory doses of these drugs were less effective than their MICs.

Since sub-inhibitory doses of antifungals are known to affect growth, morphology

and cell surface properties of *C.albicans* (Al-Bassam *et al.*, 1985), the affect of such concentrations on surface hydrophobicity of *C.albicans* was determined in this study. Hydrophobicity of stationary phase cells of AU1 was increased after growth in the presence of nystatin or Amp B, under iron-depleted conditions and further enhanced following growth in the presence of fluconazole irrespective of growth conditions.

Polyene antibiotics bind to sterols in plasma membranes and alter membrane permeability and integrity (Hamilton-Miller, 1973a). This action combined with growth under iron-depleted conditions appears to influence hydrophobicity of *C.albicans*, whereas under iron-sufficient conditions little change was apparent. The profound affect of fluconazole on hydrophobicity of *C.albicans* may be attributed to an affect of the drug on ergosterol biosynthesis which in turn alters membrane composition. Concomitantly, interaction of drug molecules with surface components could conceivably alter hydrophobicity by steric hindrance or alter surface charges.

Both polyenes and azoles appear to induce multiple effects on yeast cells, indicating that there are likely to be several target sites for these drugs (Al-Bassam *et al.*, 1985; Kerridge and Nicholas, 1986; Saag and Dismukes, 1988). Hence, interference by drug molecules of surface components may influence mechanisms of *Candida* adhesion.

When hamsters were inoculated intra-vaginally with yeasts from three *C.albicans* isolates grown under iron-sufficient and iron-depleted conditions *in vitro*, a trend in their ability to cause experimental vaginitis was apparent, i.e. C55 > AU1 > C406. The diminished ability of C406 to induce vaginitis appeared to correlate with its relatively

poor growth *in vitro*, regardless of growth conditions. Several workers have demonstrated major differences in *C.albicans* strain virulence in experimental systemic candidosis (McCourtie and Douglas, 1984), however, relatively little is known relating to factors influencing colonization of mucosal surfaces and ability to cause experimental or clinical vaginitis (Taschdjian *et al.*, 1960; Sobel *et al.*, 1984; Lehrer *et al.*, 1986).

When yeast cells are introduced into the vagina, initial colonization will depend on many factors, including the capacity of yeasts to adhere to vaginal epithelial cells (VECs) (Sobel *et al.*, 1984). Lehrer *et al.* (1986) demonstrated that yeasts of a variant strain adhered considerably less to human VECs than a wild-type strain, whereas in contrast, Sobel *et al.* (1984) in a similar study found no difference between strains. Both groups were consistent in their findings that wild-type and variant strains shared identical growth characteristics and that variant strains were shown to be less virulent in a murine vaginal candidosis model than wild-type strains. Both wild-type and variant strains used by Lehrer *et al.* (1986) produced hyphae, and revealed similar cell wall protein compositions, therefore reduction in virulence of the variant strain could not be attributed to any of these factors. In contrast, Sobel *et al.* (1984) showed that the reduced ability of their non-hyphae producing variant to colonize and infect VECs (compared to the hyphal producing wild-type) may be a result of reduced adhesion to these cells which in turn may lead to rapid yeast clearance by vaginal host defence systems.

This finding could help explain the apparent differences between the ability of AU1 and C55 to induce infection despite having similar growth kinetics. Isolate AU1 may not

be able to adhere to VECs as well as C55, and consequently may be more readily cleared from the vagina. The order of virulence found could also reflect the ability of each isolate to produce hyphae *in vivo*, irrespective of their *in vitro* growth characteristics. Yet, despite evidence suggesting an important role for hyphal production in vaginal colonization and pathogenesis, yeasts unable to produce hyphae *in vivo* can still successfully colonize vaginal tissue (Sobel *et al.*, 1984). Hence, the ability to produce hyphae is not absolutely essential to cause infection.

To investigate the affect of iron-restriction on the ability of *C.albicans* to induce experimental vaginitis, biochemical and phenotypic characteristics of the yeast inocula was taken into consideration. As these properties are influenced by growth environments, yeasts cultivated under iron-sufficient and iron-restricted conditions were used to inoculate animals. The role of iron in this infection model was not established, however, irrigation of the hamster vagina with an iron solution to simulate an iron overload condition may have contributed to a better understanding of the significance of iron in candidal vaginitis. Although there are few *in vivo* studies relating to *Candida* infections in iron overload, Abe *et al.* (1985) demonstrated a higher mortality amongst mice injected intravenously with colloidal iron (80%) than in mice without iron overload (40%). This increase in mortality was attributed to enhanced serum iron and iron saturation, decreased phagocytic activity against yeasts due to iron saturation of phagocytes, and extensive dissemination of *Candida*, particularly to kidneys where a higher tissue iron content and renal iron excretion enhanced proliferation.

The role of iron in human vaginal candidosis has not been previously investigated, however, whilst the nutritional status of microorganisms *in vivo* is often difficult to assess, and may vary from one tissue site to another one may speculate about the nature of a growth limiting nutrient for various localised sites (Brown, 1977). Nutrients believed to be important in colonization of vaginal epithelia by *Candida* include glycogen, glucose, maltose, maltotriose and maltotetraose (Gregoire, 1963). In addition, availability of keratin may be another essential component affecting growth *in vivo* (Ryley, 1986). Therefore, since iron is an essential nutrient for growth of *C.albicans*, its availability in vaginal tissues and fluids may influence colonization and pathogenicity, and add another factor to the already complex nature of vaginal candidosis.

In this study no correlation was established between agglutination of *C.albicans* by Con A and ability to induce experimental candidal vaginitis, nor between cell surface hydrophobicity and yeast agglutination, a finding consistent with studies of Kearns *et al.* (1983). In contrast, Segal *et al.* (1984) demonstrated a correlative relationship between adherence of *C.albicans* to human VECs *in vitro* and candidal vaginitis. Similarly, Douglas *et al.* (1984) found that an increased adherence of strains grown in high concentrations of certain sugars correlated with enhanced virulence in a mouse model of disseminated candidosis. Therefore some evidence suggests that a particular mode of adhesion observed *in vitro* may play a role in colonization or infection *in vivo*. Generally this is not the case, as the complexity of influential host factors present *in vivo* are rarely represented in *in vitro* adhesion models (Persi *et al.*, 1985), hence there is an

urgent need for better *in vivo* models or *in vitro* assays which mimic conditions in the host to advance our knowledge of adhesion mechanisms in *C.albicans*.

10. CONCLUDING REMARKS

This thesis and the work outlined therein stems from an increasing awareness that cell wall surfaces of *C.albicans* are initial sites of interaction between fungus and environment, and are important in mediating adhesion to host epithelial cells during colonization and infection, and presentation of antigens to hosts. Although *C.albicans* surface moieties and their antigenic determinants remain generally poorly defined, recent literature indicates that gradual progress is being achieved in this area, and it is becoming increasingly apparent that definition of surface antigens is critical to understanding structural and functional relationships of cell walls in host-*C.albicans* interactions.

The work described in this thesis has considered some of the many host factors known to influence pathogenesis of *C.albicans in vivo* and has also attempted to simulate these factors *in vitro* to investigate their affects on antigenic surfaces and on biological properties of yeast cells associated with their surfaces. SDS-PAGE and immunoblotting were the principal analytical methods used to separate and detect surface antigenic components. Antigenic profiles of yeast cells were profoundly influenced by growth environments. Growth of cells in iron-depleted medium resulted in expression of several antigens which were not expressed or barely expressed following growth in iron-sufficient medium. Although characterization of these iron-regulated proteins requires a more comprehensive biochemical analysis, lectin-blotting with concanavalin A suggested that some of these proteins are likely to be mannoprotein in nature. Furthermore, some iron-regulated proteins appeared to be located within cell walls, whereas others were

located on yeast surfaces.

Currently only one other group of workers, Ismail and Lupan (1988), have tentatively reported the presence of putative membrane and wall receptors for siderophores in *C.albicans*. Their observations along with the present findings provides some evidence, at least, for existence of iron uptake systems in *C.albicans in vitro*. The precise function of iron-regulated proteins in *C.albicans* is not known, but, based on extensive studies relating to mechanisms of iron-uptake in Gram-negative bacteria (Griffiths, 1987b), one may speculate that a possible role could involve siderophore-mediated iron-uptake. Detection of hydroxamate-type siderophores in cell-free supernatants of *C.albicans* cultures supported this view, however, this does not preclude involvement of other mechanisms of iron-uptake in the absence of siderophores.

The magnitude and variability of human antibody responses to these iron-regulated proteins/glycoproteins were dependent on the type of *Candida* infection (superficial or systemic), serum antibody class and yeast strain. Since IRMPs are expressed in Gram-negative bacteria during infection (Griffiths *et al.*, 1983; Brown *et al.*, 1984; Shand *et al.*, 1985), the possibility that receptors for siderophores could be important protective antigens has prompted a search for naturally occurring antibodies to these antigens (Griffiths *et al.*, 1983). Therefore, naturally occurring antibodies which react with iron-regulated proteins in *C.albicans* may afford a protective role against some infections and could provide a basis for investigating potential immunization strategies.

In further investigations, an attempt was made to monitor and quantify antibodies

directed against surface antigens of *C.albicans* and to determine which antigens were recognized by IgG antibodies during immunization of rabbits. Rabbit IgG antibodies recognized different antigens during early and later stages of immunization in walls from cells cultivated under iron-depleted compared to iron-sufficient conditions. It is not known whether these antigens are expressed during *Candida* infections, but these results could provide a useful basis for future investigations involving antibody recognition of surface antigens during *Candida* infections in an animal model. Given the importance of cellular (Cox, 1983) and humoral (Diamond, 1983) immune responses of a host to *Candida* infections, it would be valuable to monitor these responses to *C.albicans* during human infections and to determine whether iron-regulated proteins (or any other iron-induced fungal components) elicit these immune mechanisms.

Candidal adherence to host epithelial cell surfaces has been implicated as an important step in pathogenesis of infections, and it is clear that candidal surface components are involved in adhesion. This thesis demonstrated that growth phase rather than growth under iron-depleted or iron-sufficient conditions influenced expression of mannoproteins, which are believed to be one of the surface components that mediate adhesion in *C.albicans*. Differences between isolates were also observed in their ability to induce experimental vaginitis. Furthermore, growth under iron-depleted conditions increased the cell surface hydrophobicity of yeast cells. Therefore, growth conditions appear to alter surface moieties directly, or alter other adjacent components that may in turn affect adhesion of yeasts by influencing hydrophobicity, or masking or shedding of surface

constituents mediating adhesion. Whether these events occur *in vivo* remains to be determined, but it is clear that further studies are necessary to elucidate both specific and non-specific candidal adhesion mechanisms.

Sub-inhibitory concentrations of antifungals are likely to be attained in various host tissues during therapy, and such doses are important given the toxicity of some commonly used antifungals e.g. Amphotericin B. Consequently, such doses of polyenes and azoles have been shown to affect growth, morphology and surface properties of *C.albicans in vitro*. Furthermore, an ability of antibiotics to affect surface properties of microbial adherence to cell surfaces may be an important factor in selecting an antibiotic for therapy. Polyene antibiotics increased the surface hydrophobicity of yeast cells grown under iron-depleted conditions, and fluconazole also increased hydrophobicity irrespective of growth conditions. Interaction of these drug molecules with surface constituents may alter hydrophobicity by steric hindrance or alter surface charges. In combination with iron-depleted conditions, interference by drug molecules of surface components may consequently influence mechanisms of candidal adhesion.

Sub-inhibitory doses of nystatin induced profound changes in surface proteins of *C.albicans*, and these changes were markedly influenced by growth phase and growth in the presence and absence of iron. A most striking feature of the effect of nystatin on yeast cells was a loss of surface proteins with culture age. It is now being increasingly realised that administration of low-dosage regimens of antifungal therapy may be beneficial in certain types of superficial and systemic *Candida* infections (Husberg, 1988). This is a

critical point because sub-inhibitory doses of antibiotics coupled with host cellular and humoral responses may have therapeutic effects. In particular, polyenes are active immunoadjuvants which boost immune responses of the host to a variety of antigens, and may influence course of infection.

The data presented in this thesis emphasizes the importance of yeast cell surfaces and their associated biological characteristics in the pathogenesis of candidosis. It has been established that growth conditions and the presence of sub-inhibitory concentrations of antifungals can profoundly influence growth, adherence and antibody recognition of surface antigens of *Candida* yeast cells *in vitro*. Much of our understanding of what may be happening as pathogenic microorganisms adapt to and grow in an iron-restricted environment of a host has been derived from studies with Gram-negative bacteria. Yet mechanisms whereby *C.albicans* acquires iron *in vivo* are not known. Hence, there is a need for a better understanding of biological processes of *C.albicans* during growth *in vivo*, and the specific growth environments and host factors that influence these processes.

Given that sera from humans or experimental animals infected with *C.albicans* were used to probe yeast surfaces to identify changes in antigen expression as a result of variations in *in vitro* growth conditions, one has grounds to speculate that similar changes could be occurring during growth *in vivo*. However, in the absence of analogous studies using *in vivo* grown yeasts, definitive proof remains elusive. With the emergence of monoclonal antibodies and the potential to construct *Candida* genomic

libraries, an understanding at a molecular level could also help elucidate changes that contribute to microbial adaptation to host environments.

The ability of an organism to multiply in an iron-restricted environment of a host is just one important factor that may decide the outcome of an infection. Other host factors must also be considered and these are numerous and complex. In addition to iron-restriction, other factors that may influence microbial multiplication *in vivo* include temperature, pH, osmolarity, oxygen tension, and availability of other nutrients. Indeed, there is some evidence to suggest that mild zinc deficiency is associated with susceptibility of women to recurrent vaginal candidosis (Edman *et al.*, 1986). Special nutritional conditions occurring in certain tissues might also be expected to attribute to localization of microorganisms, or at least influence an organisms' growth rate, at specific body sites. Furthermore, given the increasing interest in the significance of bacterial biofilms during infection (Costerton *et al.*, 1987), application of appropiate methods for detection and analysis of potentially analogous structures associated with *C.albicans*, could reveal an important fungal stratergy for growth and survival during infection.

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