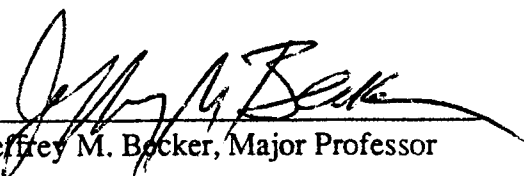
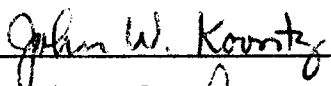
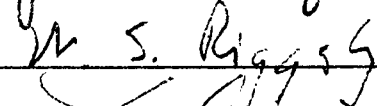


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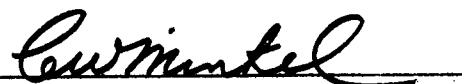
I am submitting herewith a dissertation written by Munira A. Basrai entitled "Modes of nutrient uptake in *Candida albicans* : peptide transport and fluid phase endocytosis". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.


Jeffrey M. Bocker, Major Professor

We have read this dissertation
and recommend its acceptance:


Accepted for the Council:


Associate Vice Chancellor
and Dean of The Graduate School

MODES OF NUTRIENT UPTAKE IN *CANDIDA ALBICANS* :
PEPTIDE TRANSPORT AND FLUID PHASE ENDOCYTOSIS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Munira Adnan Basrai

May 1992

DEDICATION

This dissertation is dedicated to the memory of my father, Late Hussein Poonawala, and my mother, Sharifa H. Poonawala, who inspired me to achieve my goal, and to my beloved husband, Adnan, whose support and encouragement made this possible

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ABSTRACT

Candida albicans is an important opportunistic pathogen in patients under treatments or with conditions, such as AIDS, that suppress the immune system. A basic understanding of the biology of this pathogen is essential in order to design an efficacious pathogen specific drug. The objectives of this research were to : (1) study the regulation of the peptide transport system using oxalysine-containing peptides as model compounds, (2) attempt to clone a *C. albicans* gene involved in peptide transport and (3) determine if fluid phase endocytosis is operative in the dimorphic fungus. An overview of the current literature related to the research presented here is given in Part I of this dissertation.

Part II of this dissertation summarizes the results of the growth inhibitory effect on *C. albicans* of oxalysine, a lysine analog, and oxalysine-containing di-, tri-, tetra-, and pentapeptides. Several amino acids overcame ammonium ion repression and increased the toxicity of oxalysine-containing di- and tripeptides for *C. albicans* with little or no increase in toxicity of oxalysine or oxalysine-containing tetra- and pentapeptides. The results indicate that the dipeptide and tripeptide transport system(s) of *C. albicans* are regulated by micromolar amounts of amino acids in a similar fashion to regulation of peptide transport in *S. cerevisiae* and that multiple peptide transport systems differentially regulated by various nitrogen sources exist in *C. albicans*. Oxalysine is not toxic to mammalian cells and hence it may be possible to use an oxalysine-containing peptide to design an anticandidal drug.

Part III of this dissertation describes the characterization of fluid phase endocytosis in *C. albicans*. Lucifer yellow (LY), an impermeable fluorescent dye used as a marker for fluid phase endocytosis, was internalized by *C. albicans*. LY was localized in vacuoles by a non-saturable, time-, temperature-, and energy-dependent process consistent with the characteristics of fluid phase endocytosis. Both the yeast and mold phases of this

dimorphic fungus internalized LY, and growth in complex medium appeared to be required to enable cells to internalize LY. These studies may explain how some large molecules, such as defensins, toxins, and cationic proteins, enter *C. albicans*.

Part IV of this dissertation describes attempts to clone a *C. albicans* peptide transport gene by functional complementation of a *S. cerevisiae* peptide transport mutant. The *S. cerevisiae ptr2* mutant was transformed with a genomic *C. albicans* library in a multi-copy vector and transformants with a PTR2⁺ phenotype were obtained. However, it was not possible to recover a *C. albicans* PTR2 homolog on a plasmid possibly due to integration of the *C. albicans* gene into the *S. cerevisiae* chromosome. Experimental approaches have been designed for the possible recovery of the putative *C. albicans* PTR2 homolog. Cloning of a *C. albicans* peptide transport gene should provide important information on the structure and regulation of the permease and facilitate the design of an anticandidal agent to be delivered to *C. albicans* via the peptide transport system.

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PART I

GENERAL INTRODUCTION

CHAPTER I

INTRODUCTION

Candida albicans is a diploid, dimorphic fungus with no known sexual cycle. *C. albicans* is usually encountered as a commensal on the oral, gastrointestinal and vaginal mucosae (Shepherd *et al.*, 1985) but can become an opportunistic pathogen under certain conditions (reviewed by Bodey & Fainstain, 1985). *C. albicans* is a major cause of secondary disease in patients receiving immunosuppressive therapy, antibiotic treatments, cancer chemotherapy, prolonged adrenal corticosteroid therapy or in patients undergoing surgical procedures (Meunier-Carpenter *et al.*, 1981 ; Shepherd *et al.*, 1985). *C. albicans* can be commonly isolated from blood cultures of hospitalized individuals (Shepherd *et al.*, 1985), is widespread in patients with advanced leukemia (Bodey & Fainstain, 1985) and from patients with AIDS (Chandler, 1985 ; Diamond, 1991). *C. albicans* can infect virtually any tissue but, superficial lesions of the mucosal surfaces is the most common form of candidosis. In some cases, the most serious invasive form follows dissemination of the yeast from the mucosal surfaces. Candidosis is the second most common form of vaginal infection in the United States (Sobel, 1985). Due to various limitations in the drugs available for treatment of candidosis, there is an urgent need to design a more efficacious drug specific for this disease (Drouhet & Dupont, 1987 ; Stevens, 1987).

We attempted to study nutrient uptake in *C. albicans* as it plays a central role in the growth and division of cells. A better understanding of the overall physiology of *C. albicans* is also important in the quest for an effective anticandidal agent. Studies done so far have permitted the characterization of *C. albicans* permeases for amino acids, sugars, peptides, nucleotides and nucleosides (reviewed by Prasad, 1987 ; Naider & Becker, 1987). Previous studies from our laboratory and several others have attempted to

characterize the peptide transport system(s) of both *C. albicans* and *S. cerevisiae* (Becker & Naider, 1977 & 1980 ; Davies, 1980 ; Logan *et al.*, 1979 ; Naider & Becker, 1987 ; Nisbet & Payne, 1979). Current evidence, though not conclusive, suggests the presence of at least two peptide permeases in *C. albicans* ; one transporting di- and tripeptides and the other transporting tri- and oligopeptides (McCarthy *et al.*, 1985 a & b ; Mehta *et al.*, 1984 ; Milewski *et al.*, 1988 ; Payne & Shallow, 1985 ; Yadan *et al.*, 1984). The peptide transport system of *C. albicans* is subject to regulation by the nitrogen source in the growth medium (Becker & Naider, 1977 & 1980 ; Davies, 1980 ; McCarthy *et al.*, 1985 a & b ; Naider & Becker, 1987 ; Yadan *et al.*, 1984), being repressed by ammonium ions and stimulated when cells are grown on amino acids as the sole nitrogen source (Logan *et al.*, 1979). Recently, Payne *et al.* (1991) reported stimulation of dialanine transport in *C. albicans* B2630 grown in bactopectone or trypticase peptone but not in casein hydrolysate.

Study of peptide transport is also of general importance as peptides have several roles besides serving as a nutrient (Payne, 1980). The oligopeptide permease, Opp, is involved in the recycling of peptides from the cell wall during growth in *Salmonella typhimurium* (Goodell & Higgins, 1987) and plays a role in the initiation of sporulation in *Bacillus subtilis* (Perego, 1991). Dpp, the periplasmic binding protein-dependent transport system of the enteric bacteria *S. typhimurium* and *E. coli*, is the chemoreceptor for chemotaxis towards peptides (Abouhamad *et al.*, 1991; Manson, 1986). Studies have established that resistance of tumors to chemotherapy, secretion of yeast mating pheromone α -factor, and bacterial transport have in common a family of closely related proteins all of which are believed to be associated with membranes and bind ATP (Ames, 1986 ; Gros *et al.*, 1986 ; Higgins, 1989 ; Hiles *et al.*, 1987 ; Hyde *et al.*, 1990 ; Kuchler *et al.*, 1989 ; McGrath & Varshavsky *et al.*, 1989). Such proteins have been reported to be present in bacteria,

yeasts, protozoans, insects and mammals. Furthermore, their similarities indicate that each may function by a similar mechanism.

In *C. albicans*, the study of peptide transport is also important from the standpoint of designing an effective anticandidal drug. Peptides can be used as carriers for drugs since structural requirements for transport of peptides differ between bacteria, yeast and mammalian cells (Naider & Becker, 1987). Hence, peptide conjugates can, in principle, be designed to be selective for yeast. Active transport is one of the possible ways to increase the permeation of potentially cytotoxic drugs into the cell (Allen *et al.*, 1979). This phenomenon termed "illicit transport" has been demonstrated in the enteric bacteria *E. coli* (Fickel & Gilvarg, 1973) and *S. typhimurium* (Ames *et al.*, 1973 ; Hammond *et al.*, 1987 ; Higgins, 1987) and the yeast *C. albicans* (Borowski *et al.*, 1979 ; Kingsbury *et al.*, 1983 ; Lichliter *et al.*, 1976 ; Meyer Glauner *et al.*, 1982 ; Milewski *et al.*, 1991a). The antibiotic tetaine enters *C. albicans* via the peptide permease and inhibits mannoprotein synthesis (Kenig & Abraham, 1976 ; Chmara, 1980). Peptide conjugates containing N³-(iodoacetyl)-L-2, 3-diaminopropionic acid, which inhibits mannoprotein synthesis (Andruskiewicz *et al.*, 1990), and polyoxins and nikkomycins, which inhibit chitin synthetase, have been designed to enter *C. albicans* through the peptide transport system as well (Becker *et al.*, 1983 ; Decker *et al.*, 1991 ; Krainer *et al.*, 1991 ; McCarthy *et al.*, 1985 a & b ; Milewski, *et al.*, 1991b ; Naider *et al.*, 1983 ; Yadan *et al.*, 1984).

In an attempt to further characterize the peptide transport system of *C. albicans* we used oxalysine-containing peptides as model compounds to probe the peptide transport system and study its regulation. Oxalysine, a lysine analog which is not toxic to mammalian cells, is known to cause growth inhibition of a number of bacteria (McCord *et al.*, 1957 ; Stapley *et al.*, 1967), *C. albicans* and certain other fungi (Zhang *et al.*, 1979). Our laboratory has also determined that oxalysine is a potent inhibitor of RNA synthesis, a weak inhibitor of protein synthesis, and has no effect on DNA synthesis in *C. albicans*, (Zheng *et al.*, 1992).

Part II of this dissertation describes the results of growth inhibitory effects of oxalysine-containing peptides on *C. albicans*. The nature of the nitrogen source in the growth medium had an influence on the toxicity of oxalysine-containing peptides. In addition to the nitrogen metabolism control, we observed that certain amino acids increased the toxicity of oxalysine-containing di- and tripeptides with little or no effect on the toxicity of oxalysine-containing tetra- and pentapeptides. These results suggested that the permeases transporting the peptides may be differentially regulated. Regulation by amino acids has not been reported so far in *C. albicans* ; regulation of peptide transport by amino acids has been observed in *S. cerevisiae* (Island *et al.*, 1987 & 1991).

Lucifer yellow, a marker for fluid phase endocytosis (Miller *et al.*, 1983 ; Stewart, 1978 & 1981 ; Swanson *et al.*, 1985), is internalized by *S. cerevisiae* cells into vacuoles (Riezman, 1985). These studies suggest that *S. cerevisiae* cells may be able to use fluid phase endocytosis to internalize macromolecules from the environment. Hence, we determined if fluid phase endocytosis was operative in *C. albicans* by determining if LY was internalized by this dimorphic fungus. An assay for internalization of LY by the yeast and mold phases of *C. albicans* was developed and the results of these studies are summarized in Part III of this dissertation. We observed that both the yeast and mold phases of *C. albicans* internalize LY into vacuoles by a non-saturable, time-, temperature- and energy-dependent process consistent with the characteristics of fluid phase endocytosis (Basrai *et al.*, 1990). It is known that some large molecules such as defensins, toxins, cationic proteins enter *C. albicans* and cause cell damage (Lehrer *et al.*, 1985 & 1988 ; Olson *et al.*, 1977 ; Polonelli *et al.*, 1985 ; Selsted *et al.*, 1985 ; Yphantis *et al.*, 1967). Recent reports on the passage of molecules through the yeast cell wall suggest that molecules with a mass as great as 200,000 to 400,000 diffuse through the intact yeast cell walls freely (De Nobel *et al.*, 1990 ; De Nobel & Barnett, 1991). Since the characterized oligopeptide permeases are not known to transport such large molecules across the cell

membrane (Becker & Naider, 1980 ; Naider & Becker, 1987) and, also, no receptors have been characterized for these molecules, it is possible that these molecules enter *C. albicans* by fluid phase endocytosis.

The definitive answer to the number of peptide permeases present in *C. albicans* and their regulation should result from a combined genetic and biochemical approach. The diploid nature of *C. albicans*, and a general lack of adequate genetic studies on this organism has limited a detailed analysis of its peptide transport system. However, the advent of recent techniques in molecular biology have facilitated the cloning and characterization of a number of *C. albicans* genes (reviewed by Kurtz *et al.*, 1990). One of the commonly used approaches to clone a *C. albicans* gene is by functional complementation of a *S. cerevisiae* mutant (reviewed by Kurtz *et al.*, 1990). Our laboratory has characterized peptide transport mutants of *S. cerevisiae* and results from these studies suggest that at least three genes *PTR1*, *PTR2* and *PTR3* are involved in the amino acid inducible peptide transport (Island *et al.*, 1991). The *S. cerevisiae* *PTR2* gene has been cloned and the nucleotide sequence suggests that it is a membrane protein with 10-12 membrane spanning domains (Perry *et al.* unpublished). We attempted to clone a *C. albicans* *PTR2* homolog by functional complementation of the *S. cerevisiae* *ptr2* mutant using a *C. albicans* library. *S. cerevisiae* transformants with a *PTR2*⁺ phenotype were obtained, however, a *C. albicans* *PTR2* homolog could not be recovered on a plasmid possibly due to the integration of the *C. albicans* gene into the *S. cerevisiae* chromosome. Experimental approaches were designed for the possible recovery of a putative *C. albicans* *PTR2* homolog. Attempts were also made to clone a *C. albicans* peptide transport gene by nucleic acid hybridization to cloned transporters from other systems. Results from these studies have been summarized in Part IV of this dissertation.

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PART II

TOXICITY OF OXALYSINE AND OXALYSINE-CONTAINING PEPTIDES

AGAINST *CANDIDA ALBICANS* :

REGULATION OF PEPTIDE TRANSPORT BY AMINO ACIDS

CHAPTER I

INTRODUCTION

Small peptides with highly variable amino acid composition are taken up by microorganisms via a carrier-mediated active transport system(s). The regulation of peptide transport systems in enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* has been studied extensively (Andrews *et al.*, 1986 ; Guyer *et al.*, 1985 ; Higgins, 1984 ; Hiles *et al.*, 1987 ; Payne, 1980). *S. typhimurium* and *E. coli* possess three genetically distinct systems for peptide transport with overlapping substrate specificities termed Opp, Dpp, and Tpp for oligopeptide, dipeptide, and tripeptide transport systems, respectively. The *opp*-encoded peptide transport system of *S. typhimurium* is constitutive, whereas the *tpp-B* encoded system is induced by leucine or anaerobiosis (Jamieson & Higgins, 1984). In *E. coli* however, the *opp*-encoded system rather than *tpp*-encoded system is induced either by anaerobiosis, alanine, leucine or peptides containing alanine or leucine (Andrews *et al.*, 1986 ; Andrews & Short, 1986). Recent studies by Abouhamad *et al.* (1991) showed that the absolute levels of the constitutively expressed Dpp varied widely between *E. coli* and *S. typhimurium*.

Peptide transport has been studied to a lesser extent in the fungi, but some progress has been made on the regulation of these systems. The oligopeptide transport system of *Neurospora crassa* has been shown to be constitutive (Wolfenbarger & Marzluf, 1975 ; Wolfenbarger, 1980). We have shown that the peptide transport system of *Saccharomyces cerevisiae* is regulated by micromolar amounts of amino acids (Island *et al.*, 1987 & 1991) in addition to repression by ammonium as a sole nitrogen source (Becker & Naider, 1977 & 1980 ; Moneton *et al.*, 1986 a & b ; Nisbet & Payne, 1979). Genetic analysis of peptide transport mutants of *S. cerevisiae* allowed us to define PTR1, PTR2 and PTR3 as

three components involved in the amino acid-inducible peptide transport system in this yeast (Island *et al.*, 1991).

Peptide conjugates have been used to deliver toxic moieties into *Candida albicans* via the peptide transport system (Borowski *et al.*, 1979 ; Kenig *et al.*, 1976 ; Kingsbury *et al.*, 1983 ; Lichliter *et al.*, 1976 ; Meyer Glauner *et al.*, 1982 ; Milewski *et al.*, 1991a). An understanding of the number of peptide permeases in *C. albicans* and their regulation under different physiological conditions is clearly important in order to design and deliver an efficacious, pathogen-specific drug via "illicit transport" (Fickel & Gilvarg, 1973 ; Ames *et al.*, 1973 ; Hammond *et al.*, 1987 ; Higgins, 1987) to this opportunistic pathogen. Previous investigations have concluded that the peptide transport system of *C. albicans* is regulated by nitrogen metabolism (Becker & Naider, 1977 & 1980 ; Davies, 1980 ; McCarthy *et al.*, 1985 a & b ; Naider & Becker, 1987 ; Yadan *et al.*, 1984). For example, peptide uptake is repressed by ammonium ions and stimulated when cells are grown on amino acids as the sole nitrogen source (Logan *et al.*, 1979). Recently, Payne *et al.* (1991) reported stimulation of dialanine transport in *C. albicans* B2630 grown in media containing bactopectone or trypticase peptone but not in casein hydrolysate. Physiological studies with mutants of *C. albicans* suggests the presence of multiple peptide permeases (McCarthy *et al.*, 1985 a & b ; Mehta *et al.*, 1984 ; Milewski *et al.*, 1988 ; Payne & Shallow, 1985 ; Yadan *et al.*, 1984) and no evidence has been presented on the differential regulations of these uptake systems.

Oxalysine [$\text{H}_2\text{NCH}(\text{CH}_2\text{OCH}_2\text{CH}_2\text{-NH}_2)\text{COOH}$], a lysine analog which can be produced chemically and biologically (from fermentation broth of several actinomycetes) is not toxic to mammalian cells and is known to cause growth inhibition of a number of bacteria (McCord *et al.*, 1957 ; Stapley *et al.*, 1967) and *C. albicans* (Zhang *et al.*, 1979). We report here on a homologous series of oxalysyl-(leucyl)_n-glycine peptides, where n was 1, 2, and 3, that were synthesized to test for activity against *C. albicans*. We

determined the effect of nitrogen sources and amino acids on the regulation of peptide transport using these model compounds. The studies addressed the question of multiplicity of peptide permeases and their regulation.

CHAPTER II

MATERIALS AND METHODS

Organism and growth conditions

Candida albicans H317, a well characterized (Riggsby *et al.*, 1982) clinical isolate obtained from the Centers for Disease Control (Atlanta, Georgia), was used in all experiments. The strain was maintained on YEPD agar containing (w/v) : yeast extract 1% ; Bactopeptone (Difco) 2% ; glucose 2% ; agar (Difco) 2%. The organism was transferred monthly to a fresh agar slant, incubated for 24 h at 37 °C and stored at 4 °C. Unless mentioned otherwise cells were grown overnight to exponential phase ($1-2 \times 10^7$ cells ml^{-1}) in minimal medium (MM) which was composed of yeast nitrogen base (Difco) without amino acids containing ammonium sulfate (5 mg ml^{-1}) and glucose (2%, w/v). Individual amino acids were supplemented as indicated at 0.15 mM unless stated otherwise. In some cases, the ammonium sulfate nitrogen source was replaced by either of the following ; allantoin (1 mg ml^{-1}), isoleucine (6.6 mg ml^{-1}), proline (4 mg ml^{-1}), peptone (20 mg ml^{-1}), trypticase peptone (20 mg ml^{-1} , BBL), or casein hydrolysate (20 mg ml^{-1} , Sigma). The amounts of allantoin, isoleucine, and proline used in the growth medium as the sole nitrogen source were approximately equivalent to the minimal amount required for growth.

Disk assays

Sensitivity to toxic peptides was measured by using disk assays as described by Island *et al.* (1987). Briefly, cells were grown overnight to exponential phase of growth in MM or other medium as described in the Tables, washed, and resuspended to 5×10^6 cells ml^{-1} in sterile water. One ml of this cell suspension was added to 3 ml molten Noble agar

(1.1%, w/v) and overlaid on 20 ml of the same medium used to prepare the inoculum or MM medium containing single amino acid supplements as indicated in the Tables. Oxalysine or an oxalysine-containing peptide at 0.25 μ moles unless indicated otherwise was spotted on a disk (6 mm diameter, Whatman 3MM filter paper) placed over a lawn of cells. Zones of inhibition were measured after 24-48 h incubation at 37 °C. The results represented in all Tables are averages of three independent assays. The results did not vary by more than two mm in each test. A zone of inhibition value of 7 mm represents a minimal growth inhibition value as the disk diameter is 6 mm.

Transport assays

Uptake of *L*-leucyl-*L*-[3 H]leucine was determined as described by Island *et al.* (1987). Briefly, *C. albicans* cells were grown overnight to logarithmic phase ($1-2 \times 10^7$ cells ml $^{-1}$) in MM. Cells were washed and resuspended to 2.5×10^6 cells ml $^{-1}$ in fresh MM medium with or without 0.15 mM *L*-lysine. After incubation for 4 hours, cells were harvested, washed and resuspended in glucose (1%, w/v) at 2×10^8 cells ml $^{-1}$. Five hundred microliters of cell suspension was added to an equal volume of reaction mixture and incubated at 37 °C. At various time points, 180 μ l portions were removed, placed on filters, washed and counted for radioactivity. The final concentration of components in the uptake assay was glucose (1%, w/v), 4 mM sodium citrate-potassium phosphate buffer (pH 5.5), and *L*-leucyl-*L*-[3 H]leucine (28 μ M, specific activity 20 mCi / mmole). Uptake is expressed as nanomoles per mg of cell protein.

Competition of [3 H]*L*-lysine transport by oxalysine was determined using an identical procedure as described for dileucine uptake. The final concentration of the components in the uptake assay was glucose (1%, w/v), 4 mM sodium citrate-potassium phosphate buffer (pH 5.5) containing [3 H] *L*-lysine (43 μ M, specific activity 0.6 mCi / mmole) and either no

oxalysine or a 10X, 100X or 1000X molar excess of oxalysine. The transport assays were done at least three times and the values did not differ by more than 10% of each other.

Chemicals and reagents

Oxalysine, Oxa-Leu and Oxa-Gly-Leu were a gift from Shanghai Institute of Materia Medica, Academia Sinica, Shanghai 20031, China. *L*-leucyl-*L*-[³H]leucine and oxalysine-containing peptides were synthesized by solution phase techniques (Becker & Naider 1977 ; Naider *et al.*, 1983). Briefly, α,ϵ dibenzyloxycarbonyl-*L*-oxalysine was coupled with (Leu)_n-Gly-OBzl (n = 0-3) using isobutylchloroformate in the presence of N-methylmorpholine. The protected peptides [Z-Oxa(Z)-Leu_n-Gly-OBzl (n = 0-3) where Z = benzyloxycarbonyl and OBzl = benzyl ester] were purified to homogeneity, characterized by NMR spectroscopy and the protecting groups were removed by transfer hydrogenation using Pd black and formic acid. The free peptides were > 95% homogeneous as judged by reversed phase HPLC and thin-layer chromatography. All amino acids used in the synthesis of peptides were the *L*-form.

CHAPTER III

RESULTS

Toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

Oxalysine and oxalysine-containing peptides inhibited the growth of *C. albicans* H317 in minimal medium (MM) containing ammonium sulfate as the nitrogen source (Table 1). Oxalysine-containing di-, and tripeptides were 2 to 4 times more toxic per mole of compound than oxalysine. Tetrapeptide and pentapeptide containing oxalysine were 16-fold more toxic than oxalysine. Oxa-Leu was also found to be two-fold more toxic than oxalysine when MIC was determined in MM liquid medium (data not shown).

Effect of nitrogen source on the toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

The nitrogen source in the growth medium has been shown to affect peptide transport in both *S. cerevisiae* and *C. albicans* (Becker & Naider, 1977 & 1980 ; Logan *et al.*, 1979 ; Nisbet & Payne, 1979 ; McCarthy *et al.*, 1985 a ; Moneton *et al.*, 1986 a & b; Payne *et al.*, 1985 & 1991 ; Yadan *et al.*, 1984). *C. albicans* can utilize various organic compounds, such as isoleucine, proline, allantoin, and peptone as a sole source for nitrogen. Disk sensitivity assays were performed to determine the effect of nitrogen source on the toxicity of oxalysine and oxalysine-containing peptides (Table 2). The largest zone of inhibition with oxalysine was observed with isoleucine as the sole nitrogen source. The zone of inhibition produced by oxalysine was greater in medium containing proline than in medium containing either ammonium sulfate or allantoin as the nitrogen sources. Oxalysine did not cause any growth inhibition of *C. albicans* in peptone containing medium.

TABLE 1. Toxicity of oxalysine and oxalysine-containing peptides against
C. albicans *

Compound	Minimum Inhibitory Amount § (μmoles/disk)
Oxalysine	0.125
Oxa-Leu	0.062
Oxa-Gly	0.031
Oxa-Gly-Leu	0.062
Oxa-Leu-Gly	0.031
Oxa-Leu-Leu-Gly	0.008
Oxa-Leu-Leu-Leu-Gly	0.008

* Disk sensitivity assay was done in minimal medium (MM).

§ Oxalysine or oxalysine-containing peptide from a serial two fold dilution series was spotted on a disk placed on a lawn of cells prepared as described in Methods. The least amount of the compound that showed anticandidal activity is given in the Table above.

TABLE 2. Toxicity of oxalysine and oxalysine-containing peptides against *C. albicans* : effect of nitrogen source and induction by *L*-Lysine

Assay medium*	Diameter (mm) zone of inhibition of plates containing †				
	O	OG	OLG	O(L) ₂ G	O(L) ₃ G
YNB w/ ammonium sulfate					
Without Lysine	12	12§	19	31	32
With Lysine	0	26	23	28	30
YNB w/ allantoin					
Without Lysine	12	29	26	27	27
With Lysine	0	27	24	23	26
YNB w/ proline					
Without Lysine	40	30	27	27	28
With Lysine	9§	26	19	21	22
YNB w/ isoleucine					
Without Lysine	50	45	38	36	37
With Lysine	0	25	23	26	26
YNB w/ peptone					
Without Lysine	0	25	22	0	0
With Lysine	0	25	22	0	0

*YNB w/ ammonium sulfate is MM as described in Methods, in the other media ammonium sulfate in MM was replaced with other nitrogen sources, YNB w/ allantoin is MM with allantoin (1 mg ml⁻¹) YNB w/ isoleucine is MM with isoleucine (6.6 mg ml⁻¹), YNB w/ proline is MM with proline (4 mg ml⁻¹), YNB w/ peptone is MM with peptone (20 mg ml⁻¹). *L*-Lysine was added to a final concentration of 0.15 mM where indicated.

†0.25 µmoles of oxalysine or oxalysine-containing peptide was spotted on a disk (diameter of disk is 6 mm). O = Oxalysine ; OG = Oxa-Gly ; OLG = Oxa-Leu-Gly ; O(L)₂G = Oxa-(Leu)₂-Gly ; O(L)₃G = Oxa-(Leu)₃-Gly.

§ Zone of inhibition was not clear.

Oxalysine-containing peptides showed maximum growth inhibition in medium with isoleucine as the sole nitrogen source. No significant difference in toxicity of oxalysine-containing peptides was apparent in medium with either allantoin or proline as the nitrogen source. Allantoin, an intermediate in the degradation of adenine and guanine (Cooper, 1982), is a non-repressive nitrogen source for peptide transport in *S. cerevisiae* as compared to ammonium sulfate (Island *et al.*, 1991). In contrast to other nitrogen sources oxalysine-containing di- and tripeptides were only weakly toxic in medium containing ammonium sulfate. Ammonium sulfate is known to be a repressive nitrogen source for peptide transport in *C. albicans* (Logan *et al.*, 1979). The influence of the nitrogen source on the toxicity of tetra- and pentapeptides was minor with both repressive and non-repressive nitrogen metabolites resulting in similar zones of inhibition. Only oxalysine-containing di-, and tripeptides showed toxicity in assay medium with peptone ; oxalysine-containing tetra- and pentapeptides were totally ineffective in peptone-containing medium.

Effect of *L*-lysine on the toxicity of oxalysine and oxalysine-containing peptides against *C. albicans* in medium with different nitrogen sources.

Previous studies with bacteria have shown that *L*-lysine reverses the growth inhibitory effect of oxalysine (McCord *et al.*, 1957 ; Stapley *et al.*, 1967). We found that *L*-lysine (0.15 mM) completely reversed the toxicity of oxalysine in all media tested (Table 2). With proline as a nitrogen source a hazy zone of inhibition was observed with *L*-lysine in the medium. In contrast to the result with oxalysine the effect of *L*-lysine on the toxicity of oxalysine-containing peptides was dependent on the nitrogen source. The presence of *L*-lysine (0.15 mM) increased the toxicity of oxalysine-containing di- and tripeptides in medium with ammonium sulfate as the nitrogen source ; toxicity of oxalysine-containing tetra- and pentapeptides remained unaffected or slightly depressed (Fig. 1). *L*-lysine

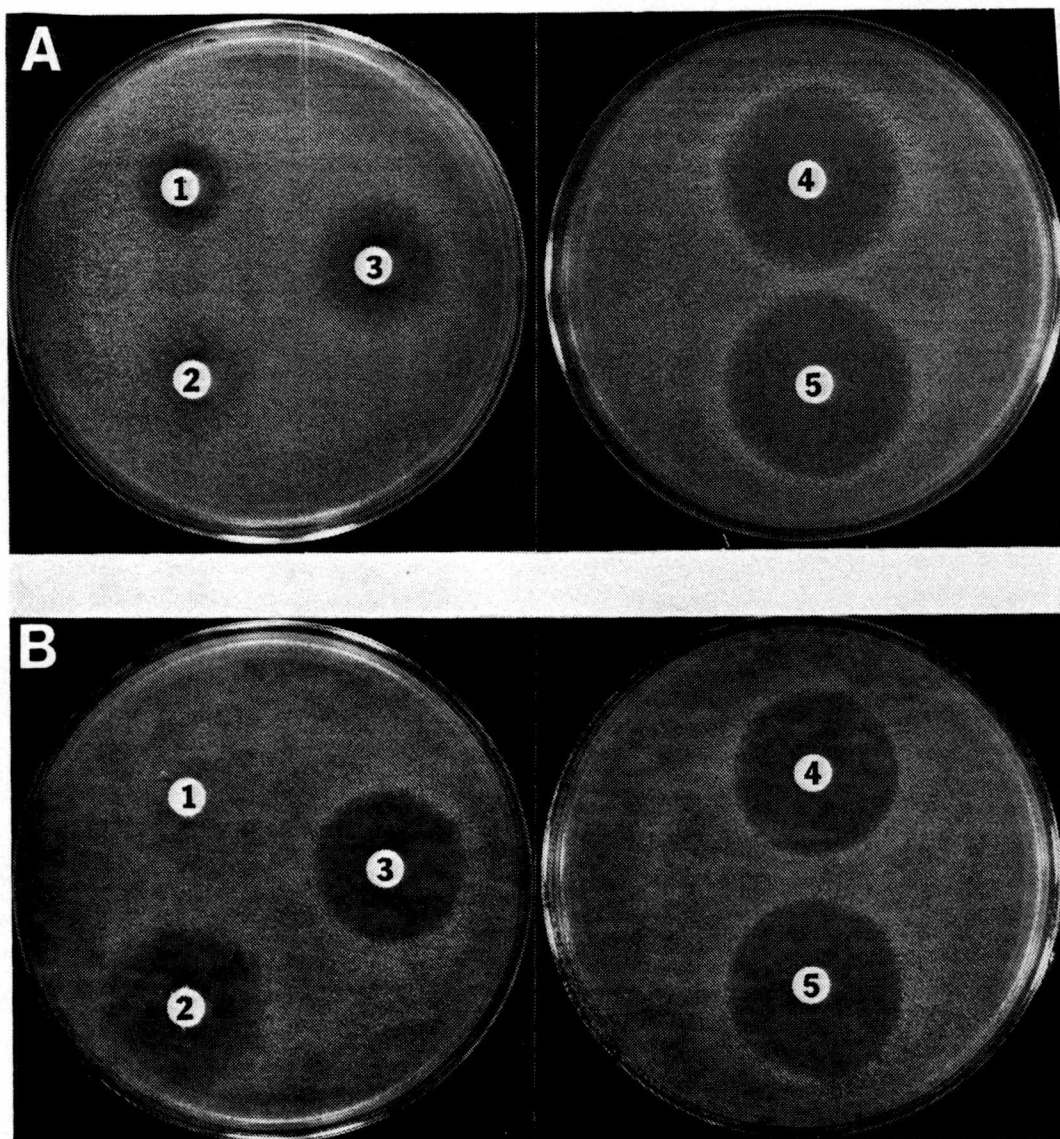


Fig. 1. Effect of *L*-lysine on toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*. Disk sensitivity assays were done in MM (A), and MM with 0.15 mM *L*-lysine (B) as described in Methods. Two hundred and fifty nmoles of (1) oxalysine, or an oxalysine-containing peptide, (2) Oxa-Gly, (3) Oxa-Leu-Gly, (4) Oxa-(Leu)₂-Gly and (5) Oxa-(Leu)₃-Gly were spotted on disks placed over a lawn of cells.

caused a slight decrease in the toxicity of oxalysine containing peptides in medium containing allantoin a slightly greater effect in medium containing proline and no effect was seen in the toxicity of Oxa-Gly or Oxa-Leu-Gly with peptone as the nitrogen source. In medium containing isoleucine as sole nitrogen source, however, *L*-lysine caused a marked reduction in the zones of inhibition caused by oxalysine-containing peptides.

Toxicity of oxalysine and oxalysine-containing peptides against *C. albicans* in the presence of amino acids

Studies from our laboratory (Island *et al.*, 1987 & 1991) showed that the peptide transport system of *S. cerevisiae* was induced by addition of several amino acids to the medium. Since ammonium sulfate was the only nitrogen source in which *L*-lysine increased the toxicity of oxalysine-containing di- and tripeptides (Table 2), we determined the effect of addition of each of 20 amino acids (0.15 mM) on the toxicity of oxalysine and oxalysine-containing peptides against *C. albicans* in MM. Several amino acids such as arginine, histidine, glutamine, aspartic acid, lysine, and serine increased the toxicity of oxalysine-containing di- and tripeptides against *C. albicans* with little or no effect on oxalysine-containing tetra- or pentapeptide toxicity (Table 3). The amino acids not listed in the table (alanine, asparagine, cysteine, glutamic acid, glycine, isoleucine, leucine, phenylalanine, threonine, tyrosine, valine, methionine, proline and tryptophan) did not significantly increase or decrease the growth inhibition by oxalysine or oxalysine-containing peptides. *L*-lysine completely reversed the toxicity of oxalysine for *C. albicans*, whereas the other amino acids had little or no effect on oxalysine toxicity. Using disk assays with different amounts of *L*-lysine on disks placed adjacent to oxalysine-containing disks it was found that the amino acid caused a dose dependent increase in toxicity of Oxa-Gly with no increase in the toxicity of Oxa-(Leu)₂-Gly (Fig. 2). As little as 25 nmoles/disk of *L*-lysine stimulated toxicity of Oxa-Gly.

TABLE 3. Effect of amino acid addition on toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

Amino acid added *	Diameter (mm) zone of inhibition of plates containing †				
	O	OG	OLG	O(L) ₂ G	O(L) ₃ G
None	14	12 [§]	20	31	32
Arginine	9	34	31	30	32
Lysine	0	26	25	28	30
Histidine	13	24	25	29	30
Glutamine	12	35	29	29	30
Aspartic acid	16	31	27	29	28
Serine	11	26	25	29	28

* The effect of addition of each of 20 amino acids (0.15mM) was determined in MM. The amino acids not listed in the table, (Ala, Asn, Cys, Glu, Gly, Ile, Leu, Phe, Thr, Tyr, Val, Met, Pro and Trp), had no significant effect on growth inhibition by oxalysine or oxalysine-containing peptides.

† 0.25 µmoles of oxalysine or oxalysine-containing peptide was spotted on a disk (diameter of disk is 6 mm). O = Oxalysine ; OG = Oxa-Gly ; OLG = Oxa-Leu-Gly ; O(L)₂G = Oxa-(Leu)₂-Gly ; O(L)₃G = Oxa-(Leu)₃-Gly.

§ Zone of inhibition was not clear.

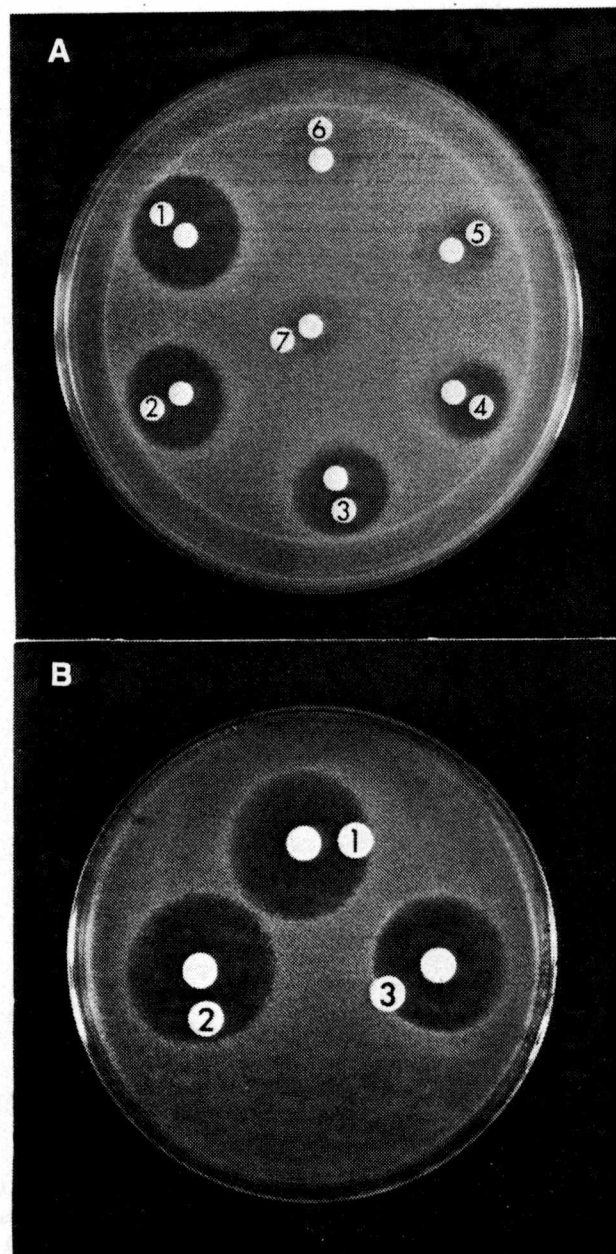


Fig. 2. Effect of *L*-lysine on the toxicity of Oxa-Gly and Oxa-(Leu)₂-Gly against *C. albicans*. Disk sensitivity assays were performed in MM as described in Methods. (A) Oxa-Gly (0.25 μmoles) was spotted on disks and adjacent to each disk was placed another disk containing different amounts of *L*-lysine, (1) 50 μmoles, (2) 25 μmoles, (3) 2.5 μmoles, (4) 0.25 μmoles, (5) 0.025 μmoles, (6) 0.0025 μmoles or (7) none. (B) Oxa-(Leu)₂-Gly (0.25 μmoles) was spotted on a disk and adjacent to each disk was placed another disk containing different amounts of *L*-lysine, (1) 2.5 μmoles, (2) 0.062 μmoles or (3) none.

Effect of *L*-lysine and *D*-lysine on the toxicity of oxalysine against *C. albicans*

Disk sensitivity assays were done in MM using different amounts of *L*-lysine or *D*-lysine on disks placed adjacent to a disk containing oxalysine (250 nmoles) to determine the least amount of *L*-lysine or *D*-lysine needed to reverse oxalysine toxicity. As little as 1.25 nmoles of *L*-lysine was sufficient to reverse the toxicity of oxalysine (Table 4, Fig. 3). A 2000-fold greater amount of *D*-lysine (2.5×10^3 nmoles) than *L*-lysine (1.25 nmoles) was required to cause the same extent of reversal of oxalysine toxicity. A small contamination (0.05%) of the *D*-lysine solution by *L*-lysine could have resulted in the apparent reversal of oxalysine toxicity in this experiment.

To further investigate the effect of *L*-lysine on the toxicity of oxalysine, we did transport competition studies of [^3H]*L*-lysine in the presence and absence of oxalysine (Fig. 4). Oxalysine at a 100- and 1000X molar excess competed with *L*-lysine for transport into the cells suggesting that the reversal of oxalysine toxicity by *L*-lysine may be due to competition for the amino acid permease.

Transport of *L*-leucyl-*L*-[^3H]leucine in *C. albicans* in the presence of *L*-lysine

We tested whether the increased sensitivity to oxalysine-containing di- and tripeptides in MM containing *L*-lysine (Table 2) could have been due to an increased transport of these peptides. Results in Fig. 5 showed that the dipeptide Leu-Leu reversed the zone of inhibition produced by Oxa-Gly, whereas Leu-Leu had no effect on the zone of inhibition produced by Oxa-(Leu)₂-Gly. These data indicated that dileucine and Oxa-Gly, but not Oxa-(Leu)₂-Gly competed with each other for transport. Direct assay of uptake of *L*-leucyl-*L*-[^3H] leucine into *C. albicans* showed that incubation of cells with *L*-lysine for 4 h resulted in a 15-fold increase in the rate of transport of the dipeptide (Fig. 6). Since Leu-Leu and Oxa-Gly share the same transport system it is reasonable to conclude that the

TABLE 4. Effect of lysine on the toxicity of oxalysine against *C. albicans* *

Amount of lysine on the disk † (μmoles)	Effect on oxalysine toxicity §	
	<i>L</i> -lysine	<i>D</i> -lysine
25.0	+	+
2.5	+	+
0.25	+	-
0.025	+	-
0.0025	+	-
0.00125	+	-
0.00025	-	-
None	-	-

* Disk sensitivity assays were done in MM as described in Methods.

† Different amounts (μmoles) of either *L*- or *D*-lysine were spotted on disks placed adjacent to disks containing 0.25 μmoles oxalysine.

§ + = reversal of oxalysine toxicity.

- = no effect.

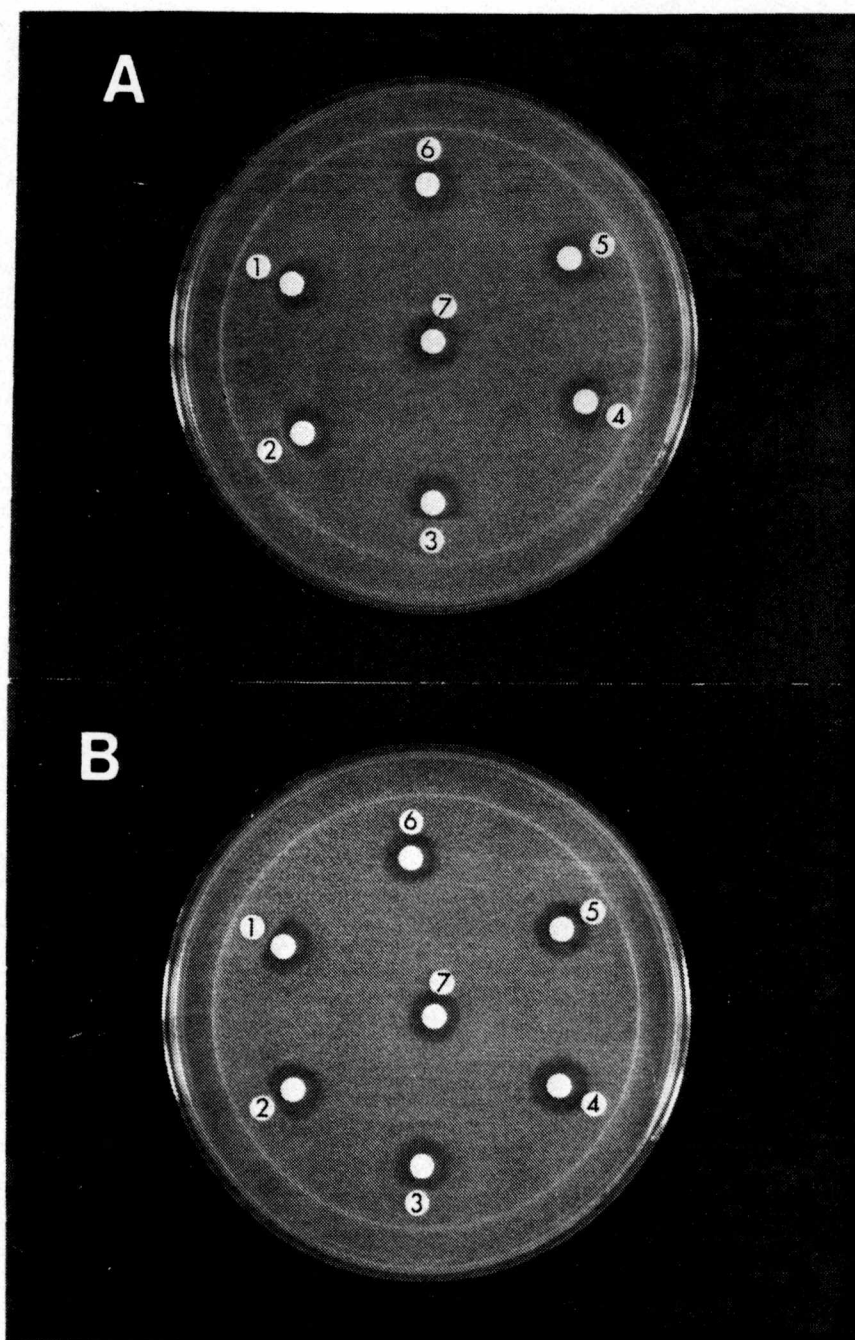


Fig. 3. Effect of lysine on the toxicity of oxalysine against *C. albicans*. Disk sensitivity assays were done in MM as described in Methods. Oxalysine (0.25 μ moles) was spotted on a disk and adjacent to each disk was placed another disk containing different molar amounts of either *L*-lysine (A), or *D*-lysine (B) ; (1) 0.25 μ moles, (2) 2.5 μ moles, (3) 0.25 μ moles, (4) 0.025 μ moles, (5) 0.0025 μ moles, (6) 0.00125 μ moles, or (7) none.

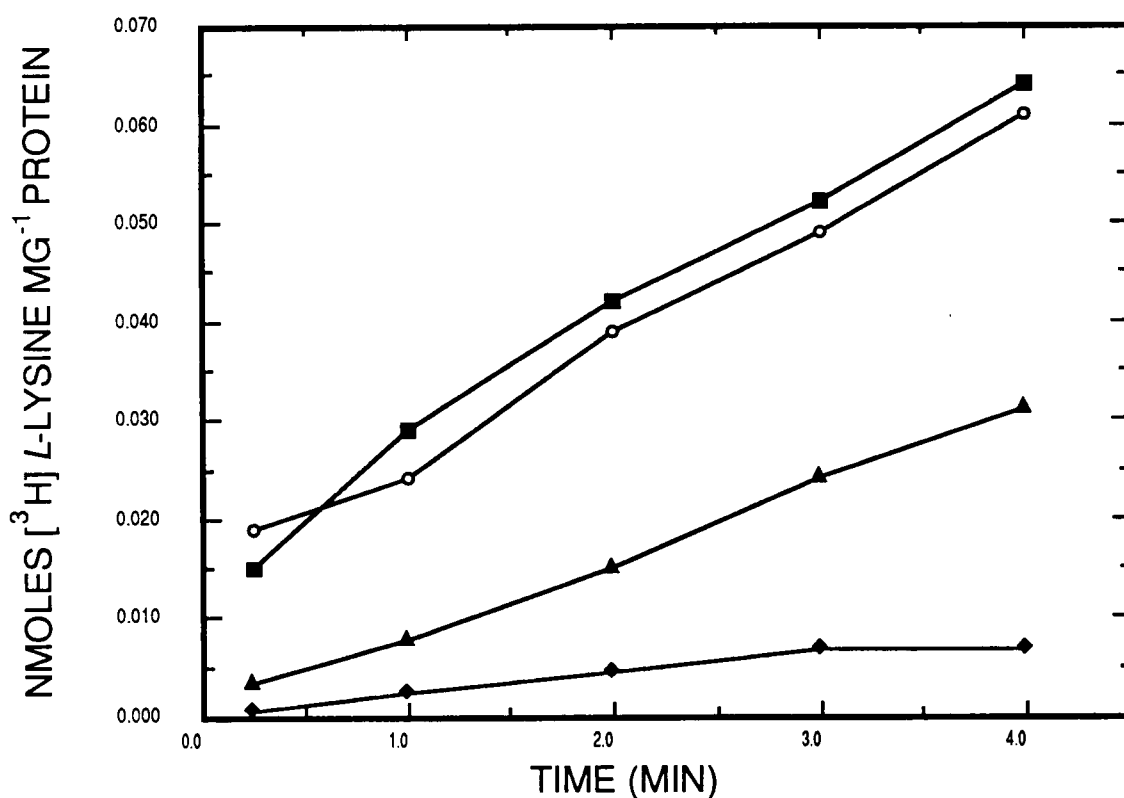


Fig. 4. Transport competition of $[^3\text{H}]$ L-lysine by oxalysine in *C. albicans*. *C. albicans* cells grown overnight in MM were used to determine transport competition of $[^3\text{H}]$ L-lysine ($43\ \mu\text{M}$, specific activity $0.6\ \text{mCi/mmol}$) by oxalysine at a 10X molar excess (■), 100X molar excess (▲), 1000X molar excess (◆) or control with no oxalysine (○) as described in Methods. Five hundred microliters of cell suspension ($2 \times 10^8\ \text{cells ml}^{-1}$) was added to an equal volume of reaction mixture and incubated at $37\ ^\circ\text{C}$. At various time points $180\ \mu\text{l}$ portions were removed, placed on filters, washed and counted for radioactivity.

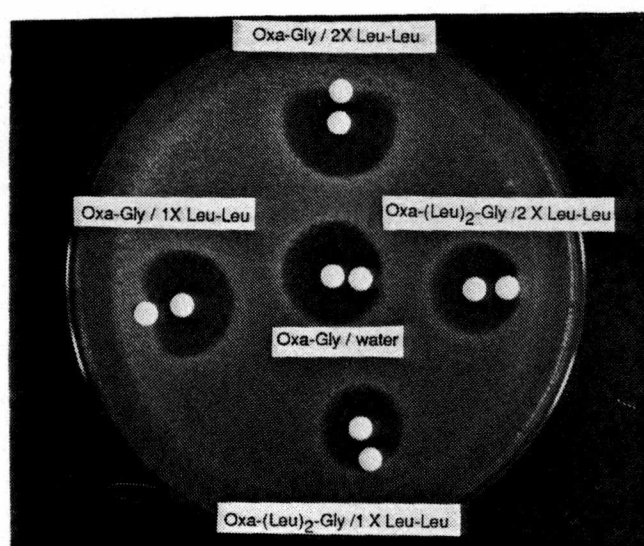


Fig. 5. Effect of Leu-Leu on the toxicity of Oxa-Gly and Oxa-(Leu)₂-Gly against *C. albicans*. Disk sensitivity assays were done in MM with 0.15 mM *L*-lysine as described in Methods. Oxa-Gly or Oxa-(Leu)₂-Gly (0.25 μ moles of each) was spotted on a disk and adjacent to each disk was placed another disk containing different molar amounts of Leu-Leu (1X Leu-Leu = 0.25 μ moles ; 2X Leu-Leu = 0.5 μ moles).

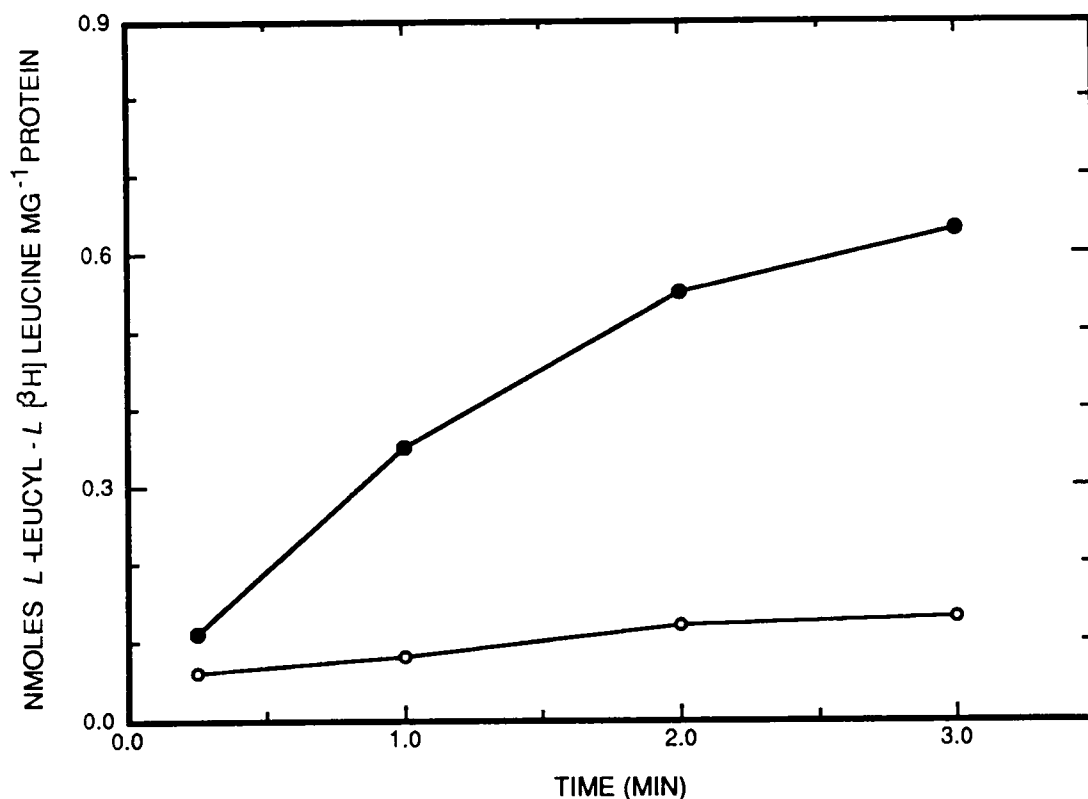


Fig. 6. Transport of *L*-leucyl-*L*-[³H]leucine in *C. albicans* in the presence of *L*-lysine. *C. albicans* cells grown overnight in MM were harvested in logarithmic phase ($1-2 \times 10^7$ cells ml^{-1}), washed and resuspended to 2.5×10^6 cells ml^{-1} in fresh medium with *L*-lysine (0.15 mM) (●), or without *L*-lysine (○). After incubation for 4 h, cells were harvested, washed and resuspended in fresh medium to 2×10^8 cells ml^{-1} . Five hundred microliters of the cell suspension was added to an equal volume of reaction mixture containing *L*-leucyl-*L*-[³H]leucine (28 μM , specific activity 20 mCi/mmol) as described in Methods. At various time points 180 μl portions were removed, placed on filters, washed and counted for radioactivity.

increased toxicity of Oxa-Gly in the presence of *L*-lysine is due to increased transport of this dipeptide.

Effect of ammonium sulfate, casein hydrolysate, trypticase peptone and peptone on the toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

We performed disk sensitivity assays to determine the toxicity of oxalysine and oxalysine-containing peptides in medium containing ammonium sulfate, casein hydrolysate, trypticase peptone (enzymatically hydrolysed peptone) and peptone (Table 5). Payne *et al.* (1991) have reported that bacto-peptone and trypticase peptone, but not casein hydrolysate, stimulated dialanine transport in *C. albicans* B2630. We observed that oxalysine-containing peptides did not cause growth inhibition of *C. albicans* H317 in medium containing casein hydrolysate. Only oxalysine-containing di- and tripeptides showed toxicity in medium containing trypticase peptone or peptone; oxalysine-containing tetra- and pentapeptides showed no growth inhibition. Oxalysine did not cause growth inhibition in media containing casein hydrolysate, peptone, or trypticase peptone. We observed that peptone could overcome the repression of dipeptide toxicity by ammonium.

Effect of amino acids on growth inhibition by nikkomycin in *C. albicans*

We tested if the amino acids that increased the toxicity of oxalysine-containing di- and tripeptides in MM had any effect on the toxicity of peptidyl nucleoside nikkomycin X + Z (Table 6). The peptidyl nucleosides enter *C. albicans* via the peptide transport system (Decker *et al.*, 1991 ; Krainer *et al.*, 1991 ; McCarthy *et al.*, 1985 a ; Yadan *et al.*, 1984). Several amino acids such as arginine, lysine, glutamine, and aspartic acid overcame repression of peptide transport by ammonium sulfate and resulted in marked growth inhibition by nikkomycin X + Z (20 µg/disc). The amino acids which had no effect on

TABLE 5. Effect of ammonium sulfate, casein hydrolysate, trypticase peptone and peptone on toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

Assay medium *	Diameter (mm) zone of inhibition of plates containing †				
	O	OG	OLG	O(L) ₂ G	O(L) ₃ G
YNB w/ (NH ₄) ₂ SO ₄	12	12 [§]	19	31	32
YNB w/ casein hydrolysate	0	0	0	0	0
YNB w/ trypticase peptone	0	11	8	0	0
YNB w/ peptone	0	25	22	0	0
YNB w/ peptone + (NH ₄) ₂ SO ₄	0	24	17	0	0

* YNB is yeast nitrogen base without any nitrogen source. Different nitrogen supplementation was added as (NH₄)₂SO₄ (0.5%, w/v), casein hydrolysate (2%, w/v), trypticase peptone (2%, w/v) or peptone (2%, w/v).

† 0.25 µmoles of oxalysine or oxalysine-containing peptide was spotted on a disk (diameter of disk is 6 mm). O = Oxalysine ; OG = Oxa-Gly ; OLG = Oxa-Leu-Gly ; O(L)₂G = Oxa-(Leu)₂-Gly ; O(L)₃G = Oxa-(Leu)₃-Gly.

§ Zone of inhibition was not clear.

TABLE 6. Effect of amino acid addition on toxicity of nikkomycin X + Z
against *C. albicans*

Amino acid added *	Diameter (mm) zone of inhibition [†]
None	0
Arginine	32
Lysine	17
Glutamine	27
Aspartic acid	28

* The effect of addition of various amino acids was determined in MM. The amino acids Ala, Asn, Cys, Glu, Gly, Ile, Leu, Phe, Thr, Tyr, Val, Met, Pro and Trp had no significant effect on growth inhibition by nikkomycin X + Z.

[†] Nikkomycin X + Z (20 µg) was spotted on a disk (diameter of disk is 6 mm).

oxalysine-containing peptide toxicity (Table 3) also did not reverse ammonium repression of nikkomycin toxicity.

CHAPTER IV

DISCUSSION

The data presented in this report indicate that oxalysine and oxalysine-containing peptides inhibit the growth of *C. albicans* H317. The nitrogen source in the medium markedly influenced the toxicity of these compounds. Zones of inhibition were larger for oxalysine-containing peptides with isoleucine and proline as the nitrogen sources as compared to the zones observed with ammonium sulfate as the nitrogen source. Increased zones of inhibition with proline as the nitrogen source could be due to increased transport of peptides : *S. cerevisiae* grown in proline as the nitrogen source transported peptides at about 10 times the rate of ammonia-grown organisms (Becker & Naider, 1977 ; Moneton *et al.* 1986 b ; Nisbet & Payne, 1979), and *C. albicans* grown in isoleucine as the nitrogen source had six to eight times the peptide transport activity (trimethionine uptake) as compared to cells grown on ammonium chloride as the nitrogen source (Logan *et al.*, 1979). Toxicity of polyoxins which are transported by the peptide transport system have been shown to be dependent on the nitrogen source (Becker *et al.*, 1983 ; Mehta *et al.*, 1984). It is clear that general nitrogen metabolism can have an important influence on the efficacy of peptide drugs.

We found that in addition to the regulation of toxicity by the nitrogen source for growth, micromolar amounts of certain amino acids can control peptide transport in *C. albicans*. Thus, addition of *L*-lysine or several other amino acids overcame repression by ammonium and increased toxicity to oxalysine-containing di- and tripeptides. A similar observation of induction of dipeptide transport in minimal medium by several amino acids has been made in *S. cerevisiae* (Island *et al.*, 1987 & 1991). At the present time, we are unable to explain why *L*-lysine (0.15 mM) caused a marked reduction in the zones of

inhibition with oxalysine-containing peptides in medium containing proline or isoleucine as the nitrogen source. Lack of effect of *L*-lysine or other amino acids on the toxicity of oxalysine-containing tetra- or pentapeptides suggests differential regulation of the permeases that transport these peptides or that these peptides may enter *C. albicans* via a process other than a peptide transport system. Current evidence, though not conclusive, indicates that *C. albicans* has at least two peptide permeases, one specific for di- and tripeptides and the other for tri- and oligopeptides (McCarthy *et al.*, 1985 a & b ; Mehta *et al.*, 1984 ; Milewski *et al.*, 1988 ; Payne & Shallow, 1985 ; Shallow *et al.*, 1991 ; Yadan *et al.*, 1984). Our results are consistent with the notion of at least two peptide permeases in *C. albicans* that are operative even under noninducing conditions. The oxalysine-containing tetra- and pentapeptides may also be able to enter cells by fluid phase endocytosis. Fluid phase endocytosis is operative in *C. albicans* (Basrai *et al.*, 1990) and we have observed that Lucifer Yellow (LY), the marker for fluid phase endocytosis, does not accumulate in cells in the presence of peptone apparently due to rapid exocytosis of the internalized LY. Exocytosis of oxalysine-containing tetra- and pentapeptides in peptone containing medium may prevent the peptides from reaching an intracellular level required to cause growth inhibition.

Oxalysine-containing di- and tripeptides caused growth inhibition of *C. albicans* H317 in medium with trypticase peptone or peptone but not in medium containing casein hydrolysate. We also observed that bacto-peptone overcomes repression of peptide transport by ammonium ions. These results are consistent with the stimulation of dialanine transport in *C. albicans* B2630 grown in medium containing bacto-peptone or trypticase peptone but not in casein acid hydrolysate (Payne *et al.*, 1991). However, casein acid hydrolysate and bacto-peptone contain a heterogeneous mixture of amino acids and peptides, respectively, and it is difficult to ascribe any effects to individual components using these complex nitrogen sources. Additionally, Payne *et al.* (1991) have shown that

bactopeptone and not proline can override ammonium repression of dialanine transport. Hence, the authors concluded that peptides and not amino acids override ammonium repression of peptide transport. We systematically analyzed the effect of 20 amino acids on growth inhibition of *C. albicans* using oxalysine-containing peptides and our results suggest that specific amino acids overcome repression by ammonium ions and cause induction of di- and tripeptide transport system(s) of *C. albicans*.

The increased sensitivity of *C. albicans* toward oxalysine-containing di- and tripeptides in the presence of *L*-lysine and other amino acids may be due to several reasons : (a) The rate of hydrolysis of the peptide intracellularly could be increased, (b) The intracellular target of the drug could have altered sensitivity in the presence of amino acids, or (c) The uptake characteristics of the peptide could be affected in the presence of amino acids. The first possibility of increased hydrolysis of the peptide in the presence of certain amino acids may be ruled out since amino acids increase sensitivity of *C. albicans* to both oxalysine-containing peptides as well as nikkomycin X + Z. The peptidyl nucleoside nikkomycin X + Z inhibits chitin synthetase activity in an intact form and is resistant to peptidase cleavage (Becker *et al.*, 1983 ; Decker *et al.*, 1991 ; Krainer *et al.*, 1991 ; Milewski *et al.*, 1991b). The second possibility of alteration of the intracellular target by certain amino acids is unlikely based on the different targets of action of the two anticandidal agents ; nikkomycin inhibits chitin synthetase, whereas oxalysine inhibits RNA synthesis (Zheng *et al.*, 1992). The increased transport of radiolabeled dileucine in the presence of *L*-lysine (0.15 mM) supports *L*-lysine mediated increase in transport. Differences in sensitivity of *S. cerevisiae* to toxic dipeptides in the presence of different nitrogen sources was also reported to be due to changes in peptide transport (Moneton *et al.*, 1986 a).

L-lysine completely reversed the toxicity of oxalysine and increased the toxicity of oxalysine-containing di- and tripeptides in MM against *C. albicans*. These data confirm previous conclusions for distinct amino acid and peptide transport systems in *C. albicans*.

They also support the argument that the peptides are not hydrolyzed extracellularly. If this were the case the released oxalysine should be competed for by *L*-lysine. As seen by the data in Table 2 this is clearly not the case.

Lysine transport in *C. albicans* is mediated by two systems ; one system is highly specific and the other is less specific and has a broad specificity (Rao *et al.*, 1986). It is possible that more oxalysine enters cells grown in the presence of proline and this may explain why a slight zone of inhibition is seen even in the presence of lysine in medium containing proline as the nitrogen source. Reversal of oxalysine toxicity by lysine was specific to *L*-lysine and the reversal observed with *D*-lysine could have been due to a very minor contamination of *L*-lysine in the *D*-lysine preparation. A partial reversal of oxalysine toxicity by high concentrations of *D*-lysine was also seen by Stapley *et al.* (1967) in studies with *E. coli* and the authors concluded this to be due to contamination of *L*-lysine in the *D*-lysine preparation.

Growth inhibition of *C. albicans* with oxalysine-containing peptides coupled with the fact that oxalysine is not toxic to mammalian cells may allow design of a pathogen specific drug to be delivered by "illicit transport" (Fickel & Gilvarg, 1973 ; Ames *et al.*, 1973 ; Hammond *et al.*, 1987 ; Higgins, 1987) via the peptide transport system. Oxalysine-containing tripeptides could be used as studies with *C. albicans* suggest that tripeptides can be transported either by the di- and/or tripeptide permease (McCarthy *et al.*, 1985 a & b ; Mehta *et al.*, 1984 ; Milewski *et al.*, 1988 ; Payne & Shallow, 1985 ; Shallow *et al.*, 1991 ; Yadan *et al.*, 1984) and the likelihood of encountering a mutant for both the permeases at the same time seems highly unlikely. Peptide transport has been used in *C. albicans* to transport poorly permeable toxic amino acids in the form of peptide conjugates (Andruskiewicz *et al.*, 1990 ; Borowski *et al.*, 1979 ; Kingsbury *et al.*, 1983 ; Lichliter *et al.*, 1976 ; Meyer Glauner *et al.*, 1982 ; Milewski *et al.*, 1991a). Also, some anticandidal agents such as polyoxins and nikkomycins which are peptidyl nucleoside

chitin synthetase inhibitors enter *C. albicans* through the peptide transport system (Decker *et al.*, 1991 ; Krainer *et al.*, 1991 ; McCarthy *et al.*, 1985 a & b ; Mehta *et al.*, 1984 ; Milewski *et al.*, 1991b ; Yadan *et al.*, 1984). Our results on regulation of peptide transport in *C. albicans* indicate that in such drug testing and screening particular attention must be given to medium composition.

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PART III

INTERNALIZATION OF LUCIFER YELLOW IN *CANDIDA ALBICANS* BY FLUID PHASE ENDOCYTOSIS *

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Journal of General Microbiology **136**, 1059-1065.

CHAPTER I

INTRODUCTION

Cells internalize their own plasma membrane and macromolecules from the environment by a mechanism termed endocytosis (reviewed by Silverstein *et al.*, 1977 ; Steinman *et al.*, 1983). It is now generally accepted that endocytosis brings about the bidirectional flow of plasma membrane into and out of the cell. There are a wide array of substances that can be internalized by this process such as nutrients, toxins, and effector molecules (growth factors, hormones, antibodies and enzymes). Two general types of endocytosis are recognized, namely, fluid phase (Silverstein *et al.*, 1977) and receptor mediated endocytosis (Goldstein *et al.*, 1979). Both processes are time-, temperature-, and energy-dependent. In fluid phase endocytosis, molecules dissolved in the extracellular medium are incorporated by bulk transport into endocytic vesicles and uptake of the ligand is not saturable. However, in receptor mediated endocytosis the ligand to be internalized binds to the cell membrane before it is incorporated into endocytic vesicles and uptake of the ligand is saturable.

Recent studies show that *Saccharomyces cerevisiae* can endocytose mating pheromones and lucifer yellow (LY) from the culture medium (Riezman, 1985). These results raise the possibility that other yeasts may also possess this type of uptake mechanism. These findings and our interest in peptide transport and drug design in *Candida albicans* (Naider & Becker, 1987) prompted us to investigate whether a similar process is operative in this dimorphic fungus, an important opportunistic pathogen (reviewed by Odds, 1988).

Earlier studies on the porosity of the yeast cell wall had been done using polyethylene glycols of different molecular sizes (Scherrer *et al.*, 1974 ; Cope, 1980) and the threshold of permeability was estimated to correspond to a molecular weight of 620 and an Einstein-

Stokes hydrodynamic radius of 0.81 nm. However, recent reports on the passage of molecules through the yeast cell wall suggest that molecules with a mass as great as 200,000 to 400,000 diffuse through the intact yeast cells walls freely (De Nobel *et al.*, 1990 ; De Nobel & Barnett, 1991). Several large molecules enter and kill *C. albicans*. The best studied examples of such molecules are defensins (mol. wt 3,500 -4000) which are variably cationic, cysteine-rich antimicrobial peptides (29-34 residues in length) and are major constituents of azurophil granules of polymorphonuclear neutrophils (Lehrer *et al.*, 1985). They are active *in vitro* against several microorganisms, including certain fungi (Selsted *et al.*, 1985). The susceptibility of *C. albicans* to human defensins depends both on the ionic environment and on the metabolic state of the target cell (Lehrer *et al.*, 1988). The mechanism of action of defensins is not clear, although it has been proposed that they act through membrane perturbation (Selsted *et al.*, 1985). Killer toxins (mol. wt 15,000-25,000), secreted into the culture medium from selected killer yeasts, cause lysis of *C. albicans* (Polonelli *et al.*, 1985). Yphantis *et al.* (1967) reported that certain cationic proteins such as ribonuclease, protamine, bovine serum albumin, cytochrome *c*, and myoglobin cause lysis of *S. cerevisiae* and *C. utilis*. All these studies indicate that these proteins may penetrate the cell wall to act directly on the cell by an undescribed mechanism.

In this paper, we report on the characteristics of uptake of LY by the yeast and mold phases of *C. albicans* using fluorescence microscopy and fluorescence spectroscopy. LY, an impermeable fluorescent dye, has been used as a marker for fluid phase endocytosis in mammalian cells and *S. cerevisiae* (Swanson *et al.*, 1985 ; Riezman, 1985). This dye offers several advantages for use as a marker for fluid phase endocytosis (Miller *et al.*, 1983 ; Stewart, 1978 & 1981) : a very high quantum yield resulting in easily visualized fluorescence after uptake by cells ; a small size which allows passage through the yeast cell wall ; stability over a wide pH range of 2.0 to 10.0 ; a negative charge at all pH values above 2.0 which prevents diffusion across the cell membrane ; it is not toxic to cells ; and

fluorescence is not quenchable by yeast cell extracts (Riezman, 1985). Our results suggest that LY is internalized by *C. albicans* by a non-saturable and time-, temperature-, and energy-dependent process consistent with the characteristics of fluid phase endocytosis. These studies may open research possibilities to a new pathway for entry of molecules into the yeast cell.

CHAPTER II

MATERIALS AND METHODS

Organism and growth conditions

Candida albicans H317, a well characterized (Riggsby *et al.*, 1982) clinical isolate obtained from the Centers for Disease Control in Atlanta, Georgia, was used in all experiments. The strain was maintained on YEPD agar containing (w/v) : yeast extract 1% ; bactopectone [Difco] 2% ; glucose 2% ; agar 2%. The organism was transferred monthly to a fresh slant, incubated at 37 °C for 24 h and stored at 4 °C.

For all experiments, unless mentioned otherwise, the yeast phase of growth was obtained in YEPD medium. Cells were inoculated at an initial cell density of 10^4 cells ml⁻¹ in 200 ml YEPD broth and incubated at 37 °C on a rotary shaker at 200 rpm. An exponentially growing culture at a cell density of $1-2 \times 10^7$ cells ml⁻¹ was used for all experiments. Cells were harvested by centrifugation at 1500 x g for 5 minutes, washed once with distilled water and twice with 50 mM potassium phosphate buffer, pH 6.0, and resuspended in phosphate buffer.

The mold phase of growth was obtained using two procedures, one a temperature shift and the other a nutritional shift. For the temperature shift procedure we used the amino acid synthetic medium (ASM) described by Lee *et al.* (1975) and the procedure of Logan *et al.* (1983). Briefly, yeast phase cells were first grown to stationary phase in ASM, pH 6.8, at 25 °C and stored at 4 °C for 12 h. Yeast phase cultures were obtained by diluting stationary phase cultures into fresh ASM medium at 25 °C. Conversion to the mold phase was initiated by diluting (1:20) the stationary phase culture into fresh ASM medium at 37 °C. The mold phase cultures (>90% conversion) were harvested 12 h after the initiation of conversion. For nutritional shift conversion (Joshi *et al.*, 1973), yeast cells

were grown on Sabouraud dextrose agar for 48 h at 37 °C. Cells were scraped from the agar and resuspended in water at 1×10^6 cells ml^{-1} , then inoculated into prewarmed (37 °C) 1% (w/v) bactopectone. The mold phase was obtained after 4-5 h at a frequency of 50-60%.

Visualization of cell-associated lucifer yellow

The procedure for the assay was similar to that described previously for *S. cerevisiae* (Riezman, 1985) with a few variations. *C. albicans* was grown overnight as described above. The assay was performed with 2×10^7 cells ml^{-1} in 50 mM potassium phosphate buffer, pH 6.0. Glucose or other additions were made to the cell suspension and, after 15 minutes at 30 °C, lucifer yellow CH (LY) (Molecular Probes, Eugene, Oregon) was added to a final concentration of 4 mg ml^{-1} . After the indicated time of incubation cells were diluted with 5 ml of ice cold Buffer A (50 mM succinic acid/NaOH, pH 5.0, containing 100 mM NaCl, 10 mM MgCl_2 , 20 mM NaN_3), collected by vacuum filtration on nitrocellulose filters (GN-6, 0.46 μm , Gelman Sciences Inc., Ann Arbor, Michigan), washed four times with 2 ml Buffer A, and once with 2 ml of 50 mM Tris-Cl, pH 7.25. The cells were eluted off the filter with the Tris-Cl buffer and the suspension was kept on ice. For visualization of cell-associated LY, the cells were mounted on a slide precoated with 20 μl of 1 mg ml^{-1} concanavalin A, which immobilized the yeast cells. Fluorescence was observed with a Leitz Orthoplan microscope with a xenon mercury lamp equipped with epifluorescence using an excitation filter BP 450-490, a beam splitting mirror TK 495 and a suppression filter K530. Photographs were taken using Ektachrome 200 ASA with exposure times of 50-60 seconds.

Quantitation of cell-associated lucifer yellow

For quantitation of cell-associated LY, cells were incubated in assay medium containing 2% (w/v) glucose and various concentrations of LY in 50 mM potassium phosphate buffer, pH 6.0. A portion of the cell suspension was removed at various time points and the cells were washed as described above. Appropriate dilutions of the cells were used to quantitate cell-associated LY using a Luminescence spectrometer Model LS-5B (Perkin Elmer, Norwalk, Connecticut), with excitation at 426 nm and emission at 535 nm (slit widths 10 and 20 nm, respectively) ; a standard curve was produced using known amounts of LY. Hemocytometer counts of each sample were determined to account for non-uniform elution of cells from the nitrocellulose filters. Each experiment was repeated at least three times with values not differing by more than 10% of each other.

Exocytosis of lucifer yellow

C. albicans cells grown in YEPD medium were harvested in the exponential phase, washed, incubated in assay medium containing 2% (w/v) glucose and LY (1 mg ml^{-1}) in 50 mM potassium phosphate buffer, pH 6.0, at 30 °C for 90 minutes, collected and washed on filters as described above, and eluted from filters into phosphate buffer. Cells were then reincubated either in potassium phosphate buffer or in 2% (w/v) peptone at 30 °C. A portion of the cell suspension was removed at various time points and centrifuged. Fluorescence associated with the cells and in the supernatant was quantitated as described above.

Column Chromatography

Column chromatography of LY and a mixture of LY and peptone was done using Sephadex G-25 (dry bead diameter 50-150 μm). The column (1.5 x 8 cm) was equilibrated with 50 mM potassium phosphate buffer, pH 6.0. The downward flow

elution rate during the packing was controlled by a peristaltic pump to give a flow rate of 1 ml min⁻¹. Two hundred microliters of a sample containing LY (37.5 µg ml⁻¹) was applied to the column, 1 ml fractions were collected and fluorescence was determined using the fluorescence spectrometer. Two hundred microliters of a second sample containing LY (37.5 µg ml⁻¹) plus peptone (500 µg ml⁻¹) was incubated at 30 °C for 90 minutes and then applied to the column. Fractions were collected and fluorescence and protein of each fraction was determined. The protein in each fraction was measured using a dye binding protein assay (Bio-Rad Laboratories, Richmond, California) with bovine serum albumin as the standard.

CHAPTER III

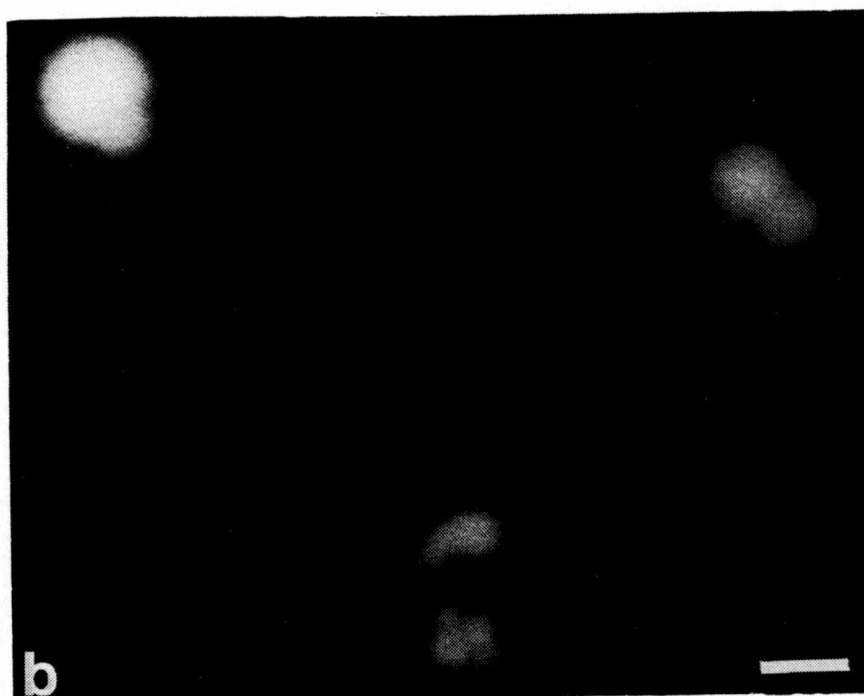
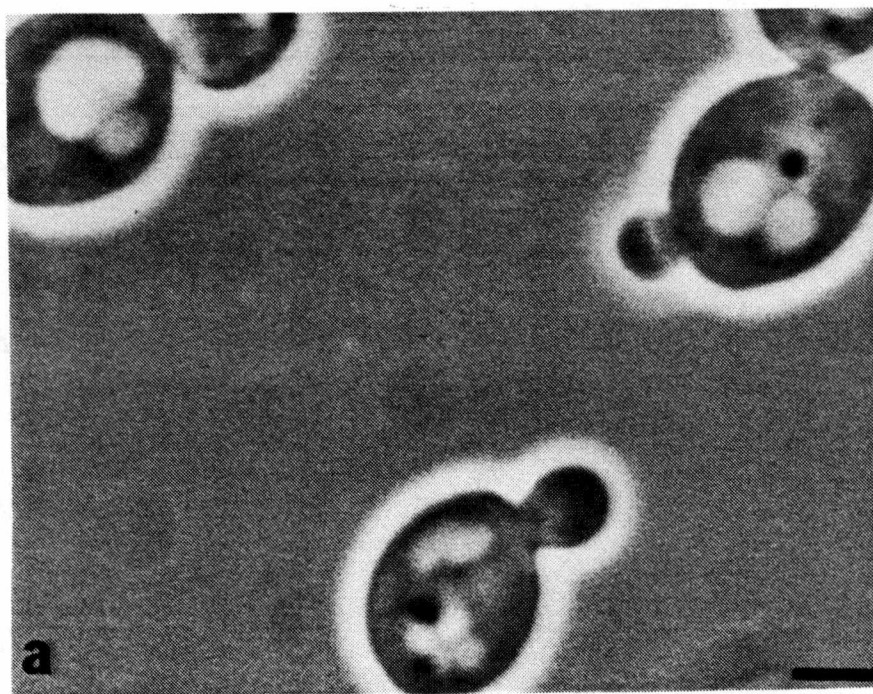
RESULTS

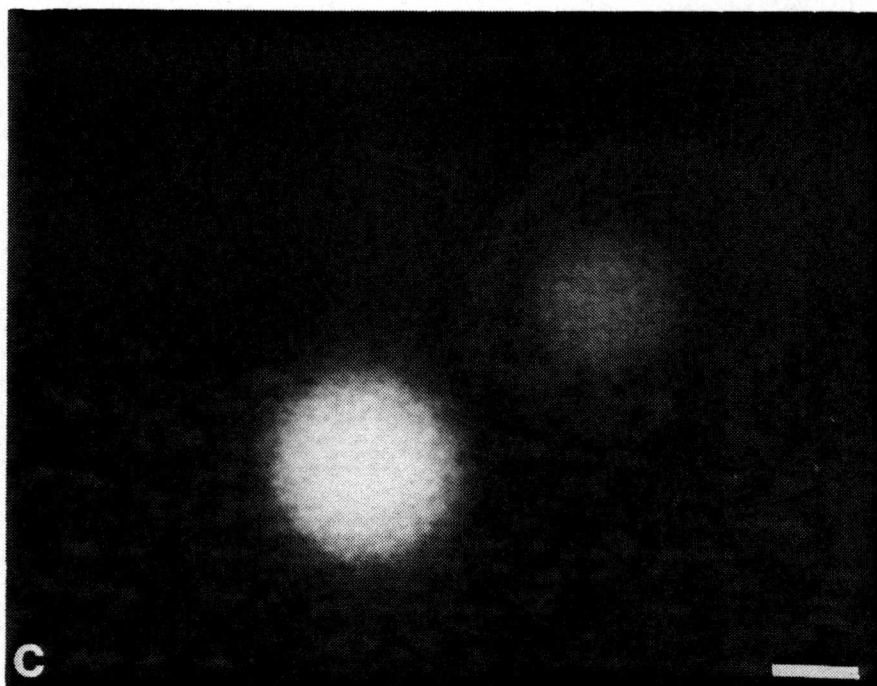
Effect of temperature and energy inhibition on uptake of lucifer yellow by the yeast phase of *C. albicans*

An optimum temperature of 30 °C for the uptake of LY by *C. albicans* was determined in buffered glucose medium during a 90 minute incubation. 80-90% of the cells internalized LY at 30 °C. Cells incubated at 22, 37, and 42 °C internalized LY at levels 50, 25, and 75% less respectively, than the levels internalized at 30 °C. Intracellular fluorescence appeared in vacuoles ; some cells showed many small vacuoles, while others had two or three large vacuoles (Fig. 1a & b). There was no internalization at 0 °C. A few cells showed entrapment of the dye between the cell wall and cell membrane at all the temperatures studied (Fig. 1c).

The energy-dependence of uptake of LY by *C. albicans* was studied during a 90 minute incubation at 30 °C. Cells incubated in 2% (w/v) glucose internalized LY into vacuoles as described above (Fig. 1a & b). Cells incubated in phosphate buffer assay medium without glucose did not internalize L (data not shown). Sodium azide (20 mM) an energy inhibitor in the presence of 2% (w/v) glucose prevented the internalization of the dye into vacuoles. However, 1-2% of cells appeared to have the dye diffuse throughout the cytoplasm and not localized in discrete vacuoles. Cells incubated with 20 mM sodium azide in the absence of glucose did not internalize the dye. Only 10-20% of the cells incubated with 2% glucose and 25 µM CCCP (carbonyl cyanide m-chlorophenyl hydrazone) an uncoupler of H⁺ gradients (Harold, 1986), accumulated LY into apparently discrete vacuoles. Cellular fluorescence was not reduced when cells were incubated with 2% glucose and 8 mM

Fig.1. Internalization of lucifer yellow by the yeast phase of *C. albicans*. Cells were incubated in assay medium containing 2% (w/v) glucose and 4 mg ml⁻¹ LY in 50 mM potassium phosphate buffer, pH 6.0, at 30 °C for 90 min. Cells were washed and visualized by phase contrast and fluorescence microscopy. (a) Phase contrast micrograph. (b) Fluorescence micrograph of the same field as in (a) showing uptake of the dye inside vacuoles. (c) Fluorescence micrograph showing entrapment of the dye as indicated by fluorescence around the cellular periphery. Bar - 1 µm.





sodium orthovanadate, which acts on membrane-associated ATPases (Hubbard *et al.*, 1985 & 1986).

Quantitative assays for lucifer yellow uptake

We quantitated cell-associated LY to determine if the uptake of the dye by the yeast phase of *C. albicans* was time-dependent and saturable. Cells were incubated for various periods of time in assay medium containing 2% (w/v) glucose and LY (0.1, 1, 2 and 4 mg ml⁻¹) in 50 mM potassium phosphate buffer, pH 6.0, at 30 °C. Quantitation of cell-associated LY for each concentration of LY at different time points suggested that the uptake of the dye by *C. albicans* increased over a 2 h incubation period (Fig. 2). The uptake of LY was not saturable over a 40-fold range of concentration tested (inset Fig. 2).

Internalization of lucifer yellow by the mold phase of *C. albicans*

Studies with the yeast phase of *C. albicans* prompted us to investigate if the mold phase of this dimorphic fungus internalized LY. Mold phase was obtained by a nutritional shift procedure using a complex medium (Sabouraud dextrose agar followed by 1% (w/v) bactopectone). Assays were performed in medium containing 2% glucose and 4 mg ml⁻¹ of LY in 50 mM potassium phosphate buffer, pH 6.0, for 90 minutes at both 30 °C, the optimum temperature for internalization of LY by the yeast phase, and also 37 °C, the temperature at which the mold phase was obtained. The mold phase internalized LY at both 30 °C and 37 °C and the dye was localized either in several small vacuoles or in large vacuoles distributed all along the length of the mycelium (Fig. 3 A & B).

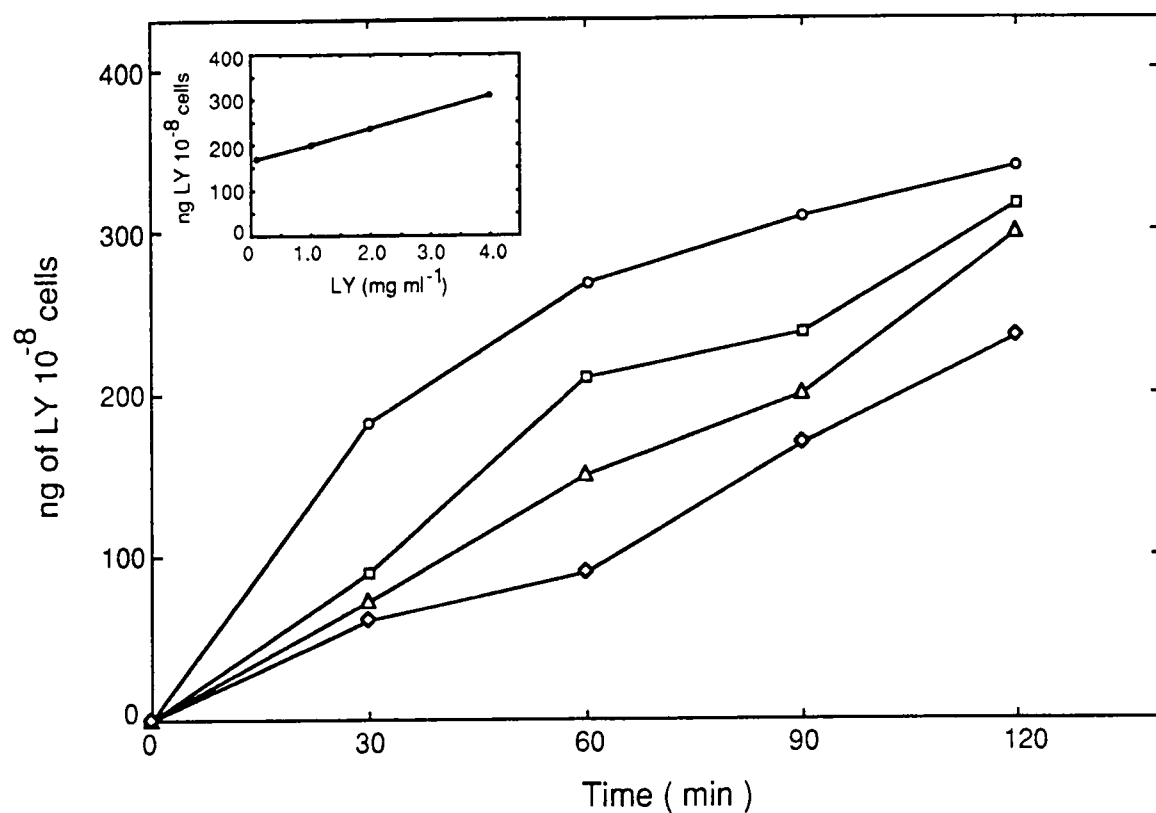
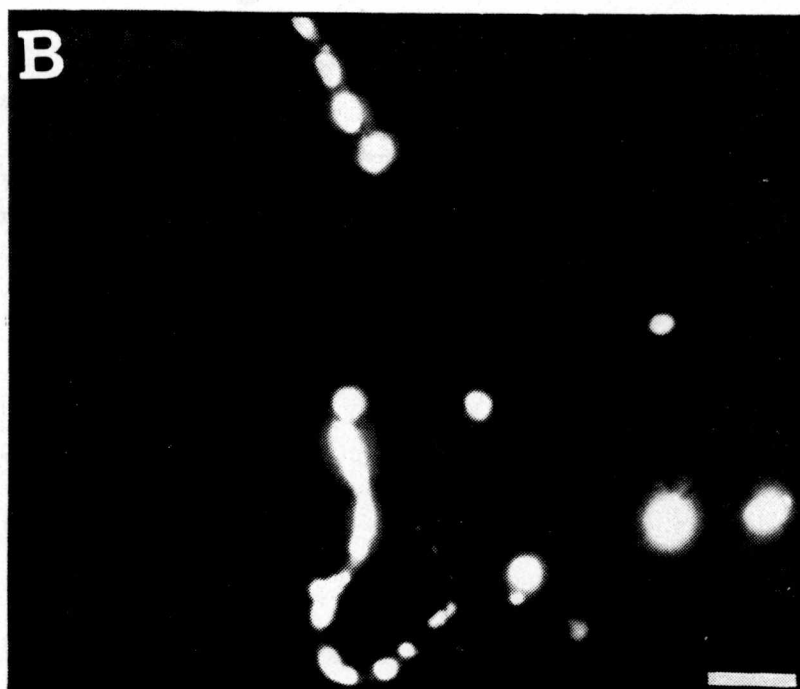
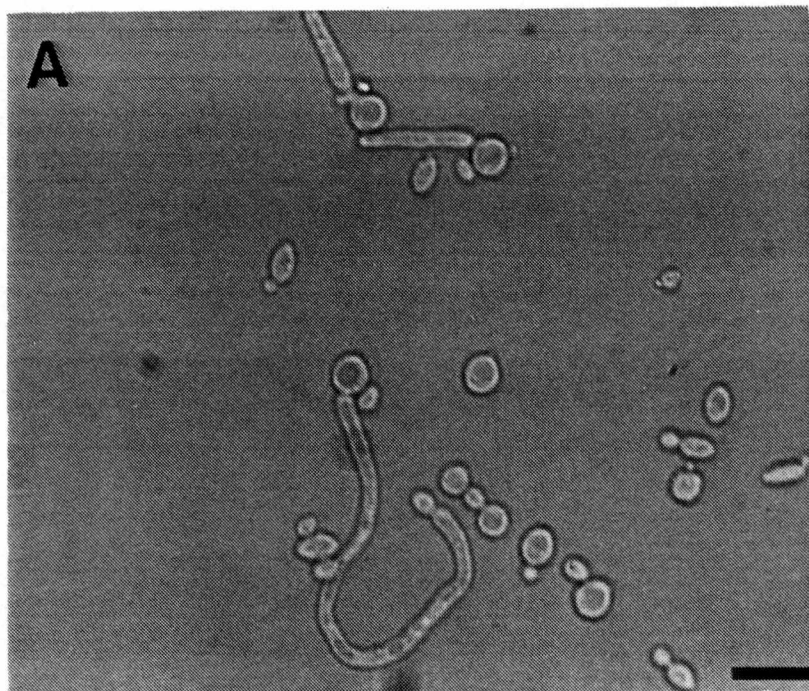


Fig. 2. Time course of uptake of lucifer yellow by *C. albicans*. Cells were incubated with various concentrations of LY (◇, 0.1 mg ml $^{-1}$; △, 1 mg ml $^{-1}$; □, 2 mg ml $^{-1}$; ○, 4 mg ml $^{-1}$) in assay medium containing 2% (w/v) glucose in 50 mM potassium phosphate buffer, pH 6.0, at 30 °C. Cell associated LY was quantified using a fluorescence spectrometer. Inset, Nonsaturable uptake of LY. Cell associated LY values for the 90 min time point (○) were replotted for each concentration of LY.

Fig. 3. Internalization of lucifer yellow by the mold phase of *C. albicans*. Cells were incubated in assay medium containing 2% (w/v) glucose and 4 mg ml⁻¹ LY in 50 mM potassium phosphate buffer, pH 6.0, at 30 °C for 90 min. Cells were washed and visualized by phase contrast and fluorescence microscopy. (A) Phase contrast micrograph. (B) Fluorescence micrograph of the same field as in (A) showing uptake of the dye inside vacuoles. Bar - 5 µm.



Effect of growth medium on the uptake of lucifer yellow by the yeast and mold phases of *C. albicans*

Yeast phase and mold phase obtained by growth in synthetic medium (ASM) were assayed for LY uptake during a 90 minute incubation in medium containing 2% (w/v) glucose and 4 mg ml⁻¹ of LY in 50 mM potassium phosphate buffer, pH 6.0. Yeast cells obtained by growth in ASM medium at 25 °C did not internalize LY at either 25 °C or at 30 °C, the optimum temperature for LY internalization by the yeast phase grown in YEPD. Also, the mold phase generated by a nutritional shift procedure in ASM medium did not internalize LY when assays were done at 30 °C or at 37 °C.

Effect of peptone and yeast extract on the uptake of lucifer yellow by *C. albicans*

The observation that the yeast phase or mold phase grown in complex medium internalized LY, whereas both phases grown in synthetic medium did not, led us to determine if peptone or yeast extract affected LY internalization. Cells were grown in complex medium and assays were done at 30 °C for 90 minutes. Only 10-20% of the cells internalized LY in the presence of 0.5-2% (w/v) peptone or yeast extract, whereas 80-90% of the cells internalized LY without the addition of peptone or yeast extract. In order to investigate if peptone complexed with LY and thus made it unavailable for endocytosis, we performed column chromatography of LY and LY-peptone mixture using a Sephadex G-25 column (Fig. 4a & b). The elution profile of LY was not affected by co-chromatography with peptone when applied as a mixture of peptone and LY. The peptone components were distributed over all the fractions collected.

Exocytosis of lucifer yellow

Since peptone in the assay medium reduced LY internalization, we determined if peptone affected exocytosis of internalized LY from the yeast phase of *C. albicans*. Cells

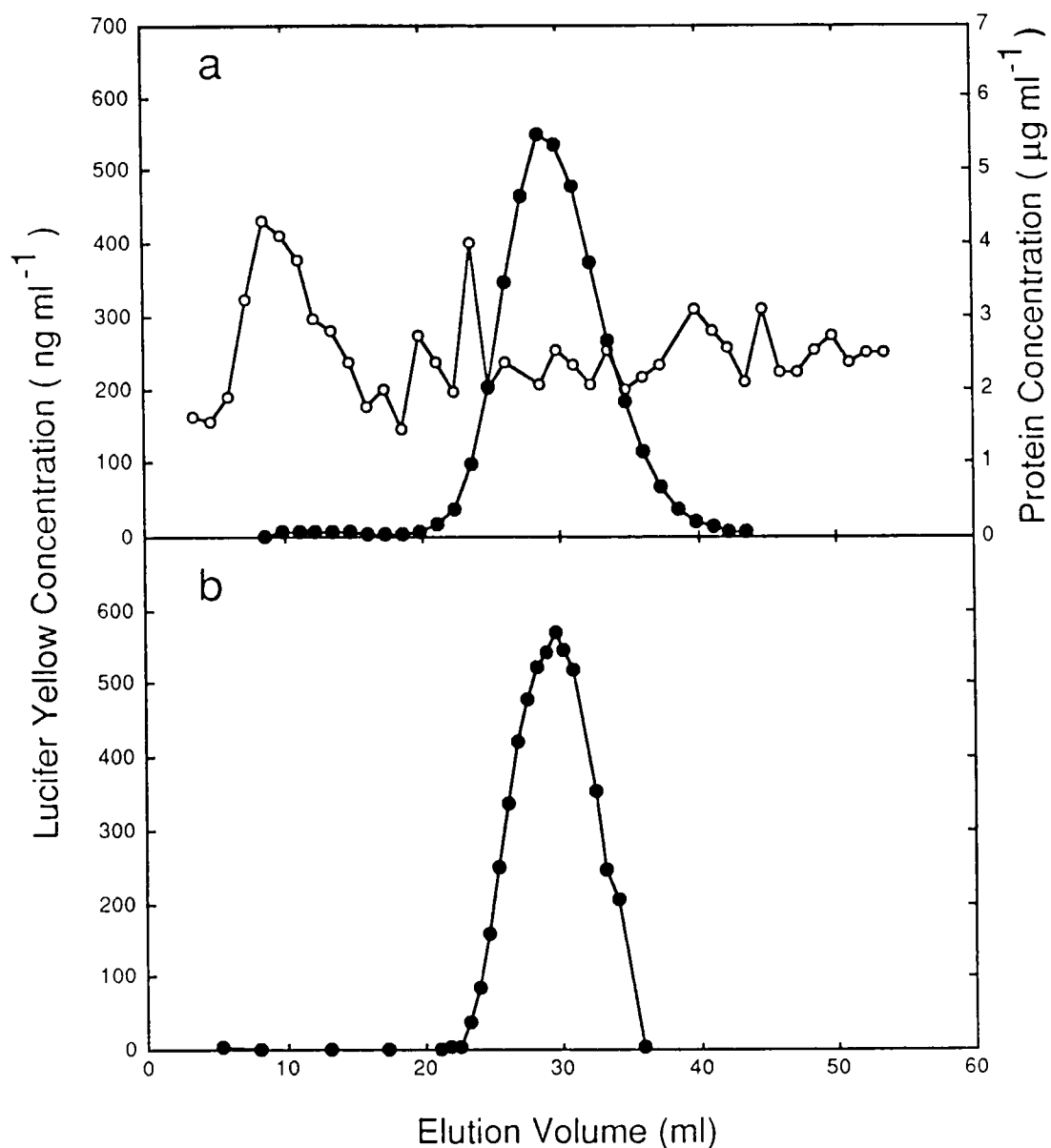


Fig. 4. Column chromatography of lucifer yellow and lucifer yellow-peptone mixture : (a) Lucifer yellow, (b) Lucifer yellow-peptone mixture were applied to a Sephadex G-25 column. Fractions were collected and fluorescence (●) was determined using a Luminescence Spectrometer, and protein (○) was determined using Bio-Rad protein assay.

were incubated in 50 mM phosphate buffer, pH 6.0, containing 2% (w/v) glucose and 1 mg ml⁻¹ LY at 30 °C for 90 minutes, washed, then reincubated in the presence or absence of 2% (w/v) peptone. Results of this experiment (Fig. 5a & b) show that yeast cells reincubated with peptone lost nearly 60% of cell-associated LY within 50 minutes with a concomitant increase in the fluorescence of the supernatant. Cells reincubated in phosphate buffer retained about 80% of the cell-associated LY even after reincubation for 2 hours.

Viability of lucifer yellow treated cells

C. albicans cells were incubated in a medium containing 2% (w/v) glucose and 4 mg ml⁻¹ LY in 50 mM potassium phosphate buffer, pH 6.0 at 30 °C for 90 minutes. After examining cells under the fluorescence microscope appropriate dilutions of cells were plated on YEPD agar plates. Control cells incubated without LY were also plated and the numbers of viable cells were compared. After incubation at 37 °C for 48 hrs, 98% of the control cells were viable as compared to 88% of the LY treated cells. These values are the averages of three experiments and show that the uptake of LY does not greatly affect viability of the cells.

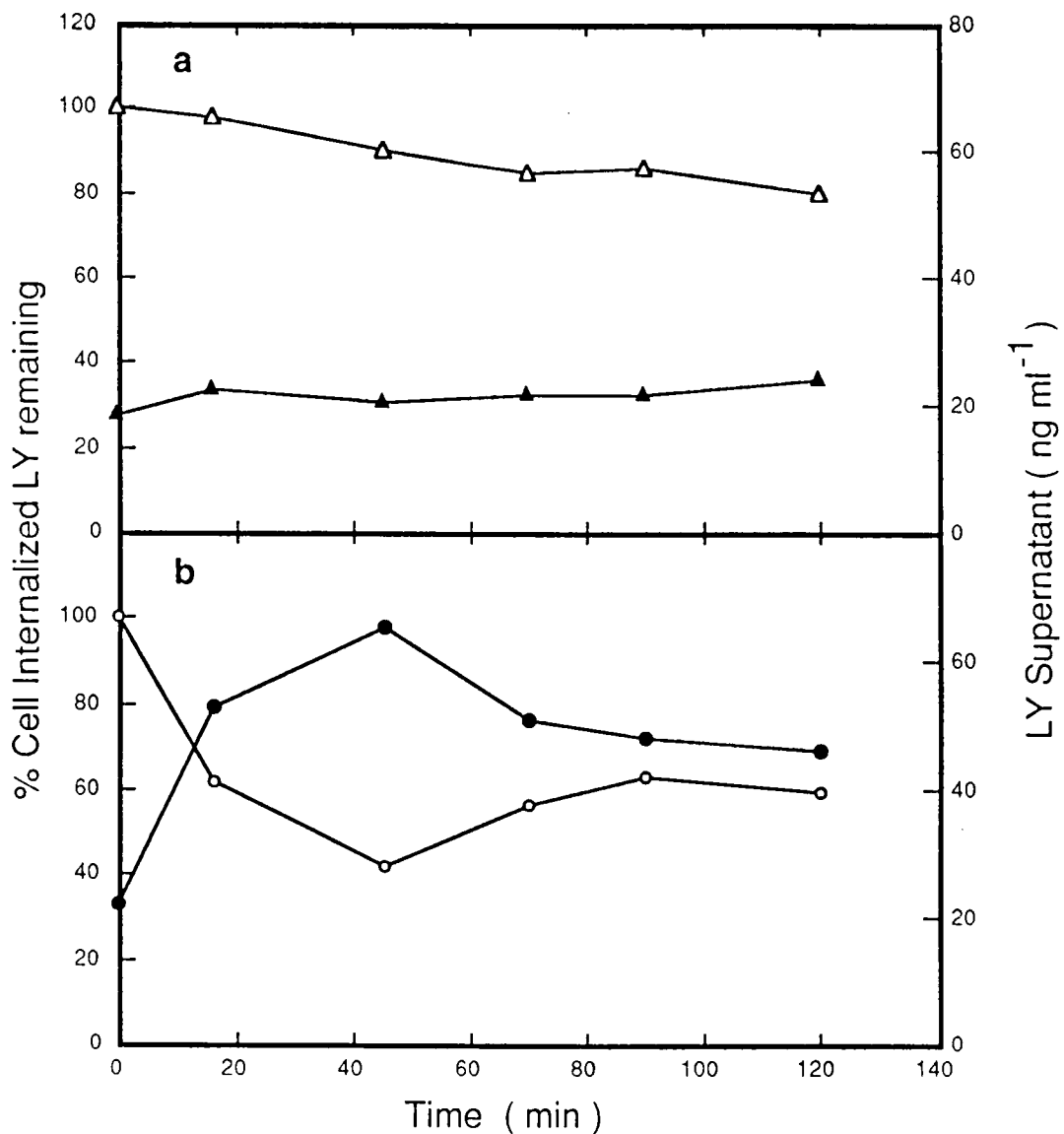


Fig. 5. Exocytosis of lucifer yellow. *C. albicans* cells incubated with 2% (w/v) glucose and 1 mg ml⁻¹ LY in 50 mM potassium phosphate buffer, pH 6.0, at 30 °C for 90 min, were washed and reincubated in phosphate buffer (a), or 2% (w/v) peptone (b), for various time periods at 30 °C. Fluorescence associated with the cells (Δ , $\circ$) and the supernatant ($\blacktriangle$, $\bullet$), was measured.

CHAPTER IV

DISCUSSION

In this report, we have shown that lucifer yellow is internalized by *C. albicans* by a non-saturable and time-, temperature-, and energy-dependent, process consistent with the characteristics of fluid phase endocytosis. The dye appeared to be concentrated inside vacuoles similar to those observed in *S. cerevisiae* (Riezman, 1985). Genetic basis for the involvement of vacuoles in endocytosis has been shown in *S. cerevisiae*. A temperature sensitive mutant, which lacked a morphologically recognizable vacuole, was defective for vacuolar enzyme biogenesis and was unable to internalize lucifer yellow (Chvatchko *et al.*, 1986 ; Riezman *et al.*, 1986 ; Dulic & Riezman, 1989).

Energy-dependence of uptake of the dye is reflected by the finding that glucose in the assay medium allowed the internalization of LY, while inhibitors of energy metabolism decreased the internalization of the dye. No internalization of LY was observed in the absence of glucose. Sodium azide, in the absence of glucose, probably blocks even the basal level of metabolism and, hence, no internalization was observed. The diffuse fluorescence in the presence of glucose and azide might indicate a non-endocytic uptake into poisoned cells, as sodium azide has been reported to interact with the yeast cell membrane causing an increase in membrane permeability (Riemersma, 1968). Sodium orthovanadate, which inhibits fungal plasma membrane ATPases (Hubbard *et al.*, 1985 & 1986 ; Goffeau & Slayman 1981 ; Willsky, 1979), might be expected to reduce cellular fluorescence of LY due to the central role of ATPases in energy metabolism. However, in both *S. cerevisiae* (Riezman, 1985) and *C. albicans*, intracellular fluorescence does not seem to be reduced in the presence of sodium orthovanadate. It is possible that the membrane associated ATPases are dispensable for endocytosis in *C. albicans* and

S. cerevisiae, or that vanadate is metabolized to vanadyl ions leading to stimulatory effects on yeast cells (Dubyak & Kleinzeller, 1980 ; Willsky *et al.*, 1984).

C. albicans is a dimorphic fungus which undergoes yeast to filamentous phase conversion when provided with the appropriate environmental conditions. Lee *et al.* (1975) developed a synthetic medium composed of amino acids, salts, glucose, and biotin which maintained this fungus in the yeast form at 25 °C, and in the mold form at 37 °C. Surprisingly, we found that neither the mold phase nor the yeast phase grown in synthetic medium internalized LY even though distinct vacuoles could be seen in the cells by phase contrast microscopy. However, both phases grown in complex medium and assayed for LY uptake in buffered glucose medium internalized LY into vacuoles. These results suggest that growth in a complex medium triggers the process of fluid phase endocytosis.

The influence of growth conditions on uptake processes in *C. albicans* has been investigated by several workers. Recently, Skowronski & Feldman (1989) reported that significant binding of estrogen to estrogen-binding protein in *C. albicans* could only be found if yeast cells were grown in complex medium whereas negligible binding was seen when cells were grown in some synthetic media. Page & Odds (1988) have reported that the growth medium affects the binding of human albumin, fibrinogen and transferrin to *C. albicans* and found that the mold phase obtained by growth in a synthetic medium bound less protein. Logan *et al.* (1979) have shown that the nitrogen source for growth affected peptide transport in *C. albicans*.

Since growth in complex media appeared to be required to enable cells to internalize LY, we investigated the effect of addition of peptone and yeast extract to the assay medium. Our results showed that peptone and yeast extract in the assay medium reduced the number of fluorescent cells, and that this was not due to a complex formation between peptone and LY. Rather, peptone caused a rapid increase in efflux of internalized LY. Exocytosis of endocytosed material has been shown to be stimulated by extracellular calcium (Besterman

et al., 1981) and chloroquine (Kotal & Kotyk, 1988). Further experiments are required to elucidate the mechanisms operating in these processes.

Studies on fluid phase endocytosis in *C. albicans* may explain how some large molecules such as defensins (Lehrer *et al.*, 1985), toxins (Polonelli *et al.*, 1985) and cationic proteins (Yphantis *et al.*, 1967 ; Olson *et al.*, 1977) enter cells and cause cell damage. Since the characterized oligopeptide permeases are not known to transport such large proteins (Becker & Naider, 1980 ; Naider & Becker 1987), and since no receptors have been reported for these large molecules, it is possible that they enter cells by fluid phase endocytosis. Sodium azide and CCCP which prevent internalization of LY also protect *C. albicans* from the killing effect of defensins (Lehrer *et al.*, 1988). These results suggest that defensins and LY may be able to enter cells by a similar mechanism. Lichtenstein *et al.*(1988) speculate that a possible endocytic uptake of defensins by the target mammalian tumor cells may be responsible for their entry into cells to produce a cytolytic effect. A definitive answer to the endocytic mechanism of defensin uptake by *C. albicans* should result from studies on uptake of radiolabeled defensins.

A relationship between endocytosis and secretion has been reported for *S. cerevisiae* (Riezman, 1985 ; Dulic & Riezman, 1989), as several of the yeast secretory mutants were defective in internalization of LY. It is possible, therefore, that studies on endocytosis may increase our understanding on how proteinases are secreted from *C. albicans*. The secreted proteinase of *C. albicans* has been implicated as a virulence factor by several workers (reviewed in Douglas, 1988), although reports to the contrary also exist (Burnie *et al.*, 1985). It will be interesting to investigate if the endocytic and secretory pathways of *C. albicans* are coupled by studying LY endocytosis in *C. albicans* proteinase deficient mutants (Kwon-Chung *et al.*, 1985).

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PART IV
ATTEMPTS TO CLONE A *CANDIDA ALBICANS*
PEPTIDE TRANSPORT GENE(S)

CHAPTER I

INTRODUCTION

Studies from our laboratory and several others have characterized the peptide transport systems of both *C. albicans* and *S. cerevisiae* (Becker & Naider, 1980 ; Davies, 1980 ; Logan *et al.*, 1979 ; Payne, 1980). Results from these studies suggest that both these yeasts contain peptide permeases with specific structural requirements different from the bacterial and mammalian permeases. Both yeasts prefer peptides containing hydrophobic residues, but, overall, several modifications of small peptides can still be recognized by their peptide transport systems (Becker & Naider, 1977 & 1980 ; Naider & Becker, 1987 ; Shallow *et al.*, 1991). The peptide transport systems of both *S. cerevisiae* and *C. albicans* are under general nitrogen control (Becker & Naider, 1977 & 1980 ; Davies, 1980 ; Logan *et al.*, 1979 ; McCarthy *et al.*, 1985 a & b ; Moneton *et al.*, 1986 a & b ; Naider & Becker, 1987 ; Nisbet & Payne, 1979 ; Payne *et al.*, 1991 ; Yadan *et al.*, 1984). We have previously shown that the peptide transport system of *S. cerevisiae* is induced by the addition of micromolar amounts of certain amino acids in a medium with a nonrepressive nitrogen source such as allantoin (Island *et al.*, 1987). Several amino acids have also been reported to overcome repression by ammonium and increase the toxicity of oxalysine-containing di- and tripeptides against *C. albicans* (Part II of this dissertation).

In an effort to define peptide transport at the genetic level, we previously isolated *S. cerevisiae* strains resistant to the growth inhibitory effects of the dipeptide Ala-Eth, yet sensitive to ethionine (Island *et al.*, 1991). These strains defined three complementation groups *ptr1*, *ptr2*, and *ptr3* suggesting that peptide transport in *S. cerevisiae* is mediated by at least three genes *PTR1*, *PTR2* and *PTR3*. Current evidence, though not conclusive, suggests the presence of at least two peptide permeases in *C. albicans*. One of the systems

transports di- and tripeptides and the other transports tri- and oligopeptides (McCarthy *et al.*, 1985 a & b ; Mehta *et al.*, 1984 ; Milewski *et al.*, 1988 ; Payne & Shallow, 1985 ; Yadan *et al.*, 1984).

The diploid nature of the *C. albicans* genome and the absence of a known sexual cycle have limited genetic studies with this organism. Cloning of the genes defining peptide transport of *C. albicans* should provide important information on the number, role and regulation of permeases under different physiological conditions. These studies will also enable us to compare the structure, physiological role and evolution of membrane proteins from different organisms. Additionally, these studies may also facilitate the design of an effective and specific anticandidal drug by exploiting the concept of "illicit transport" (Fickel & Gilvarg, 1973 ; Ames *et al.*, 1973 ; Hammond *et al.*, 1987 ; Higgins, 1987). Peptide conjugates have been used to deliver toxic moieties to *C. albicans* via the peptide transport system (Borowski *et al.*, 1979 ; Kenig & Abraham, 1976 ; Kingsbury *et al.*, 1983 ; Lichliter *et al.*, 1976 ; Meyer Glauner *et al.*, 1982).

Significant advances have been made in the past few years towards the genetic manipulation and analysis of the *C. albicans* genome. Many *C. albicans* genes have been cloned through their counterparts in *S. cerevisiae* either by functional complementation of an *S. cerevisiae* mutation, nucleotide homology, or by conferring a unique phenotype to the cells (reviewed in Kurtz *et al.* 1990). For example, many *C. albicans* genes such as *URA3*, *HIS3*, *TRP1*, *HEM3*, *ADE2*, *LEU2*, *GAL1*, *CSH1* and *TMP1* have been cloned due to the ability of the *C. albicans* genes to be functionally expressed in *S. cerevisiae*. However, no cross hybridization was reported when the *C. albicans* genes *LEU2*, *ADE2*, *URA3*, and *DFR1*, which functionally complement *S. cerevisiae* mutants, were hybridized at high stringency with *S. cerevisiae* DNA (reviewed in Kurtz *et al.*, 1988 & 1990).

Even though several *C. albicans* genes have been cloned by functional complementation of an *S. cerevisiae* mutation this by no means guarantees that every *C. albicans* gene will

be expressed and function in a heterologous system. Cloning via expression can fail as certain *S. cerevisiae* genes such as *ACT1*, *TUB2*, and *KAR1* are toxic in yeast when overexpressed (Rose *et al.*, 1987 ; Smith *et al.*, 1990). Such genes may be difficult to clone via heterologous expression since the gene product from a single copy of the gene may not be sufficient to complement the mutation (Kirsch *et al.*, 1988) and overexpression may be lethal. In situations where these problems cannot be circumvented, cloning by cross-hybridization becomes important. The actin (*ACT1*) and tubulin (*TUB2*) genes, which code for the structural proteins actin and tubulin, respectively, were cloned by cross hybridization with the corresponding *S. cerevisiae* genes (Lasker, B. L., Ph.D. dissertation, University of Tennessee, Knoxville, TN. May 1985 ; Smith *et al.*, 1988). The *SOR2*, *SOR9*, *STA1*, and *DFR1* genes were cloned as a result of their novel and selectable phenotypes in *S. cerevisiae* (reviewed in Kurtz *et al.*, 1990).

In this dissertation two alternative approaches were used in an attempt to clone a *C. albicans* peptide transport gene(s) : (1) nucleotide homology to the cloned transporters from other systems and, (2) functional complementation of a *S. cerevisiae* peptide transport mutant. In an effort to clone a *C. albicans* peptide transport gene by the approach of nucleotide homology we performed Southern blot analysis of *C. albicans* to identify putative DNA sequence similarity among various cloned genes known to be involved in the transport of peptides. Extensive sequence similarity and domain organization is shared among a superfamily of transport systems termed the ATP-binding cassette (ABC) proteins (Ames, 1986 ; Gros *et al.*, 1986 ; Higgins, 1989 ; Hiles *et al.*, 1987 ; Hyde, *et al.*, 1990 ; Kuchler *et al.*, 1989 ; McGrath & Varshavsky, 1989). Some of the proteins in this group include a oligopeptide uptake system of enteric bacteria (Opp), the P-glycoprotein associated with multidrug resistance in human tumors (MDR), the *S. cerevisiae* *STE6* gene product that exports the mating pheromone α -factor from *S. cerevisiae* MATa cells, pfMDR which is implicated in chloroquine resistance of the malarial parasite, and the product of the

cystic fibrosis gene (CF). We also reasoned that a peptide transport gene of *C. albicans* may have similarity in nucleotide sequence to the *S. cerevisiae* *PTR2* gene which is involved in amino acid inducible peptide transport (Island *et al.*, 1991 ; Perry *et al.*, unpublished). Neither the *S. cerevisiae* *PTR2* or *STE6* genes, nor the *Salmonella typhimurium* *Opp* operon, when used as probes, showed complementary sequences in whole cell DNA isolated from *C. albicans*.

In an attempt to clone the *C. albicans* peptide transport gene by functional complementation, we used a *S. cerevisiae* peptide transport mutant. One *C. albicans* genomic library in a low copy vector pYSK35 (Singer *et al.*, 1989) and two genomic libraries in a multi-copy vector YEp13 (Gillum *et al.*, 1984 ; Rosenbluh *et al.*, 1985) were screened to determine if the *ptr2* mutation in *S. cerevisiae* could be functionally complemented by a *C. albicans* gene. The *S. cerevisiae* *ptr2* mutant, PB1X-9B, which is resistant to the toxic dipeptide Ala-Eth and is unable to use defined dipeptides to satisfy its amino acid auxotrophic requirements of amino acids, was the recipient cell. No transformants with restored ability to transport peptides were recovered from the *C. albicans* library in the low copy pYSK35 vector. The *C. albicans* YEp13 libraries were useful in obtaining *S. cerevisiae* transformants with a *PTR2*⁺ phenotype. However, a functional *C. albicans* *PTR2* homolog could not be recovered on a plasmid. Although, we were not successful in recovering a *C. albicans* allele that complemented the peptide transport mutation, methods have been devised to rescue this allele from the *S. cerevisiae* transformants.

CHAPTER II

MATERIALS AND METHODS

Strains and Vectors

A list of the organisms used in this study are given in Table 1. *S. cerevisiae* PB1X-9B was used in an attempt to clone a *C. albicans* *PTR2* homolog. A list of vectors used in this study are given in Table 2.

Media

C. albicans and *S. cerevisiae* cells were maintained on YEPD medium containing (w/v) : peptone 2% ; glucose 2% ; yeast extract 1% ; agar (Difco) 2%. The minimal medium (MM) used for most studies was made by adding 10 ml of 10 X filter sterilized YNB without amino acids and without ammonium sulfate (Difco, cat #0335-15-9) to 90 ml of sterile water containing 2 g glucose, 100 mg allantoin and auxotrophic supplements depending on the strain as follows : histidine, uracil and methionine (20 mg/l), lysine and leucine (30 mg/l) (Sherman *et al.*, 1986). Allantoin, a purine catabolite (Cooper, 1982) which can serve as a sole nitrogen source for *S. cerevisiae*, was used in MM as it is neutral with respect to peptide transport activity, whereas other nitrogen sources, such as ammonium sulfate, and amino acids affect peptide transport (Island *et al.*, 1991). The mutant strain *S. cerevisiae* PB1X-9B was grown in Complete Medium (CM) which consisted of MM with histidine, uracil, lysine and leucine. *S. cerevisiae* PB1X-9B transformed with YEp13 or pRS201 based plasmids were grown on CM lacking leucine (CM-Leu), those transformed with YCp50 based plasmids were grown on CM lacking uracil (CM-Ura). Dipeptide medium [Lys-Leu, His-Lys, or His-Leu (80 μ M)] consisted of MM supplemented with auxotrophic requirements minus the amino acid components of the

TABLE 1. Organisms used in this study

Designation	Organism	Genotype	Source
HB101	<i>Escherichia coli</i>	F^- , <i>hsd20</i> (r_B, m_B), <i>recA13</i> , <i>ara-14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL20</i> (Smt^R), <i>xyl-5</i> , <i>supE44</i> , I^-	ATCC
DH5 α	<i>Escherichia coli</i>	<i>D</i> (<i>lac-proAB</i>), <i>supE</i> , <i>thi</i> / F' , <i>lacI$\phi$$\Delta$M15</i> , <i>iraD36</i> , <i>proAB</i> ⁺	BRL
PB1X-9B	<i>Saccharomyces cerevisiae</i>	<i>MATa</i> , <i>ura3-52</i> , <i>leu2-3,112</i> , <i>lys1-1</i> , <i>his4-38</i> , <i>ptr2</i>	J. P.
PB1X-2A	<i>Saccharomyces cerevisiae</i>	<i>MATa</i> , <i>ura3-52</i> , <i>leu2-3,112</i> , <i>lys1-1</i> , <i>his4-38</i> , <i>PTR2</i>	J. P.
PB13X-1B	<i>Saccharomyces cerevisiae</i>	<i>MATa</i> , <i>ptr2</i>	J. P.
SR 1181	<i>Candida albicans</i>	<i>lys</i> , <i>suf</i>	W.S.R.
RR1	<i>Escherichia coli</i>	same as <i>E. coli</i> HB101 except <i>recA</i> ⁺	ATCC

BRL = Bethesda Research Laboratories, Bethesda, MD.

ATCC = American Type Culture Collection.

J. P. = Jack Perry, University of Tennessee, Knoxville, TN.

W.S.R. = William Stuart Riggsby, University of Tennessee, Knoxville, TN.

TABLE 2. Plasmids used in this study

Designation	Bacterial markers	Yeast Markers	Yeast / <i>E. coli</i> shuttle vector	Source	Reference
pBR322	Amp ^R , Tet ^R	—	No	M. M.	A
pCH19	Amp ^R	—	No	C. H.	B
YEpl3	Amp ^R , Tet ^R	LEU2	Yes	F. L.	C
pRS201	Amp ^R	LEU2	Yes	G. C.	D
YEpSTE6	Amp ^R ,	URA3	Yes	J. M.	E
pJP9	Amp ^R	URA3, PTR2	Yes	J. P.	F
YCp50	Amp ^R , Tet ^R	URA3	Yes	J. P.	F
pYSK35	Amp ^R , Tet ^R	LEU2, TRP1	Yes	Y. K.	G

C. H. = Chris Higgins, University of Oxford, Oxford, UK.
G. C. = Guy Caldwell, University of Tennessee, Knoxville, TN.
F. L. = Frank Larimer, O. R. N. L. Oak Ridge, TN.
J. M. = John McGrath, Massachusetts Institute of Technology, Cambridge, MA.
J. P. = Jack Perry, University of Tennessee, Knoxville, TN.

M. M. = Mark Mason, formerly of University of Tennessee, Knoxville, TN.

Y. K. = Yigal Koltin, Tel-Aviv University, Tel Aviv, Israel.

A - Bolivar *et al.* 1977.

B - Hiles *et al.* 1987.

C - Broach *et al.* 1979.

D - Sikorski & Hieter. 1989

E - McGrath & Varshavsky. 1989.

F - Personal Communication.

G - Singer *et al.* 1989.

added peptides. For example the dipeptide (Lys-Leu) medium for the growth of *S. cerevisiae* PB1X-9B transformed with the *PTR2* gene on a YEp13 plasmid contained MM supplemented with Lys-Leu, histidine and uracil. Amino acid inducers such as tryptophan, isoleucine, threonine, arginine and leucine were added at a final concentration of 0.15 mM where indicated. For example, CM-Leu+Trp is CM-Leu with tryptophan. To determine the auxotrophic markers, "drop-out" plates were made which consisted of MM with all the auxotrophic supplements except the one being tested.

E. coli cells used for transformation were grown in LB medium which contained (w/v) : tryptone 1% ; NaCl 1% ; yeast extract 0.5%. Antibiotic plates contained 50 µg/ml ampicillin (Sigma). For isolation of plasmid DNA, *E. coli* transformants were grown in modified Terrific Broth (Sambrook *et al.*, 1989) which contained tryptone 12 g, yeast extract 24 g, glycerol 4 ml and 20 mM glucose in 900 ml of water to which was added 100 ml of potassium phosphate buffer (2.31 g KH_2PO_4 and 12.54 g K_2HPO_4).

C. albicans libraries

Two different genomic *C. albicans* libraries in the yeast / *E. coli* shuttle vector YEp13 were used. One of the YEp13 libraries was constructed by Rosenbluh *et al.* (1985) by partial digestion of DNA from *C. albicans* B792 with *Sau3A*, ligation of 10 kb fragments to the *BamHI* site in the tetracycline resistance gene of YEp13 followed by transformation of *E. coli* strain MC1061 to ampicillin resistance. DNA amplified from the original library was used for transformation of *S. cerevisiae* PB1X-9B. A second genomic *C. albicans* library was generated by Gillum *et al.* (1984). DNA from a clinical isolate *C. albicans* SC 5314 was partially digested with *Sau3A* and fragments of 5-20 kb were ligated into the *BamHI* site in the tetracycline gene of YEp13 and the ligation mixture was used to transform *E. coli* strain RR1 to ampicillin resistance. Plasmid DNA was obtained by growing the *E. coli* transformants in 1 liter of modified Terrific Broth containing

50 µg/ml ampicillin and incubated at 37 °C at 200 rpm overnight. Plasmid DNA was prepared by a modification of the alkaline lysis procedure (Maniatis *et al.*, 1982) as described later.

A third genomic *C. albicans* library was obtained from Yigal Koltin (Tel-Aviv University, Tel Aviv, Israel ; Singer *et al.*, 1989) in the low copy shuttle vector pYSK35. The library was prepared from *C. albicans* B792 (ATCC 36803) by partial digest of the genomic DNA with *Sau3A* and ligating 8-10 kb fragments into the low copy vector pYSK35 which is identical to pYSK41 (Smith *et al.*, 1990) except that the the *BamHI-Sall* portion of pBR322 region is present. DNA from this library was amplified in *E. coli* HB101 and an aliquot of the pooled transformants was used to inoculate 1 liter of modified Terrific Broth containing 50 µg/ml ampicillin. Plasmid DNA was isolated from the culture using the large scale isolation procedure described later which is a modification of alkaline lysis procedure (Maniatis *et al.*, 1982).

Isolation of plasmid DNA from *E. coli* transformants

Small scale plasmid DNA (mini-prep) from *E. coli* transformants was prepared as described in Maniatis *et al.* (1982) except that cultures were grown in modified Terrific Broth containing 50 µg/ml ampicillin. Briefly, 3 ml cultures grown overnight at 37 °C at 200 rpm were pelleted in a microfuge tube using two consecutive spins in the same tube. The pellet was resuspended in 100 µl of 50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA with 5 mg/ml lysozyme (added fresh). Tubes were incubated at room temperature for 5 minutes and 200 µl of a freshly prepared solution of 0.2 N NaOH and 1% SDS was added and cells were incubated for 5 minutes in a ice bucket. Then 150 µl of solution containing 3 M potassium acetate (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of distilled water) was added to the tubes which were incubated for 10 minutes on ice. The tubes were then centrifuged for 5 minutes and the supernatant was

transferred to a fresh tube. The DNA was extracted twice with 25:24:1 (v:v:v) distilled buffered phenol : chloroform : isoamyl alcohol and once with 24:1 (v:v) chloroform : isoamyl alcohol. DNA from the aqueous phase was extracted by precipitation with 2 volumes of 100% ethanol. Pellet containing DNA was resuspended in 50 µl of TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA) containing 100-200 µg/ml of DNase free RNase.

To obtain large amounts of plasmid DNA a modified procedure of the pZ523 column chromatography protocol (5Prime --> 3Prime, Inc) was used. *E. coli* cells containing plasmid DNA were grown overnight at 37 °C at 200 rpm in modified Terrific Broth containing 50 µg/ml of ampicillin. Cells were pelleted at 4000 g for 20 minutes at 4 °C and pellet was resuspended in 10 ml of ice cold solution of 50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA with 5 mg/ml lysozyme (added fresh) and incubated for 10-15 minutes at room temperature. Twenty milliliters of 0.2 NaOH and 1% SDS was added to the tubes and mixed gently but thoroughly by swirling or inversion and incubated on ice for 10 minutes. Fifteen milliliters of 3M potassium acetate (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of distilled water) was added and tubes were incubated for 10 minutes at room temperature after mixing was complete. The tubes were centrifuged at 16,000 g for 15 minutes at 4 °C and the resulting supernatant was transferred to a fresh tube. Isopropanol (0.6 volumes) and 0.1 volume of 3 M sodium acetate and 1 mM EDTA was added to the tube and incubated for 20-30 minutes at room temperature. The tubes were centrifuged at 16,000 g for 5 minutes, the supernatant was discarded, and the DNA pellet was resuspended in TE (pH 8.0). The DNA was extracted twice with 25:24:1 (v:v:v) distilled buffered phenol : chloroform : isoamyl alcohol and once with 24:1 (v:v) chloroform : isoamyl alcohol. DNA from the aqueous phase was extracted by precipitation with 0.6 volumes of isopropanol and 0.1 volume of 3 M sodium acetate and 1 mM EDTA. The DNA pellet was resuspended in 1 ml of TE (pH 8.0) and DNase-free

RNase was added to a final concentration of 100-200 µg/ml and incubated for 2 hours at 37 °C. The DNA was precipitated using 0.6 volumes isopropanol and 0.1 volume of 3 M sodium acetate and 1 mM EDTA. The DNA pellet was resuspended in 1.8 ml of TE (pH 8.0) and NaCl was added to a final concentration of 1 M. The sample was carefully layered on top of pZ523 column and centrifuged at 2000 rpm at room temperature in a table top centrifuge for 24 minutes. DNA from the eluate was precipitated using 0.6 volumes isopropanol and 0.1 volume of 3 M sodium acetate and 1 mM EDTA. The final DNA pellet was resuspended in TE (pH 8.0) and stored at -20 °C.

Isolation of plasmid DNA from *S. cerevisiae* transformants

Plasmid DNA from *S. cerevisiae* transformants used for transformation of *E. coli* was obtained by a modification of the procedure described by Strathern & Higgins (1991). Cells were grown overnight in 10 ml of medium selective for maintenance of the plasmid and harvested the next day. Cell pellet was resuspended in 200 µl of buffer containing 100 mM NaCl, 0.1% SDS, 1 mM EDTA and 10 mM Tris-Cl, pH 8.0. Sterile glass beads (0.45 mm in diameter) were added until just below the level of the liquid and tubes were vortexed at high speed for 1.5 minutes. Phenol (200 µl) was added and the tubes were vortexed for an additional 2 minutes followed by centrifugation for 2 minutes in an eppendorf. The DNA in the aqueous phase was extracted two times with an equal volume of phenol and once with an equal volume of chloroform : isoamyl alcohol 24 : 1 (v/v). DNA from the aqueous phase was precipitated at -20 °C for 30 minutes with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate and 1 mM EDTA. The DNA pellet was washed once with 80% ethanol and the dried pellet was dissolved in 20 µl TE. One microliter of the original or a 10 to 100-fold dilution of the same was used for transformation of *E. coli*.

Isolation of whole cell DNA from *S. cerevisiae*

Whole cell DNA from *S. cerevisiae* was obtained by a slight modification of the procedure described by Ausubel *et al.* (1990). Briefly, yeast cells were grown in 5 ml YEPD at 30 °C at 200 rpm for 16-18 hours. Cells were pelleted and resuspended in 0.5 ml sorbitol solution (0.9M sorbitol, 0.1M Tris-Cl, pH 8.0, and 0.1M EDTA). Fifty microliters of 0.28 M β -mercaptoethanol was added and tubes were incubated at 37 °C shaking at 200 rpm for 30 minutes. Fifty microliters of 5 mg/ml of yeast lytic enzyme from *Arthrobacter luteus* (70,000 + u/gm, ICN) in sorbitol solution was added and tubes were incubated for an additional 30 minutes till "ghosts" were visible on microscopic examination of lysed cells. Spheroplasts were recovered by centrifugation and resuspended in 0.5 ml of TE pH 8.0. Spheroplasts were lysed by adding 50 μ l of 10% SDS and tube was inverted several times to mix. The tube was incubated at 65 °C for 20 minutes and 200 μ l of 5M potassium acetate was then added to the tube and mixed well. The tube was kept on ice for 60 minutes and centrifuged for 5 minutes in an eppendorf centrifuge. The supernatant was poured into a clean microfuge tube and the tube was filled with 100% ethanol. The DNA was allowed to precipitate overnight at -20 °C. Tubes were centrifuged and DNA pellet was dried and resuspended in 300 μ l TE pH 8.0. To the DNA was added DNase free RNase (100-200 μ g/ml) and incubated for 2 hours at 37 °C. DNA was precipitated with 0.6 volumes of isopropanol and 0.1 volume of 3 M sodium acetate and 1 mM EDTA at room temperature for 30 minutes or overnight at -20 °C. DNA pellet was resuspended in 125 μ l of TE pH 8.0. Fifteen microliters of the whole cell DNA preparation was used for restriction enzyme digestion and subsequently for Southern blot analysis. Five or ten microliters of whole cell DNA from the *S. cerevisiae* transformants isolated by the procedure outlined above was used in some cases to recover plasmid DNA from yeast for transformation of *E. coli*.

Bacterial transformation

E. coli cells were transformed using the CaCl_2 procedure described in Maniatis *et al.* (1982). Briefly, *E. coli* cells were grown overnight in 100 ml LB broth at 37 °C at 200 rpm. One ml of the overnight culture was used to inoculate 100 ml of fresh LB broth and incubated at 37 °C at 200 rpm to a cell density of $\sim 5 \times 10^7$ cells/ml ($\text{OD}_{550} = 0.4 - 0.5$). The culture was placed on ice for 10 minutes and centrifuged for 5 minutes at 4000g at 4 °C. The supernatant was discarded and cells were resuspended in half the original volume of ice-cold solution of 50 mM CaCl_2 , and 10 mM Tris-Cl (pH 8.0). Cells were placed on ice for 15 minutes and the cell suspension was centrifuged for 5 minutes at 4000g at 4 °C. The supernatant was discarded and cells were resuspended very carefully in 1/15th the original volume in the Tris- CaCl_2 buffer described earlier. The competent cells were dispensed in 200 μl portions and placed at 4 °C till ready to use (generally overnight). For transformation, the appropriate amount of DNA (20-60 ng) was added to the tubes and cells were placed on ice for 45 minutes and heat shocked for 2 minutes at 42 °C. One ml of LB broth was added to the cells and incubation was done at 37 °C for one hour. Appropriate amount of each transformation mixture was plated on LB agar plates containing ampicillin (50 $\mu\text{g/ml}$) and incubated overnight at 37 °C.

Yeast transformation

Yeast transformation was done using a modified alkali ion transformation procedure (Ito *et al.*, 1983). Cells grown overnight to exponential phase of growth ($1-2 \times 10^7$ cells/ml) in YEPD broth at 30 °C were harvested at 2000 rpm for 5 minutes and washed once with sterile distilled water. The pellet was resuspended very carefully in 0.1M lithium acetate at one third of the original volume. The culture was shaken slowly (about 40 rpm) at 30 °C for 30 minutes and cells were pelleted at 2000 rpm for 5 minutes. The pellet was resuspended to 5×10^8 cells/ml in 0.1 M lithium acetate and 100 μl of these cells were

placed in microcentrifuge tubes. DNA (1-5 μ g) was then added to the cells and after incubation at 30 °C for 30 minutes (no shaking) an equal volume of 70% PEG 4000 (Sigma) was added and incubated for another hour at 30 °C (no shaking). The cells were then placed for 5 minutes at 42 °C and pelleted for 20 seconds in a microcentrifuge. The pellet was resuspended in 1 M sorbitol and 200-300 μ l of cells were plated on appropriate media. Plates were incubated at 30 °C for 4 days or longer.

Southern blotting

Southern blotting (Southern, 1975) was done as described in Maniatis *et al.* (1982) with a few modifications. Briefly, genomic DNA was digested for at least 3 hours or longer with restriction enzymes and electrophoresed on 0.8% agarose gels (1-1.5 volts/cm for 14-18 hours). Lambda DNA digested with *Hind III* was used as a size marker. The gel was placed on two 3MM Whatman paper wicks on a platform in a Pyrex dish with the wick ends in 0.4 N NaOH. A nylon membrane (ICN) was placed over the gel followed by two pieces of 3MM Whatman paper and a stack of paper towels cut to the same dimension. Parafilm was placed over the edges of the platform to prevent the towels from contacting the transfer buffer. The entire assembly was covered with Saran wrap and left overnight at room temperature. A glass weight was placed on the top and left overnight at room temperature. After transfer was complete the gel was stained with ethidium bromide to ensure that DNA was completely transferred to the nylon membrane. The membrane was dipped in 2X SSC and placed between two 3MM Whatman papers and dried in a vacuum oven for one hour at 70 °C. The membrane was stored at room temperature until ready to use.

Prehybridization was done at 45 °C for 12-18 hours in a rotary oven with 20 ml of the following solution : 6X SSC, 0.1% SDS or Sarkosyl, 5X Denhardt's solution and 100 μ g/ml denatured calf thymus DNA. A 20X SSC stock solution was made by combining

175.3 g NaCl and 88.2 g sodium citrate in distilled water and adjusting the pH to 7.0 and making the volume to 1 liter. A 50X stock of Denhardt's solution was made by combining 5 g ficoll, 5 g polyvinylpyrrolidone and 5 g BSA in distilled water to a final volume of 500 ml and the stock solution was divided into portions and frozen at -20 °C.

The *S. cerevisiae* *PTR2* gene was labeled with $\alpha^{32}\text{P}$ -dCTP using the BRL Nick Translation System. Briefly, this consisted of adding the DNA to a solution of unlabeled deoxynucleotides (0.2 mM dATP, dGTP, dTTP), 5X nick translation buffer, $\alpha^{32}\text{P}$ -dCTP (ICN) and distilled water. DNaseI-DNA polymerase mixture was added to the reaction tube and incubated at 15 °C for 1 hour. The plasmid pCH19 which contained the *OPP* operon (Hiles *et al.*, 1987) and the *SacI*-*Sall* fragment from YEpSTE6 containing the *S. cerevisiae* *STE6* gene (McGrath & Vasrshavsky, 1989) were labeled with $\alpha^{32}\text{P}$ -dCTP using the random primer labeling kit from USB. Briefly, the DNA (1-5 μg) was denatured for 10 minutes at 95 °C and chilled on ice. The deoxynucleotides dATP, dGTP and dTTP were added followed by the addition of a random hexanucleotide mixture and $\alpha^{32}\text{P}$ -dCTP (ICN) and Klenow enzyme. The reaction mixture was incubated at 37 °C for 2 hours and then terminated by heating at 65 °C for 10 minutes. The radioactive probes were retrieved using a Sephadex G-50 spun column previously equilibrated with column buffer (50 mM Tris pH 7.9, 250 mM NaCl, 2 mM EDTA and 0.5% SDS). The yield was approximately $3\text{-}4 \times 10^7$ cpm/ μg of DNA. The probe was stored at -20 °C until ready to use. The probe (5×10^6 cpm) was made single stranded by adding it to 0.5 ml of distilled water, placing in a boiling water bath for 2 minutes and then quickly adding it to the bag containing prehybridization solution. The bags containing the probe were placed in another bag and heat sealed to limit any leakage of radioactive material. The blots containing the probe were incubated for 20-24 hours at 65 °C in a rotary oven. Blots were washed with different concentrations of SSC with 0.1% SDS at the indicated temperatures for 30 minutes (2X). Autoradiography was performed at -70 °C in Kodak film holders using Kodak XAR-5 X-

Ray film placed between two DuPont Cronex Lighting-Plus intensifying screens. Exposure times varied according to the probe.

Agarose gel electrophoresis of DNA

For quick gel electrophoresis the mini-gel apparatus supplied by BRL was used. For Southern blotting procedures the horizontal gel apparatus by Hoeffer which accommodates 5.5 cm x 3.8 cm gels was used. All agarose gels were prepared in 1X TAE buffer which contained 40 mM Tris-acetate and 1 mM EDTA (pH 8.0). Agarose gels containing 0.6% to 1.2% agarose were used depending on the analysis to be done. DNA samples (about 20 μ l) containing 4 μ l of the loading dye (25% Ficoll, 0.1% Bromophenol blue and 1mM EDTA) were loaded on the gels. Electrophoresis was done at 80 Volts until the dye front was about 0.5 inches from the edge. Gels were stained with ethidium bromide (0.5 μ g/ml) and photographed with either Polaroid Type 57 or Type 667 film and Kodak number 23A Wratten gelatin filter upon illumination by a Mineralight 302 nm, long wave ultraviolet light transilluminator (Fisher).

Growth assays

Growth assays to determine the phenotype of the cells were done as described in Island *et al.* (1991). Briefly 3 μ l of culture from a suspension of $1 \cdot 10^7$ cells/ml was applied to the surface of the medium and plates were incubated at 30 °C for 48 hours or longer.

Screening of *S. cerevisiae* transformants with a PTR2⁺ phenotype

S. cerevisiae PB1X-9B was transformed with the YEp13 library of *C. albicans* generated by Rosenbluh *et al.* (1985) and about 7,800 LEU⁺ transformants were selected on CM-Leu medium. About 8,000 *S. cerevisiae* LEU⁺ transformants should ensure a 99% probability that each DNA sequence is represented in the library at least once (Maniatis

et al., 1982) considering an average insert size of 10 kb and the genome size of *C. albicans* $\approx 2 \times 10^7$ bp (Riggsby *et al.*, 1982). *S. cerevisiae* transformants from CM-Leu medium were pooled and 1 ml portions were frozen in 15% glycerol (final concentration). A slightly different procedure was used to screen the *C. albicans* YEp13 library of Gillum *et al.* (1984). *S. cerevisiae* PB1X-9B was transformed with the amplified *C. albicans* YEp13 library and transformants were plated on either CM-Leu+Trp or CM-Leu plates. *S. cerevisiae* transformants obtained on CM-Leu medium were pooled into three pools, Pool 1, 2, and 3 each of which contained 3,000, 4,200, and 4,800 transformants, respectively. Transformants obtained on CM-Leu+Trp medium were pooled into Pools 4 and 5 which contained 5,400 and 9,000 transformants, respectively. For selection of *S. cerevisiae* transformants with a PTR2⁺ phenotype the LEU⁺ transformants were plated on medium containing dipeptides as described and incubated at 30 °C for 4-5 days. Transformants that could utilize the defined dipeptides to satisfy the amino acids auxotrophic requirements were picked and restreaked on dipeptide medium. The auxotrophic markers of the transformants were checked using "drop out" plates and growth assays as described elsewhere. Sensitivity of the transformants to ethionine and Ala-Eth was also determined. *S. cerevisiae* transformants obtained after transformation of *S. cerevisiae* PB1X-9B with the *C. albicans* library will be referred to as primary transformants. The mutant strain *S. cerevisiae* PB1X-9B transformed with the vector YEp13 was resistant to Ala-Eth and was unable to grow on any of the dipeptide medium tested (reversion frequency $> 10^{-8}$).

Disk sensitivity assays

Sensitivity to ethionine and Ala-Eth was determined by the method described by Island *et al.* (1987). Briefly, cells were grown overnight to exponential phase of growth in MM with appropriate supplements as needed, washed, and resuspended to 5×10^6 cells /ml in

sterile water. One ml of this cell suspension was added to 3 ml molten Noble agar (0.8% final concentration) and overlayed on 20 ml of the same medium used to prepare the inoculum. A disk (6 mm diameter, Whatman 3MM filter paper) was placed on the plate and either ethionine or Ala-Eth (0.38 μ moles each) was spotted on the disk. Zones of inhibition were measured after 24-48 h incubation at 30 °C.

Effect of limiting amounts of tryptophan on growth of *S. cerevisiae* PTR2⁺ transformants

The *S. cerevisiae* PTR2⁺ transformants J7, PT1 and PT5 which were dependent on tryptophan for growth were grown in CM-Leu+Trp broth overnight at 30 °C shaking at 200 rpm. Cells were harvested, washed once with distilled water and resuspended to 5 x 10⁶ cells/ml in distilled water. A microtiter plate containing 24 wells was used to prepare base agar that contained CM-Leu+Trp medium with a 2-fold serial dilution of tryptophan such that the first well contained 1.5 x 10³ μ g/ml and the last well contained 1.47 μ g/ml of tryptophan. A lawn of cells (2.5 x 10⁵ cells) in Noble agar (0.8%) was poured over the base agar. Plates were incubated at 30 °C for 48 hours and presence or absence of growth in each of the wells was scored. Plates were placed under a transilluminator to determine the fluorescence associated with the accumulation of a fluorescent intermediate.

Plasmid curing experiment

S. cerevisiae transformants were grown nonselectively in YEPD broth for about 40 generations. Cells were then plated on YEPD plates to obtain isolated colonies. Isolated colonies from YEPD agar plates were checked for the auxotrophic markers, sensitivity to ethionine and Ala-Eth, and the ability to grow on dipeptides.

Tetrad analysis

The crosses were performed as previously described by Sherman *et al.* (1986). Haploid strains were mated on YEPD agar at 30 °C for 6-9 hours and diploids were isolated by micromanipulation. Diploid cells were grown on presporulation medium at 30 °C for 2 days and then transferred to sporulation medium and incubated at 30 °C for 3-5 days. The tetrads were dissected by micromanipulation and the ascospores were allowed to germinate on YEPD agar at 30 °C. The phenotype of the cells was assayed using growth assays as described earlier.

Chemicals and reagents

Ala-Eth was synthesized by standard solution phase techniques (Becker & Naider, 1977 ; Naider *et al.*, 1983), Ethionine, His-Leu, Lys-Leu, Lys-Met and His-Lys were obtained from Sigma Chemical Company, St Louis, MO.

CHAPTER III

RESULTS

ATTEMPTS TO CLONE A *C. ALBICANS* PEPTIDE TRANSPORT GENE BY NUCLEIC ACID HYBRIDIZATION

We determined if genes involved in transport from other organisms hybridized to whole cell DNA from *C. albicans*. Three different probes were used : *PTR2* the amino acid inducible dipeptide transport gene of *S. cerevisiae* (Perry *et al.* unpublished) ; *STE6* of *S. cerevisiae* which is involved in the export of mating pheromone α -factor from MATa cells (Kuchler *et al.*, 1989 ; McGrath & Varshavsky, 1989) ; and *OPP* operon of *Salmonella typhimurium* which encodes the oligopeptide permease, a periplasmic binding protein-dependent transport system that transports peptides containing two to six amino acids (Hiles *et al.*, 1987).

Southern blot analysis with *Saccharomyces cerevisiae* *PTR2* gene as a probe

A nick translated 2.2 kb *HindIII-EcoRI* fragment from plasmid pJP9 (Table 2) containing the *PTR2* gene of *S. cerevisiae* was used as a probe for Southern blot analysis. Whole cell DNA from *C. albicans* and *S. cerevisiae* digested with different restriction enzymes were electrophoresed on an agarose gel (Fig. 1) An autoradiogram (Fig. 2) showed that the *S. cerevisiae* *PTR2* probe hybridized to the expected 3.2 kb *BamHI* fragment (Lane 6), and a 4.5 kb *EcoRI* fragment (Lane 5) from whole cell DNA of *S. cerevisiae*. A larger *BamHI* (>20 kb) fragment was also observed to hybridize to the probe and this may be due to an upstream *BamHI* site. No hybridization of the *S. cerevisiae* *PTR2* probe to whole cell *C. albicans* DNA was observed (Lanes 2 to 5) even when the blot was washed at low stringency of 6X SSC and 0.1% SDS at 50°C for

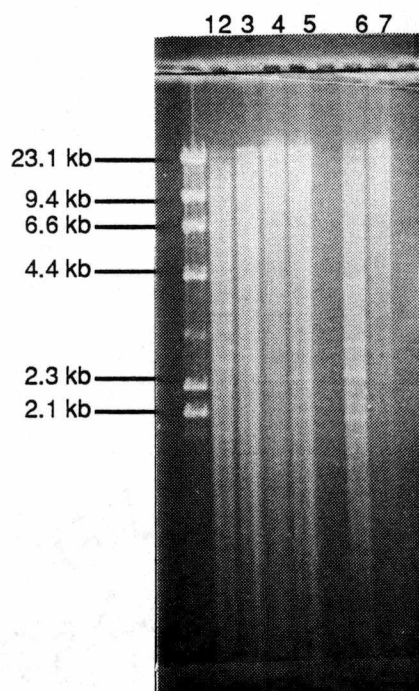


Fig. 1. Agarose gel electrophoresis of whole cell DNA from *S. cerevisiae* and *C. albicans*. Lane 1- DNA size standard marker (Lambda *HindIII* digest), Lanes 2-5 contain whole cell DNA from *C. albicans* digested with *EcoRI* (Lane 2), *HindIII* (Lane 3), *SalI* (Lane 4), and *PstI* (Lane 5). Lanes 6 and 7 contain whole cell DNA from *S. cerevisiae* digested with *EcoRI*(Lane 6), and *BamHI* (Lane 7).

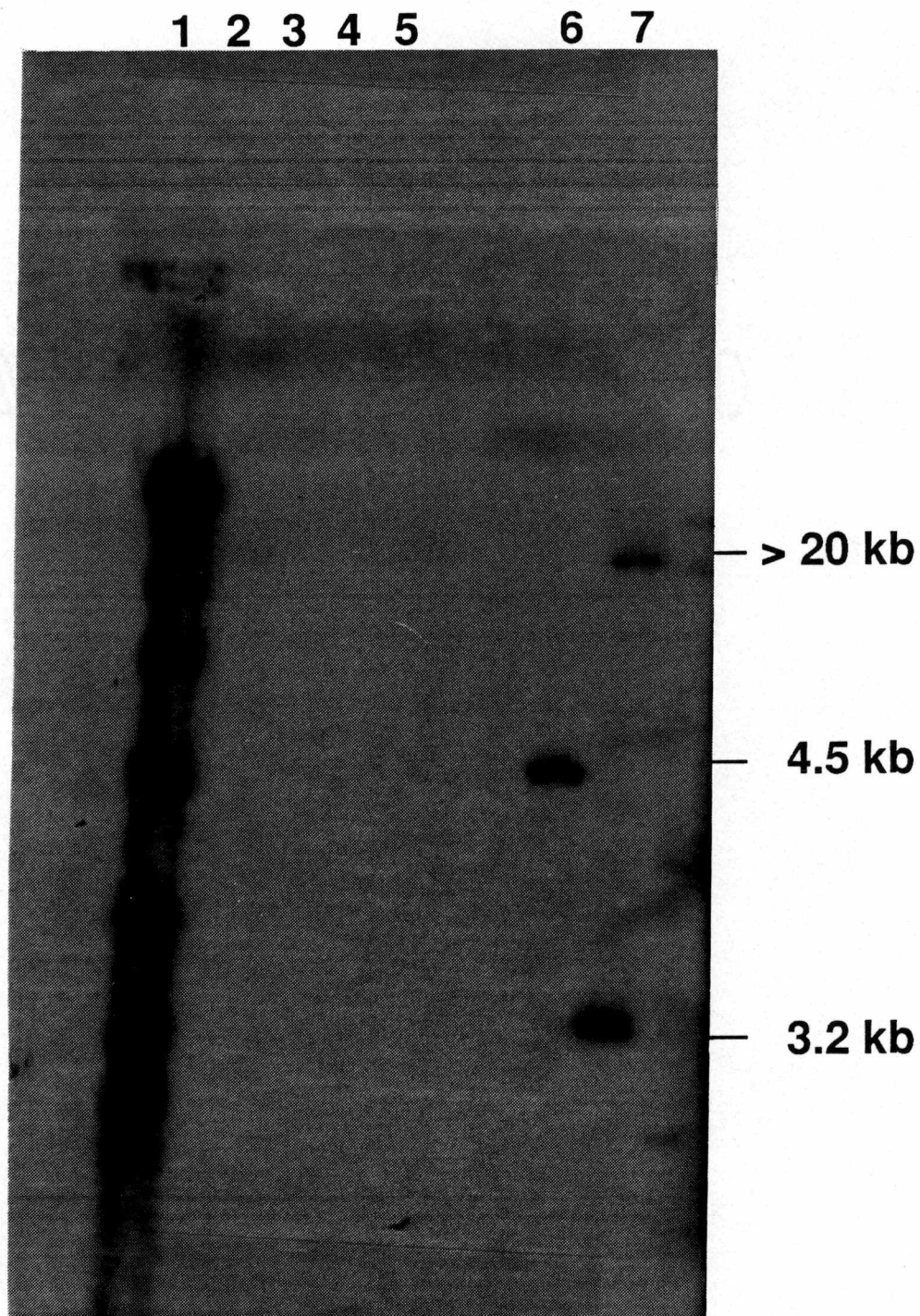


Fig. 2. Southern blot analysis of whole cell DNA from *C. albicans* and *S. cerevisiae* probed with *S. cerevisiae* *PTR2* gene. The gel in Fig. 1 was blotted and probed with a 2.2 kb *EcoRI-HindIII* fragment containing the *S. cerevisiae* *PTR2* gene. The blot was washed in 6X SSC and 0.1% SDS at 50 °C for 30 minutes (2X) and the film was exposed for 48 hours.

30 minutes (2X). These results suggest that *PTR2*, the amino acid inducible peptide transport gene of *S. cerevisiae*, cannot be used as a probe to clone a *C. albicans* *PTR2* homolog by nucleic acid hybridization.

Southern blot analysis with *Saccharomyces cerevisiae* *STE6* gene as a probe

Whole cell DNA from *C. albicans* and *S. cerevisiae* digested with different restriction enzymes was electrophoresed on an agarose gel (Fig. 3). A *SacI-SalI* fragment of YEp352 (Table 2) containing the *S. cerevisiae* *STE6* gene was used as a probe for Southern blot analysis. An autoradiogram (Fig. 4) showed that the *STE6* probe hybridized to the expected 1.2 kb *EcoRI* fragment from *S. cerevisiae*. A larger *EcoRI* fragment was also observed to hybridize to the probe and this may be due to a upstream *EcoRI* site. The *STE6* probe also hybridized to a >23 kb *HindIII* fragment of *S. cerevisiae* and this may be due to an upstream *HindIII* site. The *S. cerevisiae* *STE6* gene did not hybridize to whole cell DNA from *C. albicans* even when the blot was washed at very low stringency of 10X SSC and 0.1% SDS at 45 °C for 30 minutes (2X).

Southern blot analysis with *Salmonella typhimurium* *Opp* operon as a probe

Whole cell DNA from *C. albicans* and *S. cerevisiae* digested with different restriction enzymes was electrophoresed on an agarose gel (Fig. 3). The radiolabeled plasmid pCH19 (Table 2) which contains *Opp A*, *OppB*, *Opp C* and a part of *Opp D* gene was used as a probe. The labeled plasmid pCH19 did not show hybridization to whole cell DNA from *S. cerevisiae* or *C. albicans* even when the blot was washed at low stringency of 10X SSC and 0.1% SDS at 45 °C for 30 minutes (2X) (data not shown).

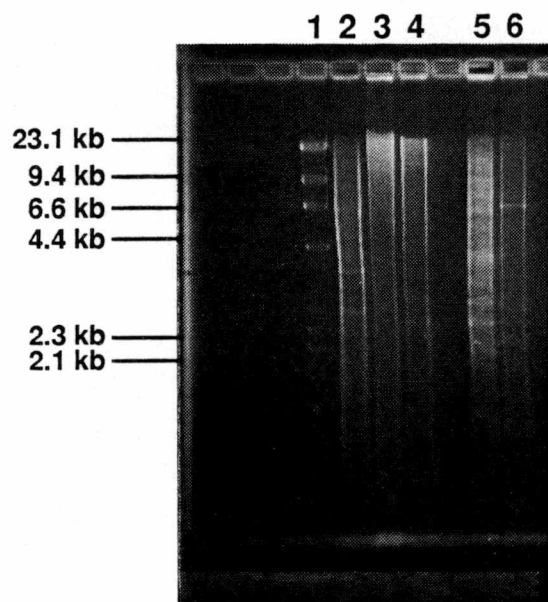


Fig. 3. Agarose gel electrophoresis of whole cell DNA from *S. cerevisiae* and *C. albicans*. Lane 1-DNA size standard marker (Lambda *HindIII* digest), Lanes 2-4 contain whole cell DNA from *C. albicans* digested with *EcoRI* (Lane 2), *HindIII* (Lane 3), and *PstI* (Lane 4). Lanes 5 and 6 contain whole cell DNA from *S. cerevisiae* digested with *EcoRI*(Lane 5), and *HindIII* (Lane 6).

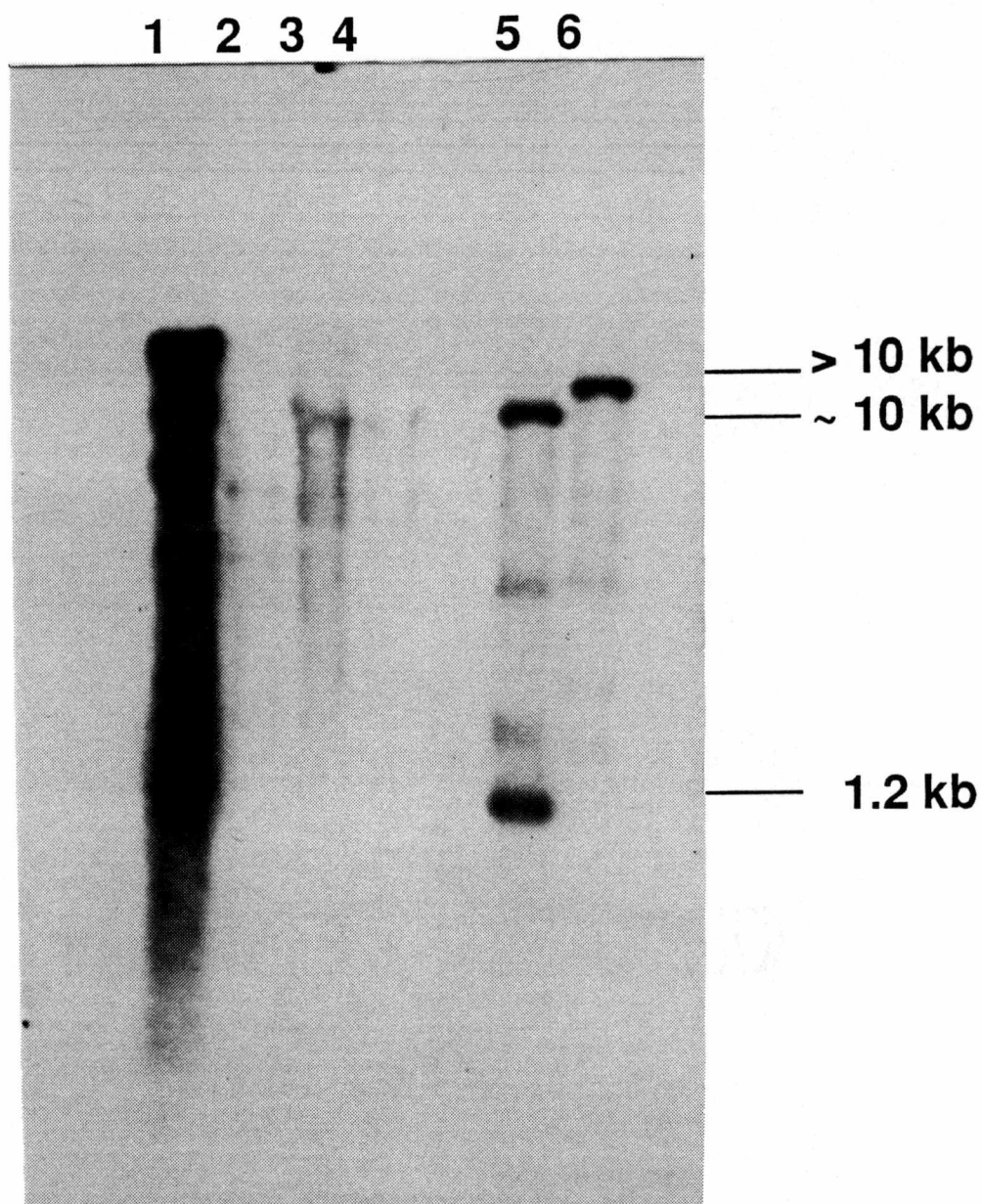


Fig. 4. Southern blot analysis of whole cell DNA from *C. albicans* and *S. cerevisiae* probed with *S. cerevisiae* *STE6* gene. The gel in Fig. 3 was blotted and probed with a 5.9 kb *SacI-SalI* fragment containing the *S. cerevisiae* *STE6* gene. The blot was washed in 10X SSC and 0.1% SDS at 45 °C for 30 minutes (2X) and the film was exposed for 20 hours.

ATTEMPTS TO CLONE A *C. ALBICANS* GENE BY FUNCTIONAL COMPLEMENTATION

Three *C. albicans* libraries which included one library in a low copy vector pYSK35 (Singer *et al.*, 1989) and two libraries in the multi-copy vector YEp13 (Gillum *et al.*, 1984 ; Rosenbluh *et al.*, 1985) were screened to determine if the *ptr2* mutation in *S. cerevisiae* could be functionally complemented by a *C. albicans* gene. The *S. cerevisiae ptr2* mutant, PB1X-9B, which is resistant to the toxic dipeptide Ala-Eth and is unable to use defined dipeptides to satisfy its amino acid auxotrophic requirements of amino acids, was the host for transformation.

Attempts to clone a *C. albicans* PTR2 homolog using a centromere containing plasmid library

Since cloning of a *C. albicans* gene that restores dipeptide transport in the mutant strain *S. cerevisiae* PB1X-9B involves plating the transformants on dipeptide medium, it was necessary to determine the range of dipeptide substrates that could be used by *C. albicans* and *S. cerevisiae*. Auxotrophic strains *C. albicans* WD18-6 and *S. cerevisiae* PB1X-2A could use the dipeptides Lys-Met, Lys-Leu, His-Leu or His-Lys to satisfy the amino acid requirements of lysine, methionine, histidine or leucine in dipeptide medium with the appropriate supplements.

Sequence analysis of the *S. cerevisiae* PTR2 gene suggests that it may be a membrane protein with 10-12 hydrophobic membrane spanning domains (Perry *et al.*, unpublished). It is possible that overexpression of a membrane protein may have an adverse effect on the cell. Hence, we attempted to clone a *C. albicans* PTR2 homolog by using a *C. albicans* library in the low copy vector pYSK35 (Singer *et al.*, 1989). The *S. cerevisiae* shuttle vector pYSK35 carries the *TRP1* and *LEU2* genes of *S. cerevisiae* as well as the CEN sequence to maintain the plasmid at a low copy number. The mutant strain *S. cerevisiae*

PB1X-9B was transformed with the *C. albicans* library in pYSK35 and about 5,500 LEU⁺ transformants were obtained on CM-Leu medium. The *S. cerevisiae* LEU⁺ transformants were pooled and a portion of these was plated on dipeptide medium containing either His-Lys (for both histidine and lysine requirement) or His-Leu (for histidine requirement) + Lys-Leu (for lysine requirement) with tryptophan (0.15 mM). The tryptophan in the medium served as an inducer for peptide transport (Island *et al.*, 1987 & 1991). No transformants were obtained on either of the dipeptide media. Thus, a *C. albicans* PTR2 homolog was not cloned from the *C. albicans* centromere library in pYSK35. As a control, the *S. cerevisiae* LEU⁺ transformants were also plated on CM-Leu-Lys. Two transformants were obtained on CM-Leu-Lys and these may contain a *C. albicans* LYS1 homolog.

Attempts to clone a *C. albicans* PTR2 homolog using the YEp13 library of Rosenbluh *et al.* (1985)

The inability to clone a *C. albicans* gene involved in peptide transport using the low copy centromere library in the vector pYSK35 may be due to poor expression of the gene in *S. cerevisiae* (Kurtz *et al.*, 1990). Hence, we decided to use a *C. albicans* library in a multi-copy plasmid vector YEp13. The mutant strain *S. cerevisiae* PB1X-9B was transformed with the *C. albicans* YEp13 library of Rosenbluh *et al.* (1985) and about 7,800 LEU⁺ transformants were obtained on CM-Leu medium. The pooled *S. cerevisiae* LEU⁺ transformants were plated on dipeptide medium containing His-Lys or Lys-Leu + His-Leu containing the amino acid inducers arginine, isoleucine, threonine or tryptophan (0.15 mM). Isoleucine, threonine and tryptophan are good inducers for the peptide transport system of *S. cerevisiae* (Island *et al.*, 1987 & 1991) and arginine is a good inducer for the peptide transport system of *C. albicans* (Part II, this dissertation). Colonies were obtained only on dipeptide medium containing tryptophan. Three *S. cerevisiae* transformants J7,

PT1 and PT5 were chosen for further studies ; these were sensitive to ethionine and Ala-Eth and were able to grow on the dipeptides Lys-Met, Lys-Leu, His-Leu or His-Lys with tryptophan (0.15 mM) (Table 3). It was possible that all the transformants identified represented one original clone as we plated pooled *S. cerevisiae* LEU⁺ transformants on the dipeptide medium.

Whole cell DNA isolated from the primary *S. cerevisiae* transformant PT5 was used to transform *E. coli* HB101. Restriction enzyme analysis of the plasmid pCaPTR2 from the *E. coli* transformant showed the expected YEp13 vector sequences and an insert of about 7 kb (Fig. 5). Plasmid DNA from the *E. coli* transformant was amplified and used to transform the mutant strain *S. cerevisiae* PB1X-9B. No *S. cerevisiae* transformants were obtained on dipeptide medium, however, *S. cerevisiae* LEU⁺ transformants were obtained on CM-Leu medium.

A plasmid curing experiment was done to determine if the PTR2⁺ phenotype of the primary *S. cerevisiae* transformants was due to a plasmid borne gene. All three primary *S. cerevisiae* transformants J7, PT1 and PT5 when subjected to plasmid curing retained the *LEU2* marker, could grow on dipeptide, were sensitive to ethionine and Ala-Eth and still needed tryptophan for growth. We were not able to cure the PTR2⁺ phenotype of the primary *S. cerevisiae* transformants.

Peculiarly, we found that the primary *S. cerevisiae* transformants J7, PT1 and PT5 all had an absolute requirement for tryptophan for growth (Table 3) even though *S. cerevisiae* PB1X-9B, the transformation recipient did not have a *trp* marker. Since the *S. cerevisiae* transformants J7, PT1 and PT5 were dependent on tryptophan for growth, it was of interest to determine whether a particular step in the tryptophan biosynthetic pathway, was blocked (for review Braus, 1991). Mutants of *S. cerevisiae* that accumulate anthranilic acid, an intermediate in the tryptophan biosynthetic pathway fluoresce when placed under a transilluminator if grown in limiting amounts of tryptophan. We used a microtiter plate

TABLE 3. Growth of *S. cerevisiae* transformants obtained from the *C. albicans* library of Rosenbluh *et al.*(1985) ^a

Media ^b	<i>S. cerevisiae</i> with YEp13 ^c	<i>S. cerevisiae</i> PTR2 ⁺ transformants ^d
CM	+	+
CM-Leu	+	-
CM-Leu +Trp	+	+
CM-Leu +Trp +AE	R	S
CM-Leu +Trp +E	S	S
Dipeptide medium	-	+

^a + = growth ; - = no growth ; R = resistant to toxic effect of either ethionine or Ala-Eth ; S = sensitive to toxic effect of either ethionine or Ala-Eth.

^b Media were as described in Methods. CM-Leu+Trp+E = CM-Leu+Trp plus ethionine (80 μ M) ; CM-Leu+Trp+ AE = CM-Leu+Trp plus Ala-Eth (40 μ M) ; Dipeptide medium contained the dipeptides Lys-Met, Lys-Leu, His-Leu or His-Lys with tryptophan.

^c *S. cerevisiae* PB1X-9B transformed with the vector YEp13.

^d *S. cerevisiae* PTR2⁺ transformants J7, PT1 and PT5 were obtained as described in Methods after screening the *C. albicans* YEp13 library of Rosenbluh *et al.*(1985). Each isolate gave an identical phenotype.

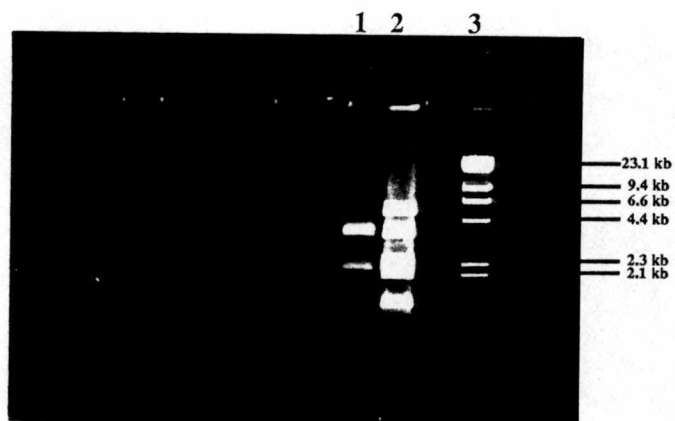


Fig. 5. Restriction enzyme analysis of plasmid pCaPTR2. A *HpaI*-*PvuII* digest of the DNA to be analyzed was run on a 1% agarose gel. Lane 1-YEp13 vector. Lane 2- plasmid pCaPTR2 isolated from the *S. cerevisiae* transformant PT5 and amplified in *E. coli* HB101. Lane 3-DNA size standard marker (Lambda *HindIII* digest).

assay and determined that the *S. cerevisiae* transformants J7, PT1 and PT5 fluoresced when grown in the presence of limiting amounts of tryptophan ($\leq 1.9 \times 10^{-2}$ $\mu\text{g/ml}$) but not at higher concentrations of tryptophan (Table 4). Cell growth and fluorescence was observed even at tryptophan concentrations as low as 1.5 $\mu\text{g/ml}$. *S. cerevisiae* PB1X-9B transformed with the YEpl3 vector did not show any fluorescence (data not shown). Disk assays were done to determine if anthranilic acid or chorismic acid which are intermediates of the tryptophan biosynthetic pathway could support the growth of the *S. cerevisiae* transformants. Neither anthranilic acid (81.75 μg , 8.18 μg , 1.64 μg or 0.33 μg) nor chorismic acid (108 μg , 21.6 μg , 4.32 μg or 0.86 μg) spotted on disks placed over a lawn of cells could support the growth of the primary *S. cerevisiae* transformants J7, PT1 or PT5 (data not shown).

In order to further characterize the *S. cerevisiae* transformants with PTR2^+ phenotypes we determined the sensitivity of *S. cerevisiae* J7, PT1 and PT5 to ethionine and Ala-Eth. Disk assays showed that the sensitivity of *S. cerevisiae* J7 to ethionine was comparable to that of *S. cerevisiae* *PTR2* transformed with YCp50 or pRS201 vectors (Table 5). Ala-Eth sensitivity, however, varied with the composition of the medium and the transformant. The *ptr2* mutant, *S. cerevisiae* PB1X-9B, transformed with either of the vectors pRS201 or YCp50 was sensitive to ethionine and resistant to Ala-Eth. The wild type *S. cerevisiae* PB1X-2A transformed with pRS201 vector displayed complete sensitivity to Ala-Eth in CM-Ura and CM-Ura+Trp medium but minimal sensitivity in CM-Leu medium. The zone of inhibition with Ala-Eth in assay medium containing tryptophan (CM-Leu+Trp) was also not very clear and growth could be observed in the zone of inhibition. CM-Ura medium contains histidine, lysine and leucine, and the latter amino acid is a good inducer for dipeptide transport in *S. cerevisiae* (Island *et al.* 1987). On the other hand CM-Leu contains uracil, histidine and lysine ; the lack of leucine in this medium may explain why a very hazy zone of inhibition was observed with Ala-Eth. The *S. cerevisiae* transformant J7

TABLE 4. Effect of limiting amounts of tryptophan on growth of *S. cerevisiae* PTR2⁺ transformants ^a

Tryptophan ^b (µg/ml)	Growth ^c	Fluorescence ^c
1.5 x 10 ³	+	-
7.5 x 10 ²	+	-
3.8 x 10 ²	+	-
1.9 x 10 ²	+	-
9.4 x 10 ¹	+	+
4.7 x 10 ¹	+	+
2.3 x 10 ¹	+	+
1.1 x 10 ¹	+	+
5.9	+	+
2.9	+	+
1.5	+/- ^d	+
None	-	-

^a *S. cerevisiae* PTR2⁺ transformants J7, PT1 and PT5 were obtained as described in Methods after screening the *C. albicans* YEp13 library of Rosenbluh *et al.* (1984). All three transformants gave similar results.

^b Lawn of *S. cerevisiae* PTR2⁺ cells was made over base agar that consisted of CM-Leu medium with different concentrations of tryptophan as indicated.

^c + = presence of growth or cellular fluorescence ; - = absence of growth or cellular fluorescence.

^d +/- = barely visible growth.

TABLE 5. Disk sensitivity assays of *S. cerevisiae* transformants

Strain used ^a	Plasmid ^b	Media ^c	Zone of inhibition (mm) ^d	
			Eth	Ala-Eth
<i>S. cerevisiae</i> PB1X-2A [isogenic to PB1X-9B except <i>PTR2</i>]	YCp50	CM-Ura	49	45
		CM-Ura+Trp	46	42
	pRS201	CM-Leu	43	20 ^e
		CM-Leu+Trp	48	40 ^f
<i>S. cerevisiae</i> PB1X-9B [isogenic to PB1X-2A except <i>ptr2</i>]	YCp50	CM-Ura	52	0
		CM-Ura +Trp	ND ^g	ND
	pRS201	CM-Leu	50	0
		CM-Leu+ Trp	52	0
<i>S. cerevisiae</i> <i>PTR2</i> ⁺ transformant J7		CM-Leu	NG ^h	NG
		CM -Leu+Trp	45	43

^a The genotype of the *S. cerevisiae* strains which are as described in Table 1. *S. cerevisiae* *PTR2*⁺ transformant J7 is *S. cerevisiae* PB1X-9B carrying a putative *C. albicans* *PTR2* homolog from the YEp13 library of Rosenbluh *et al.* (1985). Strains PT1 and PT5 responded the same as the J7 strain.

^b The plasmids used for transformation can be found in Table 2, the pRS201 plasmid has *LEU2* as the selectable marker and YCp50 has *URA3* as the selectable marker.

^c The composition of the media is as described in Methods, for example, CM-Ura is MM with histidine, lysine and leucine without uracil, CM-Ura +Trp is CM-Ura plus tryptophan. CM-Leu is MM with histidine, lysine and uracil without leucine. CM-Leu+Trp is CM-Leu plus tryptophan.

^d Disk sensitivity assays were done as described in Methods. Ethionine or Ala-Eth (0.38μ moles) was spotted on disks (diameter of disk is 6 mm).

^e Zone of inhibition was extremely hazy and growth was observed within the zone.

^f Zone of inhibition was hazy, outline of the zone was not well defined and some growth was observed within the zone.

^g ND- not determined.

^h NG- no growth as the cells need tryptophan to grow.

responded with well-defined zones of inhibition to Ala-Eth in CM-Leu+Trp medium. Sensitivity of *S. cerevisiae* transformants PT1 and PT5 to ethionine and Ala-Eth was not significantly different (data not shown) from that of the *S. cerevisiae* transformant J7.

Attempts to clone a *C. albicans* PTR2 homolog using the YEp13 library of Gillum *et al.* (1984)

Attempts to clone a *C. albicans* PTR2 homolog from the *C. albicans* centromere library in pYSK35 (Singer *et al.*, 1989) as well as the YEp13 library of Rosenbluh *et al* (1985) failed to yield a plasmid containing a functional *C. albicans* PTR2 homolog. Therefore, we decided to screen yet another *C. albicans* YEp13 library generated by Gillum *et al.* (1984). *S. cerevisiae* PB1X-9B transformed with the amplified *C. albicans* YEp13 library was plated on either CM-Leu+Trp or CM-Leu plates, and LEU⁺ transformants were pooled as described in Methods. A portion of each of the pooled transformants was plated on CM-Leu-Lys or CM-Leu-Ura as a control to determine whether the *LYS1* or *URA3* homologs of *C. albicans* could be cloned from the library. LYS⁺ and URA⁺ transformants were obtained on these media. A portion of the transformants from each of the five pools was also plated on dipeptide medium with His-Lys or His-Leu+Lys-Leu with tryptophan (0.15 mM). Several colonies were obtained from each of the five pools on the dipeptide medium. Colonies were streaked again on dipeptide medium and several of the colonies from Pool 5 grew on this medium. A primary *S. cerevisiae* transformant PMB5 was chosen for further studies and it was found to be sensitive to ethionine and Ala-Eth, could utilize the dipeptides Lys-Met, Lys-Leu, His-Leu and His-Lys, and had the correct auxotrophic markers (Table 6). Plasmid DNA isolated from *S. cerevisiae* PMB5 was used to transform two different *E. coli* strains : *E. coli* HB101 and *E. coli* RR1. The *E. coli*

TABLE 6. Growth of *S. cerevisiae* transformant obtained from the *C. albicans* library of Gillum *et al.*(1984) ^a

Media ^b	<i>S. cerevisiae</i> with YEp13 ^c	<i>S.cerevisiae</i> PMB5 ^d
CM	+	+
CM-Leu	+	+
CM-Leu + AE	R	S
CM-Leu + E	S	S
Dipeptide medium	-	+

^a + = growth ; - = no growth ; R = resistant to toxic effect of either ethionine or Ala-Eth ; S = sensitive to toxic effect of either ethionine or Ala-Eth.

^b Media were as described in Methods. CM-Leu+Trp+E = CM-Leu+Trp plus ethionine (80 μ M) ; CM-Leu+Trp+ AE = CM-Leu+Trp plus Ala-Eth (40 μ M) ; Dipeptide medium contained the dipeptides Lys-Met, Lys-Leu, His-Leu or His-Lys with tryptophan.

^c *S. cerevisiae* PB1X-9B transformed with the vector YEp13.

^d *S.cerevisiae* PMB5 transformant was obtained from Pool 5 as described in Methods after screening the *C. albicans* YEp13 library of Gillum *et al.* (1984).

RR1 strain was used to make the *C. albicans* YEp13 library of Gillum *et al.* (1985). Plasmid DNA recovered from both *E. coli* strains showed similar restriction enzyme pattern that indicated presence of YEp13 and an additional fragment that may correspond to a *C. albicans* insert. Plasmid DNA amplified in the *E. coli* strains RR1 and HB101 was used to transform *S. cerevisiae* PB1X-9B. Transformed cells were plated on CM-Leu, CM-Leu+Trp and dipeptide medium. Transformants were obtained on both CM-Leu and CM-Leu+Trp but none on dipeptide medium.

A plasmid curing experiment was done to determine if the $PTR2^+$ phenotype of the primary *S. cerevisiae* transformant PMB5 was due to a plasmid borne gene. The primary *S. cerevisiae* transformant subjected to a plasmid curing experiment lost *LEU2*, the marker on YEp13 plasmid, however, the cured cells still had the ability to grow on dipeptide medium and were sensitive to ethionine and Ala-Eth. Thus, the $PTR2^+$ phenotype could not be cured from the primary *S. cerevisiae* transformant.

The phenotype of the *S. cerevisiae* transformant PMB5 was characterized further by determining the sensitivity of the transformant to ethionine and Ala-Eth. The results of the disk sensitivity assays in CM-Leu+Trp medium were comparable to those reported earlier for the primary *S. cerevisiae* transformants J7, PT1 or PT5 (Table 5). Zones of inhibition of the *S. cerevisiae* PMB5 for ethionine and Ala-Eth in CM-Leu+Trp medium were 45 mm and 40 mm, respectively. The transformants also displayed sensitivity to Ala-Eth in CM-Leu medium, zones of inhibition were 52 mm and 40 mm for ethionine and Ala-Eth, respectively (data not shown). The wild type cells *S. cerevisiae* PB1X-2A displayed little or no sensitivity to Ala-Eth in CM-Leu medium.

A functional *C. albicans* *PTR2* homolog could not be recovered on a plasmid using three different libraries ; it seemed possible that a *C. albicans* *PTR2* homolog : (1) integrated into the chromosome, (2) recombined with the endogenous 2 μ m plasmid, or (3) the original *ptr2* mutation reverted to $PTR2^+$. In order to investigate this further we

crossed the *S. cerevisiae* transformant PMB5 with *S. cerevisiae* PB13X-1B, a *S. cerevisiae ptr2* strain of the opposite mating type, sporulated the diploid, and analyzed the phenotype of the 4 spores. If a *C. albicans PTR2* homolog integrated into the chromosome then a 2:2 segregation of the $PTR2^+$ phenotype would result. If on the other hand the gene was carried on a plasmid, then all the 4 spores should have the $PTR2^+$ phenotype. Spores resulting from a cross of *S. cerevisiae* PMB5 with *S. cerevisiae* PB13X-1B showed a 2:2 segregation of the auxotrophic markers *LEU*, *URA*, and *LYS* (Table 7). The phenotype resulting from *ptr2* mutation also segregated 2:2 ; these spores could grow on dipeptide medium and were sensitive to ethionine and Ala-Eth. The tetrads from the control cross of *S. cerevisiae* PB1X-9B containing YEp13 plasmid with *S. cerevisiae* PB13X-1B showed 2:2 segregation of the auxotrophic markers *HIS*, *LYS* and *URA*. In only one of the tetrads did the *LEU* marker segregate 4:0 as expected for a plasmid-borne trait. In six tetrads the *LEU* marker segregated 2:2 probably due to loss of plasmid under conditions nonselective for *LEU* during mating, sporulation and spore analysis. The spores from the tetrad that showed 4:0 segregation of the *LEU* marker was subjected to a plasmid curing experiment. Two of the spores showed loss of the *LEU* marker suggesting that the *LEU* marker in those spores was plasmid borne. Results from the cured cells of the control *S. cerevisiae* transformed with YEp13 also showed rapid loss of the *LEU* marker when cells were grown nonselectively (data not shown). Additionally, the control cross showed a 4:0 segregation of the phenotype resulting from the *ptr2* mutation as both parents of the cross are *ptr2* mutants.

TABLE 7. Tetrad analysis.

Cross ^a	# of tetrads analyzed	SEGREGATION PATTERN ^b				
		<i>HIS</i>	<i>LYS</i>	<i>URA</i>	<i>LEU</i>	<i>ptr2</i>
<i>S. cerevisiae</i>						
PB13X-1B						
X	7	2:2	2:2	2:2	2:2	2:2
<i>S. cerevisiae</i>						
PMB5						
<i>S. cerevisiae</i>						
PB13X-1B	6 ^c	2:2	2:2	2:2	2:2	4:0
X						
<i>S. cerevisiae</i>						
PB1X-9B	1 ^d	2:2	2:2	2:2	4:0	4:0
(YEp 13)						

^a Crosses were performed as described in Methods. *S. cerevisiae* PMB5 is the PTR2⁺ transformant from Pool 5 obtained after screening the *C. albicans* YEp13 library of Gillum *et al.* (1984). *S. cerevisiae* PB1X-9B (YEp13) is *S. cerevisiae* PB1X-9B transformed with YEp13 vector. *S. cerevisiae* PB13X-1B is a *ptr2* mutant of opposite mating type i.e. MAT α as described in Table 1.

^b Segregation of the auxotrophic markers was determined by dissecting the asci and the phenotype of the four ascospores was assayed as described in Methods ; the phenotype of *ptr2* was analyzed by determining the sensitivity or resistance to the toxic dipeptide Ala-Eth and the ability to grow on defined dipeptides.

^c Six out of the 7 tetrads analyzed showed 2:2 segregation of the auxotrophic markers *HIS*, *LYS*, *URA* and *LEU*.

^d One tetrad out of the 7 tetrads analyzed showed 2:2 segregation of *HIS*, *LYS*, *URA* and 4:0 segregation of the *LEU* marker.

CHAPTER IV

DISCUSSION

A *C. albicans* peptide transport gene could not be cloned by nucleic acid hybridization to transporters from other organisms. Southern blot analysis of whole cell DNA from *C. albicans* did not show hybridization to either the *S. cerevisiae* *PTR 2*, the amino acid inducible dipeptide permease (Island *et al.*, 1991 ; Perry *et al.*, unpublished), the *S. cerevisiae* *STE6* gene which pumps out the mating pheromone α -factor from MATa cells (McGrath & Varshavsky, 1989 ; Kuchler *et al.*, 1989), or the *OPP* operon of *Salmonella typhimurium* (Hiles *et al.*, 1987). This rules out the possibility of cloning the *C. albicans* peptide transport gene using these probes. The *OPP* operon of *S. typhimurium* did not show any hybridization to whole cell DNA from *S. cerevisiae* even though there is homology between the STE6 protein and OPPD at the amino acid level (Gros *et al.*, 1987). This is not surprising considering the degeneracy of the genetic code and codon bias usage in the two organisms.

Attempts were also made to clone a *C. albicans* *PTR2* homolog by functional complementation of the *S. cerevisiae* *ptr2* mutant PB1X-9B. *S. cerevisiae* transformants with a $PTR2^+$ phenotype were obtained by screening two *C. albicans* YEp13 libraries, one made by Rosenbluh *et al.* (1985) and the other by Gillum *et al.* (1984). The primary *S. cerevisiae* $PTR2^+$ transformants were sensitive to ethionine and Ala-Eth and amino acid auxotrophic requirements could be satisfied by dipeptides in the growth medium. Plasmid DNA isolated from the *S. cerevisiae* $PTR2^+$ transformants when amplified in *E. coli* revealed the presence of the expected vector fragments and an insert. A maxiprep of the *E. coli* plasmid DNA could not restore the phenotype of dipeptide utilization to the mutant strain *S. cerevisiae* PB1X-9B although LEU^+ transformants were obtained. The $PTR2^+$

phenotype could not be cured from the primary *S. cerevisiae* transformants. Peculiarly, the *S. cerevisiae* PTR2⁺ transformants J7, PT1 and PT5 obtained after screening the YEp13 library of Rosenbluh *et al.* (1985) exhibited a requirement of tryptophan for growth even though the mutant strain *S. cerevisiae* PB1X-9B does not have a *trp* marker.

Based on the above results we propose that the PTR2⁺ phenotype of the primary *S. cerevisiae* transformant was due to (1) integration of a *C. albicans* PTR2 homolog into the *S. cerevisiae* chromosome, or (2) recombination of the YEp13 plasmid containing a *C. albicans* gene with endogenous 2 μ m plasmids, or (3) reversion of the chromosomal *ptr2* defect to PTR2. The latter possibility was unlikely because the *S. cerevisiae* transformant PMB5 displayed sensitivity to Ala-Eth under conditions in which the wild type strain displayed little or no sensitivity and a revertant of the mutant has not been encountered either in these studies or by Perry *et al* (unpublished) during the cloning of the *S. cerevisiae* PTR2 gene (reversion frequency > 10⁻⁸). These observations suggest that the observed PTR2⁺ phenotype of the *S. cerevisiae* transformants is not likely due to reversion of the chromosomal *ptr2* mutation.

Results of tetrad analysis of spores from a cross of the *S. cerevisiae* transformant PMB5 with a mutant strain in the opposite background *S. cerevisiae* PB13X-1B showed a 2:2 segregation of the auxotrophic markers *LEU*, *URA*, and *LYS*. The PTR2⁺ phenotype also segregated 2:2 which may rule out the recombination of the YEp13 plasmid containing a *C. albicans* gene with endogenous 2 μ m plasmids. Results of plasmid curing experiments suggested that *S. cerevisiae* J7, PT1, PT5 and PMB5 retained the PTR2⁺ phenotype even after cells were grown for over 40 generations under conditions non selective for maintenance of the plasmid. Additionally, *S. cerevisiae* PMB5 showed sensitivity to Ala-Eth in CM-Leu medium in which the wild type cells *S. cerevisiae* PB1X-2A displayed little or no sensitivity. Thus, results of tetrad analysis, plasmid curing

experiments and disk sensitivity assays suggest a possible integration of a *C. albicans* *PTR2* homolog into the *S. cerevisiae* chromosome.

Since the *S. cerevisiae* transformants J7, PT1 and PT5 obtained from the library of Rosenbluh *et al.* (1985) have a requirement for tryptophan, it was possible that a *C. albicans* *PTR2* homolog integrated at the *trp* locus of *S. cerevisiae* resulting in the auxotrophic requirement for tryptophan. Additional experiments showed that the cells grown in limiting amounts of tryptophan fluoresced probably due to accumulation of anthranilic acid, an intermediate of the tryptophan biosynthetic pathway (for review Braus, 1991). That neither anthranilic acid, nor chorismic acid supported the growth of the transformants suggests that the cells may be blocked at a step in the biosynthetic pathway after anthranilate synthesis. Complementation of the primary *S. cerevisiae* *PTR2*⁺ transformants with either *TRP1*, *TRP3*, or *TRP4* may shed light on the likely mutation in the transformants. These experiments may allow us to determine the possible site of integration of a *C. albicans* *PTR2* homolog into the *S. cerevisiae* chromosome, as well. Integration at the *TRP* locus could be examined by probing a Southern blot of whole cell DNA from the *S. cerevisiae* *PTR2*⁺ transformant with the *TRP* gene shown to complement the mutation. The integration at the *TRP* locus can also be determined by using primers to the *TRP* gene and the PCR technique can be used to determine if the size of the *TRP* gene changes with the integration of the *C. albicans* *PTR2* homolog. If the *C. albicans* gene has integrated into the *TRP* locus of *S. cerevisiae*, then primers to the *TRP* gene can be used to rescue the integrated *C. albicans* gene. Alternatively, a gapped plasmid can be used to rescue the integrated *C. albicans* gene with the established methods of gap repair (Ausubel *et al.*, 1990). If the above approach fails to recover a functional *C. albicans* *PTR2* homolog, then another approach to clone the gene would be to screen either a plasmid or lambda library of *C. albicans* with the insert recovered from the primary *S. cerevisiae* transformants J7, PT1, PT5 or PMB5.

Once a *C. albicans* *PTR2* homolog is cloned, then the nucleotide sequence of the cloned *C. albicans* gene can be analyzed and compared to the *S. cerevisiae* *PTR2* gene and transporters from other systems. Knowledge of the open reading frame should allow us to do a homozygous gene disruption in *C. albicans* using the procedure described recently by Gorman *et al.* (1991) which is based on the method described by Alani *et al.* (1987) for *S. cerevisiae*. The phenotype of *C. albicans* cells with the disrupted or deleted gene should enable us to define the role of the cloned gene in *C. albicans*. The cloning of the gene will also enable us to determine the number and role of the peptide permeases in *C. albicans* and will make it possible to genetically analyze the results obtained previously by physiological studies.

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VITA

Munira A. Basrai was born in Poona, India on April 4, 1957. She attended the Convent of Jesus and Mary's St. Anne's High School and graduated with honors in General Science. She attended Abasaheb Garware College, Poona, and completed her Bachelors and Masters Degree in Microbiology in May 1977 and 1979, respectively from the University of Poona. She was ranked first in order of merit by the University of Poona at both the B. S. and M. S. examinations and was awarded several prizes.

Munira worked as a lecturer in Microbiology at Abasaheb Garware College, University of Poona for five years and taught the undergraduate and graduate classes. After marrying Adnan Y. Basrai in April 1984, she came to the United States. In January 1987, Munira joined the Department of Microbiology at the University of Tennessee, Knoxville, and performed her dissertation research in the laboratory of Dr. Jeffrey M. Becker. She was the recipient of Science Alliance Achievement Awards of the University of Tennessee in 1990, 1991 and 1992. She was also the recipient of the University of Tennessee Chancellors Citation for "Extraordinary Professional Promise" in April 1992. In May 1992 she received a Doctor of Philosophy degree in Microbiology. Munira plans to do her post-doctoral research in the laboratory of Dr. Philip Hieter, Associate Professor at the John Hopkins University, School of Medicine, Baltimore, MD.