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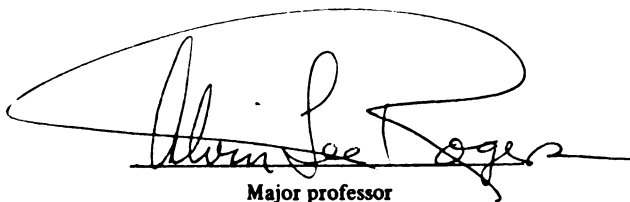
Cell Wall Ultrastructure of Candida albicans
Susceptible and Resistant to 5-Fluorocytosine

presented by

Donna Marie Duberg

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**CELL WALL ULTRASTRUCTURE OF CANDIDA ALBICANS
SUSCEPTIBLE AND RESISTANT TO 5-FLUOROCYTOSINE**

By

Donna Marie Duberg

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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1986

ABSTRACT

CELL WALL ULTRASTRUCTURE OF CANDIDA ALBICANS SUSCEPTIBLE AND RESISTANT TO 5-FLUOROCYTOSINE

By

Donna Marie Duberg

Differences in the cell wall ultrastructure of Candida albicans were investigated by transmission electron microscopy (TEM) using strains susceptible and resistant to 5-fluorocytosine (5-FC).

Three modified glutaraldehyde-osmium tetroxide fixation with resin embedding procedures were used to prepare the yeast cells for TEM.

Cell layer 1 when present was similar in appearance and thickness in 5-FC susceptible and resistant strains.

Measurements of the cell wall showed that the cell wall thickness was not uniform in all cells. The ratio of cell wall thickness to cell diameter was also variable.

These variabilities were not restricted to cells of a particular strain or susceptibility to 5-FC. This variation in cell wall thickness could be due to distortion during fixation and embedding, the plane at which the cell was sectioned or possible shifting of the cell layers as seen in budding.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to the members of my graduate committee for all their support and guidance. To Dr. Alvin Rogers, many thanks for his patience, understanding and hard work that have made this thesis a reality. Sincere appreciation is extended to Dr. Everett Beneke for his wealth of knowledge and experiences which he is so willing to share and for his wonderful sense of humor. Special thanks to Dr. Karen Klomparens for her expert advice in the area of transmission electron microscopy and especially for her ever positive and optimistic attitude that has been the mainstay of my research days - and nights! Warmest, heartfelt thanks to Dr. Sharon Zablotney for her friendship and mentoring throughout my graduate years and for her confidence in my ability to succeed. I am especially grateful to have worked with Mrs. Martha Thomas whose caring and love of students is reflected in her teaching and advising. She has always been there with a helping hand, good advice or a big hug.

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Most of all, I would like to thank my parents, Joseph and Josephine, for their continuous support and encouragement of everything I have tried, for their understanding of the demands of graduate school but especially, for their abundance of love.

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INTRODUCTION

Candida albicans is an opportunistic yeast that exists as a commensal inhabitant of the mucous membranes and digestive tracts of many normal individuals (Odds, 1979). Candidiasis has many varied clinical manifestations from oral thrush to deep-seated systemic infections. The condition may be acute, subacute or chronic. It was first believed that the yeast stage of C. albicans was the saprophytic form and the mycelial stage the pathogenic form. More recently it has been shown that the presence of yeast cells indicates an early lesion or colonization with mycelial elements becoming more prominent as the colonization and invasion of a tissue or organ continues (Rippon, 1982). An increase in both the number and in the severity of the infections has been noted due to the widespread use of antibiotics, immunosuppressive drugs and parenteral feedings (Shepherd et. al., 1985).

Numerous research articles have been published on the yeast, Saccharomyces cerevisiae, which has generated techniques and information that have been applied to other yeast, in particular, Candida albicans. There are two distinctions that should be made in regards to Candida species. First, C. albicans is a commensal organism of

humans and is frequently encountered in the digestive system and the vaginal tract. Second, C. albicans is considered an imperfect fungus. Much of the current research has focused on three areas: genetics, cell wall structure, and dimorphism. The genetics of C. albicans is being extensively investigated to better understand the many characteristics of the organism. The cell wall is of concern to researchers because of its antigenicity and the mode of action of antifungals. Lastly, the transition of C. albicans from yeast to mycelium (dimorphism) is directly associated with its pathogenicity (Shepherd et. al., 1985).

This present research focused on the topic of the cell wall ultrastructure of Candida albicans strains that were susceptible and resistant to the antifungal agent 5-fluorocytosine (5-FC). The study concentrated on the differences in the outermost layer labelled 1 by Djaczenko and Cassone (1971) which correspond to Poulain's layer C1 (1978) and also compared the cell wall thickness to the cell wall diameter.

LITERATURE REVIEW

CANDIDA ALBICANS-THE ORGANISM

Candidiasis is a primary or secondary yeast infection caused by organisms in the genus Candida, primarily Candida albicans (Emmons et.al., 1977). Clinically, the disease can range from acute to chronic with the most common manifestations seen as superficial lesions or infections of the mucous membranes of the mouth and vagina (Odds, 1979). Thrush, an infection of the oral mucosa, was described as a clinical condition by Hippocrates in his "Epidemics" written about 400 BC. Throughout history studies have supported Hippocrates' observation that candidiasis was associated with debilitated patients - Galen, about 200 AD; Pepys, 1665; Bennett, 1838. Since a sexual phase has not been shown, the genus Candida has been classified as Fungi Imperfecti (Deuteromycota) (Rippon, 1982).

Candida albicans, the most frequent etiologic organism of candidiasis, was first named by Berkhout in 1923. It can be isolated from infected material including scrapings, sputum or other mucous secretions, tissue specimens and body fluids such as cerebrospinal or thoracentesis fluid and urine (Cooper and Silva-Hunter, 1985). C. albicans will grow on most common laboratory media, but Sabouraud's

agar with antibacterial antibiotics is recommended to reduce bacterial contamination that would interfere with biochemical testing of the yeast (Emmons et.al., 1977; Beneke and Rogers, 1980). Incubation of the inoculated media can be at room temperature or 37C with cream-colored, smooth, pasty colonies appearing in three to four days (Beneke and Rogers, 1980). Microscopically, Candida albicans appears as round, oval or oblong, 2.5 by 3 to 14 μm occurring singly or in clusters or chains.

Identification procedures include the germ tube test, chlamydospore formation and carbohydrate fermentation and assimilation tests. The germ tube test consists of incubating the unknown yeast in serum at 37C for two to four hours (Cooper and Silva-Hunter, 1985). After incubation the preparation is observed for the formation of sprout mycelia, also called germ tubes (Reynolds-Braude phenomenon) (Rippon, 1982). A number of media are available for chlamydospore formation, including corn meal-Tween 80 agar, rice Tween 80 agar and Wolin-Bevis agar. The media are inoculated by furrowing two or three parallel lines of yeast into the agar and incubating at room temperature (23-25C) for 18 to 48 hours (Cooper and Silva-Hunter, 1985). Microscopic observation using the low power objective should reveal branching mycelia with clusters of blastoconidia attached along the sides with round, thick-walled chlamydospores, about 8-12 μm in

diameter, at the end of hyphal strands (Beneke and Rogers, 1980). Germ tube and chlamydospore formation are seen in most strains of Candida albicans and occasionally in Candida stellatoidea. The sucrose assimilation test and sensitivity to cyclohexamide will distinguish between these two species. C. albicans assimilates sucrose and is resistant to cyclohexamide while C. stellatoidea does neither (Cooper and Silva-Hunter, 1985).

Carbohydrate assimilation may be performed by swabbing a suspension of the yeast on yeast nitrogen agar or by incorporating the yeast suspension into the medium as a pour plate. Disks impregnated with a 20% solution of each carbohydrate to be tested are placed on the agar and the plates incubated at 24-30C for 24 to 72 hours. Growth around the disk indicating utilization of the carbohydrate is considered positive (Beneke and Rogers, 1980). Commercial kits are available (API 20C Clinical Yeast System, Analytab Products). Cyclohexamide-containing media are also commercially available i.e. Mycosel (BBL Microbiology Systems).

GENETICS OF CANDIDA ALBICANS

Consensus at this time is that naturally occurring Candida albicans is diploid (Shepherd et. al., 1985). Whelan and Magee (1981) first suggested that C. albicans was naturally diploid. Their mutagenesis studies showed

that following UV irradiation, some, but not all, strains of C. albicans developed a biased auxotrophic spectrum and these biased auxotrophic strains were "heterozygous for fully recessive defective biosynthetic alleles". Furthermore, they proposed that this heterozygous state was brought to homozygosity by induced mitotic crossing-over due to exposure to UV irradiation. Confirmation for this phenomenon has come from Poulter (unpublished results as reviewed in Shepherd et. al., 1985). Whelan et. al., (1981) studied 5-FC resistant strains of C. albicans to determine if 5-FC resistance could be heterozygous. Using UV irradiation to stimulate mutagenesis, Whelan demonstrated that 5-FC resistance is due to an almost fully recessive allele. Strains that are heterozygous for this 5-FC resistant allele are partially resistant and grow slowly on 5-FC. Homozygous, highly resistant, strains do occur at a high frequency from spontaneous mitotic crossing-over (DeFever et. al., 1982) and could provide C. albicans with genetic adaptability in the absence of a sexual cycle (Shepherd et. al., 1985).

CELL WALL ULTRASTRUCTURE OF CANDIDA ALBICANS

The cell wall of C. albicans has been subdivided into five to eight layers depending on the age of the cell, the type of medium used to culture the organism and the method used to visualize the structures (Djaczenko and Cassone,

1971; Poulain et. al., 1978). Djaczenko and Cassone (1971) used Tris-(1-aziridinyl) phosphine oxide (TAPO) to visualize the cell layers after the cells had been fixed in aldehydes. They used six varied combinations of reagents for fixation. The acrolein - TAPO (1% - 1%) used for prefixation with postfixation in 4% unbuffered osmium tetroxide (OsO_4) gave better visualization of the cell wall layers than the other combinations. The five cell wall layers they observed were numbered beginning at the outermost portion of the cell. Poulain, et. al., (1978) observed eight main layers in the cell wall of C. albicans using the periodic acid - thiocarbohydrazide silver (PATAg) method (Thi  ry, 1967). This technique allows the visualization of polysaccharide macromolecules as electron opaque structures. In the Poulain study (1978) both the media used to grow the yeast cells and the age of the cells used were varied to observe the effect of these changes on the number of layers observed in the cell wall. Poulain and his coworkers identified eight main layers which they numbered C1 through C8, also beginning at the outermost portion of the cell wall. Layer C1 contains fibrillar high electron dense structures extending perpendicularly and scattered with fine granules. This layer varies according to the preparation method used and the growth medium (Poulain et. al., 1978). Layers C2 to C4 also demonstrate high contrast and contain mannans, proteins and some lipid

material. Layers C5 and C7 showed a lower electron density suggesting that they contain mostly glucans and chitin which do not react with the PATAg (Poulain et. al., 1985). Layer C6 which was unpredictably present stained with both PATAg and heavy metal staining indicating the presence of both proteins and polysaccharides. Layer C8 which rests immediately adjacent to the plasma membrane had marked PATAg activity indicating large amounts of polysaccharides. In addition to these eight layers, various other layers were observed depending on the fixation regimen used for electron microscopy (Poulain et.al., 1978).

The layers described below are numbered using the system proposed by Djaczenko and Cassone (1971) with the corresponding label from Poulain's study (1978) given in parentheses (Figure 1 as modified from Poulain et.al., 1978).

Layer 1 (C1) measured 55 - 80 nm and was composed of thin filaments that lay perpendicular to the cell wall. They were embedded with small, 2.5 - 3.0 nm, granules. This layer was of medium electron density with the electron density of the granules being slightly higher than that of the filaments. Layer 2 (C4) measured 35 - 45 nm, was the most electron dense of all the layers and contained an amorphous matrix of high electron opacity. Layer 3 (C5) measured 50 - 70 nm and had electron dense filaments as

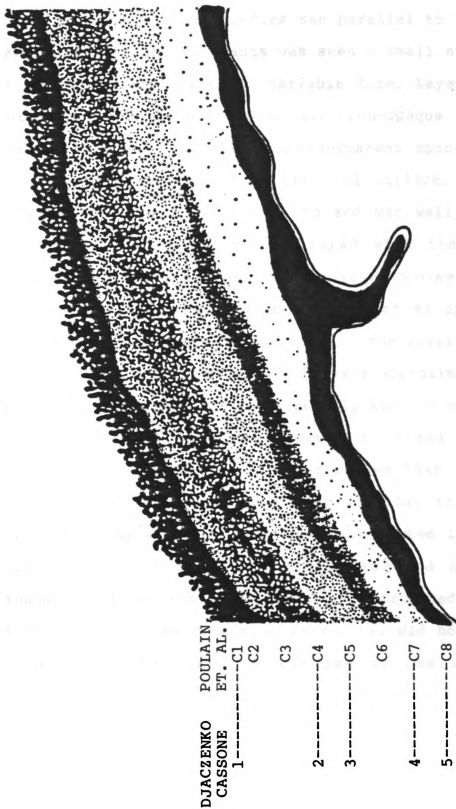


Figure 1

The Scheme of the Cell Wall of Candida albicans Blastoconidia.
Modified From Poulain, et. al. (1978) to include
Layers Corresponding to Djaczenko and Cassone

seen in layer 1 but these bundles ran parallel to the cell surface. Between the filaments was seen a small number of electron-transparent spaces of variable form. Layer 4 (C7) measured 40 - 50 nm and showed electron-opaque material scattered between 5 nm electron-transparent spaces which were oriented perpendicular to the cell surface. Layer 5 (C8) varied in size from 10 - 25 nm and was well delineated. It was most closely associated with the plasma membrane and followed the highly convoluted course of this membrane. The electron density was almost as opaque as layer 2 and appeared very homogeneous. The layers C2 and C3 described by Poulain et.al., 1978, were approximately 10 nm and 40 nm, respectively, and were only seen in old cells from cultures that had been allowed to stand at room temperature for 2 months. It was observed that layer C1 was apparently lost as the cell ages and that this layer had intense PATAg activity which may have masked layers C2 and C3. Layer C2 was well delineated and showed a continuous electron density while layer C3 produced a lower electron staining. The remaining layer, C6, did not appear with any predictability at any age of the culture, regardless of the growth media used nor even among different yeast cells from the same isolate under the same conditions. When layer C6 was present, it appeared unevenly distributed in the same cell and was not present

in each cell of the same population whether susceptible or resistant to 5-FC.

Biochemical studies of the cell wall have shown the presence of the following main polysaccharides (Figure 2 modified from Poulain et.al., 1978):

1. α -1-6 mannan
2. α -1-2 mannan
3. β -1-6 glucan
4. β -1-3 glucan
5. β -1-4-N-acetyl-glucosamine (chitin)

The black spheres represent the silver precipitates from PATAg staining which are seen as electron opaque structures in the transmission electron microscope (TEM) (Figure 2). According to Poulain (1978), layers C1 through C4 and layer C6 are composed of mannans and/or β -1-6 glucans (variations of electron opacity were observed). Layers C5 and C7 were mainly β -1-3 glucans and chitin (primarily electron transparent). Layer C8 showed a marked degree of PATAg reactivity which indicates large amounts of polysaccharides probably of low polymerization in addition to chitin. Chattaway et. al. (1976) used a double staining with heavy metal ions, uranium and lead. Chattaway's group reported that the innermost layer, C8, would probably contain chitin and protein, compounds that may supply the rigidity of the cell structure. Poulain's group (1978) further suggested that the C8 layer may be involved with

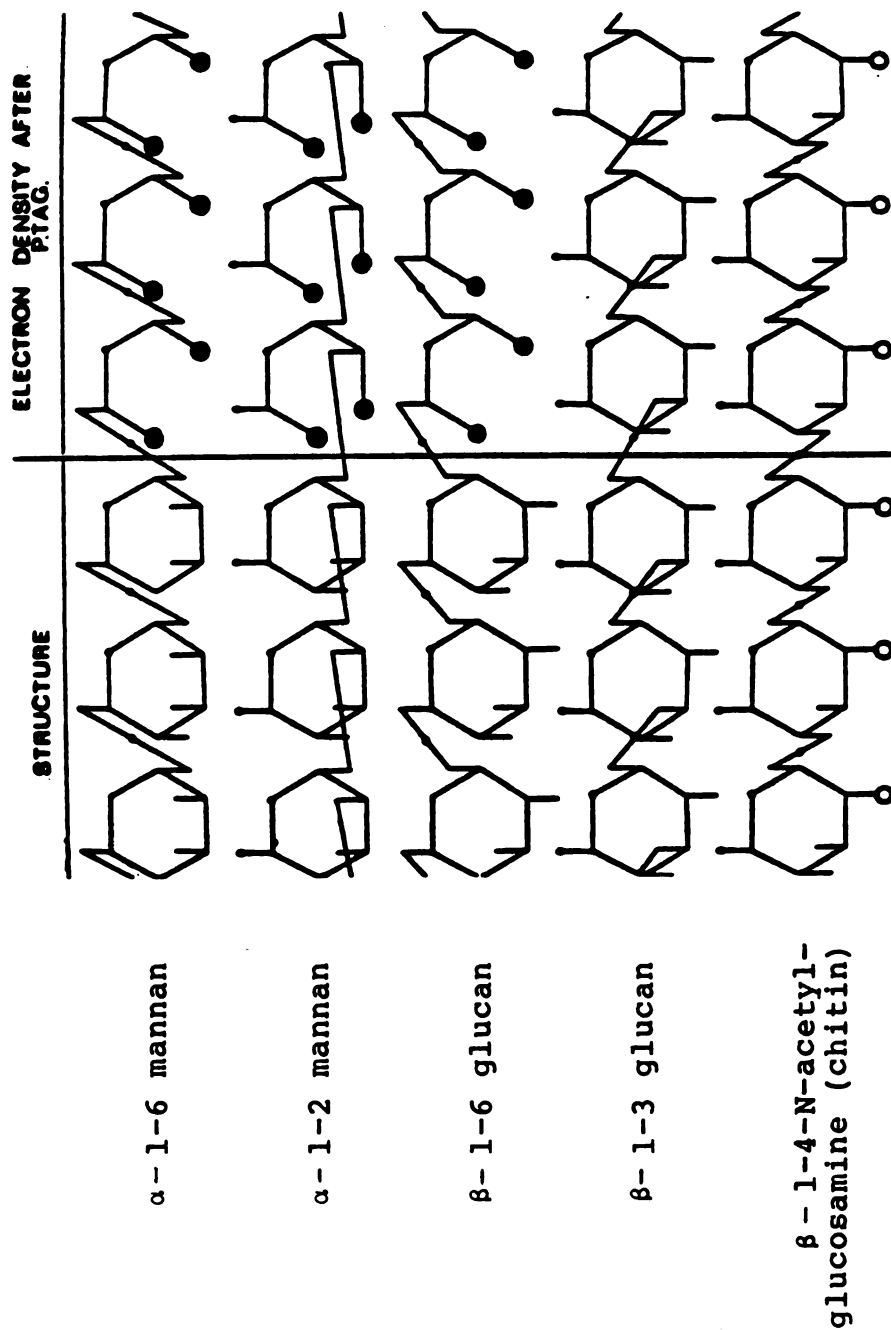


Figure 2

Structure and Reactivity, with Thiéry Method (PTAG), of Polysaccharides Known to be Present in the Cell Wall of *C. albicans* *Blastoconidia* (Modified from Poulain, 1978)

the mechanisms by which polysaccharides may be incorporated into the more superficial layers of the cell wall.

5 - FLUOROCYTOSINE

The antifungal agent, 5-fluorocytosine (5-FC), is a substituted pyrimidine which was first synthesized in 1957 (Ashe and Van Reken, 1977). The advantages of 5-FC over other antifungal agents, especially amphotericin B, are that it can be orally administered and it has reduced renal toxicity. The 5-FC compound is absorbed well though the walls of the digestive tract with 90% being cleared by the kidneys and penetration into the cerebrospinal fluid of levels up to 70% of serum. This drug is considered a secondary agent in the treatment of some systemic infections including those caused by Cryptococcus neoformans, various Candida species, strains of Torulopsis glabrata and species in the genus Aspergillus. It is suggested that it be used in conjunction with amphotericin B which is the drug of choice for most of these infections (Herman and Keys, 1983). It has not been confirmed if the effect of the combination therapy is synergistic or additive (Polak, 1978). When this dual drug regimen is used, the dosage of amphotericin B can be decreased which limits the nephrotoxicity and other side effects of the amphotericin B. Treatment of Candida species with 5-FC is limited by the occurrence of isolates demonstrating primary

resistance to this antifungal agent (10 - 50% of untreated strains) and the development of resistant strains during treatment (Stamm and Dismukes, 1983).

Mode of Action of 5-FC

The compound, 5-FC, is transported into the cell by cytosine permease (Figure 3 from Medoff and Kobayashi, 1983). Once inside the cell, the 5-FC is deaminated to 5-fluorouracil (5-FU) by cytosine deaminase. This is directly metabolized to 5-fluorouridine monophosphate (5-FURPh) by means of uridine monophosphate (URPh) pyrophosphorylase. After successive phosphorylations, 5-fluorouridine triphosphate (5-FURPhPhPh) is available in the main amino acid pool to be incorporated into RNA where it replaces up to 50% of the uracil normally found in the RNA. This substituted RNA does not function properly (Ashe and Van Reken, 1977), resulting in the synthesis of abnormal proteins and ultimately cell death (Polak and Scholar, 1975). A second mode of action of 5-FC is on DNA synthesis. 5-FURPh inhibits thymidylate synthetase, an enzyme used to make thymidine which is needed in DNA synthesis (Diasio, 1978). Mammalian cells lack the cytosine permease necessary to transport 5-FC internally accounting for the decreased toxicity to the host (Ashe and Van Reken, 1977).

Mechanisms of Resistance to 5-FC in Yeasts

Jund and Lacroute, (1970), have distinguished four types of resistance to 5-FC in their studies of Saccharomyces cerevisiae that may be applied to resistance seen in C. albicans. These four types are:

1. deficiency in cytosine permease
2. deficiency in cytosine deaminase
3. deficiency in URPh pyrophosphorylase
4. de novo synthesis of pyrimidines due to a loss of feedback regulation of aspartic transcarbamylase by uridine triphosphate (URPhPhPh).

A fifth mechanism was proposed by Drouhet et. al. (1974) who suggested that the increase in de novo synthesis of the pyrimidines is due to stimulation of orotidylic acid pyrophosphorylase and orotidylic decarboxylase. The increased pyrimidines, cytosine and adenine, antagonistically compete with 5-FC for the cytosine permease necessary to gain entry into the cell. Uracil affects incorporation of 5-FU into RNA. Uridine is slow to block this incorporation step since it must first be converted to uracil which requires several enzymatic steps. The disturbance of protein synthesis has also resulted in a change in the internal amino acid pool. Polak (1974) found an increase in the incorporation of ^{14}C -alanine and a marked decrease in ^{14}C -histidine suggesting an influence on protein synthesis. This was supported by accumulations of ^{14}C -histidine in the pool. Both the shift in amino acids from pool to protein and vice versa are likely to account for the growth inhibitory and lethal action of the drug.

MATERIALS AND METHODS

ORGANISMS

Candida albicans was isolated from clinical specimens processed at Texas-Baylor Hospital (DA 06844), Dallas, TX, Olin Health Center (OHC 910 and OHC 986) and the Medical Mycology Laboratory (MSU #1), Michigan State University, East Lansing, MI. The morphological and biochemical characteristics of these isolates were studied by germ tube formation in human serum and fetal calf serum, by chlamydospore formation on corn meal agar with 1% Tween 80, by growth on cyclohexamide medium and by assimilation studies performed using the API 20C Clinical Yeast System, Analytab Products, Sherwood Medical, Plainview, NY. Stock cultures of the isolates were stored on Sabouraud's agar slants at room temperature.

MEDIA

Susceptibility testing with 5-fluorocytosine (5-FC) was performed using minimal medium (MIN) and minimal medium supplemented with 5-FC (Sigma Chemical Co., St. Louis, MO) to a final concentration of 50 ug/ml (MFC50). MIN medium contained yeast nitrogen base without amino acids, 4.0 g; dextrose, 12 g; agar, 12 g; and distilled water, 600 ml. The medium was sterilized by autoclaving (15 minutes; 15

lb/in²) and cooled to 56C before pouring plates or the addition of 5-FC. Stock 5-FC was prepared in small quantities (0.05 g 5-FC in 10 ml distilled water), filtered sterilized and stored at 4C.

SUSCEPTIBILITY TESTING WITH 5-FC

Susceptibility testing with 5-FC was performed according to Whelan et. al., (1981). Stock yeast cultures were transferred to MIN medium for overnight growth at 37C. A suspension was prepared from the overnight growth in 0.9% physiological saline to yield a final concentration of approximately 10^6 viable cells per ml. Counting was performed using an improved Neubauer chamber with 0.1% methylene blue added to the suspension (0.9 ml suspension to 0.1 ml of methylene blue). Viable yeast cells do not stain with methylene blue while non-viable cells appear dark blue. MIN and MFC50 media were each inoculated with a 0.1 ml aliquot of the yeast cell suspension which was spread with a sterile bent glass rod. Resultant cultures were incubated at 37C for five to seven days. The number of colonies on each plate were counted daily beginning with day 2. Candida albicans strains that demonstrated no visible growth on the MFC50 medium and too numerous to count (TNTC) growth on the MIN medium were considered susceptible. Resistance to 5-FC was indicated by TNTC growth on both the MIN and the MFC50 media of the isolate.

susceptible. Resistance to 5-FC was indicated by TNTC growth on both the MIN and the MFC50 media of the isolate.

TRANSMISSION ELECTRON MICROSCOPY (TEM PROCEDURES)

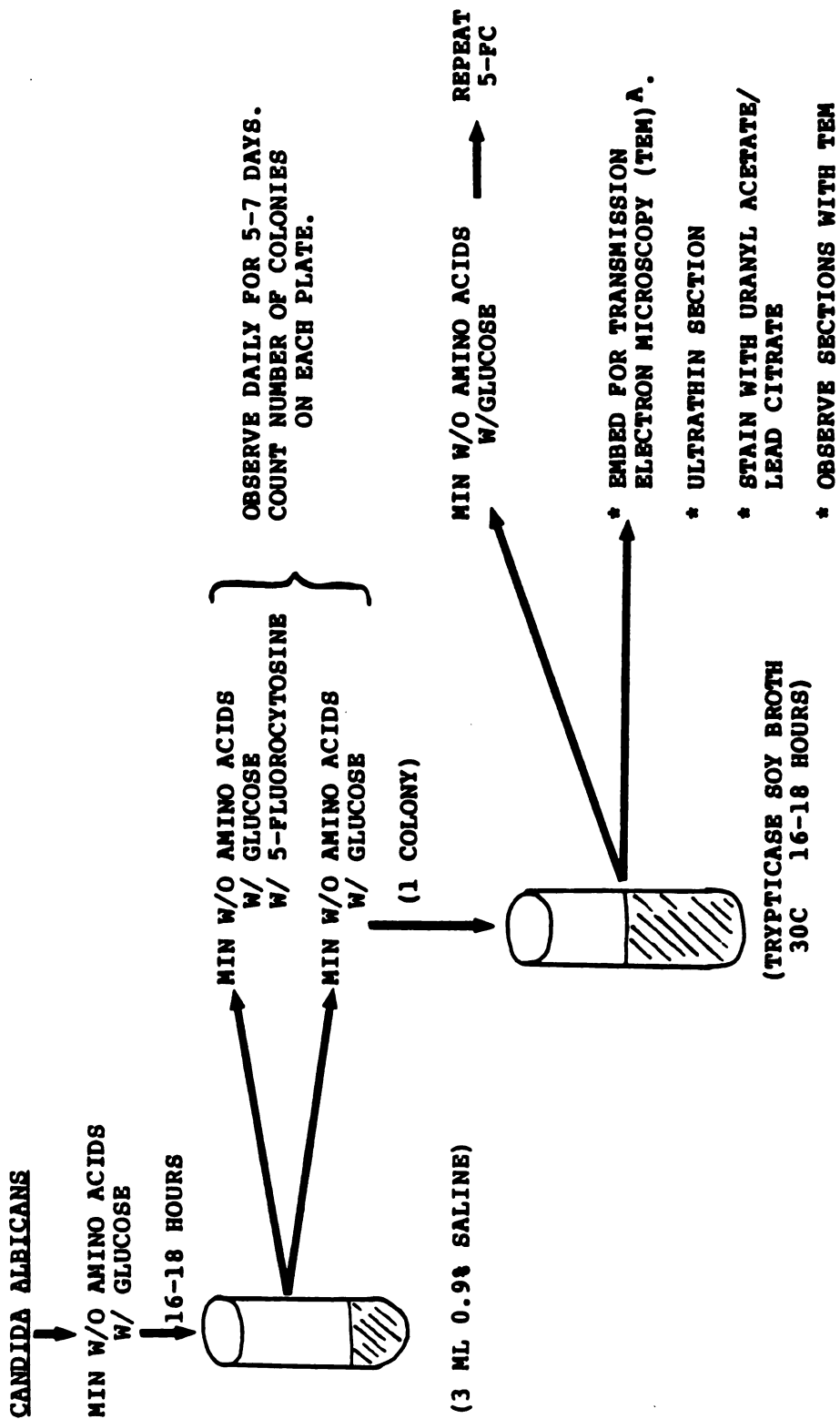
Method 1

After 24 hours of incubation of the 5-FC susceptibility testing, one colony was removed from the MIN control plate and suspended in a small drop of 4% agar that had been melted and cooled slightly. The solidified agar block was cut into approximately one mm³ pieces and fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for two hours (on ice). The pieces were then rinsed in the same buffer three times, 20 minutes each (on ice). Post fixation with 1% osmium tetroxide in 0.1 M phosphate buffer for one hour at room temperature was followed by two buffer rinses (on ice) for 15 minutes each. Dehydration in graded ethanols-25%, 50%, 75% and 100%-for 15 minutes each was followed by another 100% ethanol rinse for one hour (all dehydration steps on ice). A third rinse in 100% ethanol was placed in the cold (0-5C) for overnight dehydration. A final 100% ethanol rinse was in the cold for 30 minutes. Before embedding, the ethanol was replaced with acetone by suspending the specimens first in a 2:1 100% ethanol/acetone mixture and then in a 1:2 100% ethanol/acetone mixture. Each change was incubated at room temperature for 15 minutes on a rotator followed by two changes with 100%

acetone at room temperature for 30 minutes each. From acetone to Spurr's-Mollenhauer resin required two transition steps-acetone/resin (3:1) and then acetone/resin (1:3)-incubated at room temperature on a rotator for four to eight hours each. Specimens were placed in 100% resin for eight hours to overnight on a rotator (room temperature). One section was placed in each mold slot containing fresh resin and a label added for reference. Molds were placed in a 68-70C oven for 12 to 48 hours to polymerize. Resin blocks were removed from cooled molds and stored in a desiccator until sectioned.

Method 2

Method 2 was a modification of Method 1 and attempted to enhance the preservation and visualization of the cell wall ultrastructure. Figure 4 is a flowchart of this method. For each strain of C. albicans, one colony from the MIN medium was suspended in Tryptic Soy Broth with 4% glucose in sterile screw-capped tubes for overnight growth at 30C. A loopful of this growth was streaked on MIN medium for isolation and then retested for 5-FC susceptibility. The tubed suspension was centrifuged at 4500 rpm for five minutes and the supernatant discarded. The pellet of yeast cells was resuspended in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4) and fixed for 20 minutes (on ice). This mixture was centrifuged as before and the supernatant discarded. A



A. MODIFIED PROCEDURE FROM HOOPER ET AL 1979

Figure 4

Flowchart for Investigating the Cell Wall Ultrastructure of
5-FC Susceptible and Resistant *Candida albicans* Strains

portion of the pellet was suspended in 4% agar and cut into 2 to 3 small blocks approximately one mm³ for TEM embedding. The sections were fixed with 2% glutaraldehyde in cacodylate buffer for 1 to 1 1/2 hours and then rinsed in the same buffer three times, 20 minutes each time (on ice). Post fixation with 0.5% osmium tetroxide in cacodylate buffer for 30 to 60 minutes at room temperature was followed by two buffer rinses (on ice) for 15 minutes each. The specimens were dehydrated in graded ethanols-25%, 50%, 75% and 100%-for 15 minutes each (on ice). Another 100% ethanol rinse for one hour was followed by an overnight dehydration with 100% ethanol- both of these steps occurring in the cold. Instead of acetone, the transition medium in this method was propylene oxide but the times and temperatures for these steps are the same as in Method 1. The propylene oxide/resin changes were incubated for a minimum of six to eight hours and there were two 100% resin changes held for eight hours to overnight each. Polymerization and storage were as in Method 1.

Method 3

Specimen handling, fixation and dehydration steps were exactly the same as Method 2. In the embedding procedure, however, Spurr's (1969) ERL Medium was used, which did not require a transition medium. After overnight incubation in 100% ethanol, the specimens were rinsed in fresh 100%

ethanol in the cold for 30 minutes. Two replacement steps, 100% ethanol/resin (3:1) and 100% ethanol/resin (1:3), were held at room temperature on a rotator for a minimum of six to eight hours followed by two changes of 100% resin held overnight after each change also on the rotator. After placement of the specimens in the molds with fresh resin, they were polymerized for a maximum of 24 hours. Blocks were removed from the molds and stored as previously described.

Thin sections were cut with glass or diamond knives on a Sorvall Porter-Blum, MT-2 ultramicrotome and collected on 300 mesh uncoated copper grids. Grids with sections were stained with uranyl acetate and Reynold's (1963) lead citrate. For specimens embedded with Spurr's-Mollenhauer saturated uranyl acetate was used and rinsed with distilled water. Those sections in Spurr's ERL Medium were stained with a 0.5% uranyl acetate in mixed alcohols (75 ml methanol + 25 ml 75% ethanol) and rinsed with the mixed alcohol solution. Stained grids were examined with a Philips 201 or with a JEOL 100CX II transmission electron microscope at accelerating voltages of 60 to 100 kV. Photomicrographs were taken of intact yeast cells and enlargements made of each.

The ratio of the width of the cell wall to the diameter of the yeast cell was computed using measurements taken across the length and width of the cell. For each

isolate, the average percentage and the range were computed for each ratio.

RESULTS

ORGANISM IDENTIFICATION

The strains of C. albicans used in this study formed germ tubes in both fetal calf and human sera, formed chlamydospores on corn meal agar with 1% Tween 80 and grew on cyclohexamide-containing medium (Mycosel). They assimilated sucrose as well as other substrates in the API 20C Clinical Yeast System. These biochemical assimilation results and interpretations are given in Tables 1A and 1B, respectively. MSU #1, OHC 910 and DA 06844 had identical assimilation patterns. Strain K 8559 differed only in its inability to utilize methyl-D-glucoside and OHC 986 was unable to assimilate adonitol, N-acetyl-D-glucosamine and trehalose.

Referring to the API index, the probability of the pattern of assimilation demonstrated by strains MSU #1, OHC 910 and DA 06844 corresponding to an identification of Candida albicans is excellent. The probability for K 8559 is very good and for OHC 986 acceptable.

Table 1A

Biochemical Testing of *Candida albicans* Strains Susceptible and Resistant to 5-Fluorocytosine (5-FC) Using the API 20C Clinical Yeast System (Analytabs Products, 1984).

Organism	Biochemical Substrates ^a															
	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC
<i>Candida albicans</i>	100 ^b	13	100	2	93	90	97	100	0	98	95	98	0	0	100	99
5-FC Susceptible:																
MSU #1	+	0	+	0	+	+	+	+	0	+	+	+	0	0	+	+
OHC 910	+	0	+	0	+	+	+	+	0	+	+	+	0	0	+	+
5-FC Resistant:																
DA 06844	+	0	+	0	+	+	+	+	0	+	+	+	0	0	+	+
K 8559	+	0	+	0	+	+	+	+	0	+	0	+	0	0	+	+
OHC 986	+	0	+	0	+	0	+	+	0	+	+	0	0	0	+	+

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^aBiochemical Substrates:

GLU - Glucose	SOR - Sorbitol (Glucitol)
GLY - Glycerol	MDG - Methyl-D-glucoside
2KG - 2-Keto-D-gluconate	NAG - N-Acetyl-D-glucosamine
ARA - L-Arabinose	CEL - Cellulose
XYL - Xylose	LAC - Lactose
ADO - Adonitol (Ribitol)	MAL - Maltose
XLT - Xylitol	SAC - Sucrose
GAL - Galactose	TRE - Trehalose
INO - Inositol	MLZ - Melezitose
	RAF - Raffinose

^bFigures indicate the percentage of positive reactions after 72 hours of incubation at 30C.

^cPositive assimilation is indicated by +; negative assimilation by 0.

Table 1B

Interpretation of Biochemical Testing of Candida albicans
Strains From Table 1A

<u>Organism</u>	<u>API Profile #^a</u>	<u>Identification^b</u>	<u>Comment^c</u>
<u>5-FC Susceptible:</u>			
MSU #1	2576170	<u>C. albicans</u>	Excellent
OHC 910	2576170	<u>C. albicans</u>	Excellent
<u>5-FC Resistant:</u>			
DA 06844	2576170	<u>C. albicans</u>	Excellent
K 8559	2572170	<u>C. albicans</u>	Very Good
OHC 986	2566030	<u>C. albicans</u>	Acceptable

^aThis seven digit number was derived from the results of the API 20C Clinical Yeast System by assigning a numerical value to positive reactions.

^bIdentification was made by comparing the API Profile # to a computer data base of probabilities called the profile index.

^cThe comment descriptors were used in the profile index to indicate the likelihood the identification listed was correct.

SUSCEPTIBILITY TESTING WITH 5-FC

MSU #1 and OHC 910 were tested for 5-fluorocytosine susceptibility five and three times, respectively, (Table 2). In each experiment there was no visible growth on the MFC50 medium and too numerous to count (TNTC) growth on the MIN medium control indicating susceptible strains. DA 06844 was tested six times with four of these showing TNTC growth on both the MFC50 and MIN media. In the other two trials, colonies on the MFC50 medium showed a slight variation in size with half growing slightly larger than the rest with an even growth observed on MIN. Of the four times K 8559 was tested only once did this strain show an even TNTC growth pattern on MFC50 and MIN media. The remaining three tests consistently demonstrated a mixed pattern with most of the colonies growing slightly larger than the rest. Strain OHC 986 produced TNTC growth on both MFC50 and MIN media of similar sized colonies. The patterns of growth described above for strains DA 06844, K 8559 and OHC 986 indicate resistance to 5-FC.

TRANSMISSION ELECTRON MICROSCOPY

Method 1

Specimen blocks from Method 1 were easy to trim. The thickness of the sections necessary for the study of the cell wall ultrastructure was difficult to determine.

Table 2

Results of 5-Fluorocytosine Testing on
Susceptible and Resistant Strains
of Candida albicans

Organism	Tests ^a					
	1	2	3	4	5	6
<u>Susceptible:</u>						
MSU #1	NG	NG	--	NG	NG	NG
OHC 910	--	--	--	NG	NG	NG
<u>Resistant:</u>						
DA 06844	TNTC-E	TNTC-E	TNTC-E	TNTC-M	TNTC-M	TNTC-E
K 8559	--	--	TNTC-M	TNTC-M	TNTC-M	TNTC-E
OHC 986	--	--	--	TNTC-E	TNTC-E	--

^aNG = No growth on MFC50 medium and too numerous to count growth on MIN medium.

TNTC-E = Too numerous to count growth on MFC50 medium and MIN medium. All colonies were of similar size.

TNTC-M = Too numerous to count growth on MFC50 medium and MIN medium. Colonies were of 2 sizes.

Sections that were thin enough to observe the cell wall layers were too thin for the resin to support the cells in the electron microscope beam. Thicker sections were too uniformly electron dense to distinguish any particular cellular structures and showed areas of compression (Plate 1, Fig.1).

Internal structures and cell wall ultrastructure were difficult to discern (Plate 1, Figs. 1 and 2). Since the inoculum from the MIN medium was so small, the number of yeast cells embedded in the block was minimal and only a rare cell could be found on the grids. Thus, a second method was tried.

Method 2

Inoculating tryptic soy broth for overnight growth of the yeast yielded a large number of cells for study. As in Method 1 there was difficulty in adjusting the thickness of the ultrathin sections to achieve a balance between sections that were thin enough to observe fine detail and still thick enough to maintain the integrity of the cell while exposed to the electron beam (Plate 2, all figures).

Areas of compression were apparent (Plate 2, Fig. 3). Staining of the cell was not as intense as in method 1 (Plates 1 and 2). Internal structures when observed were not well delineated (Plate 2, all figures). Cell wall

Plate 1

Figures 1-2. Transmission electron micrographs of Candida albicans strain K 8559 resistant to 5-fluorocytosine, processed according to Method 1 - 4% glutaraldehyde, 1% osmium tetroxide, 0.1 M phosphate buffer, Spurr's-Mollenhauer resin.

Sections are thick with areas of compression. Internal structures are difficult to distinguish.

Figure 1. Whole yeast cell with areas of compression (arrows).

Figure 2. Enlargement of the cell wall showing invaginations of the plasma membrane.

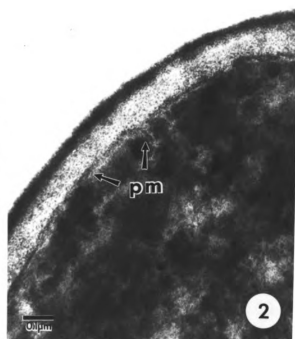
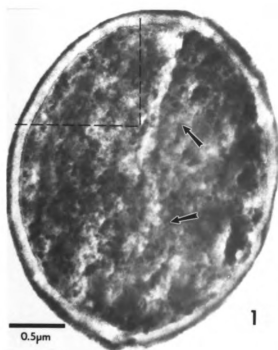


Plate 2

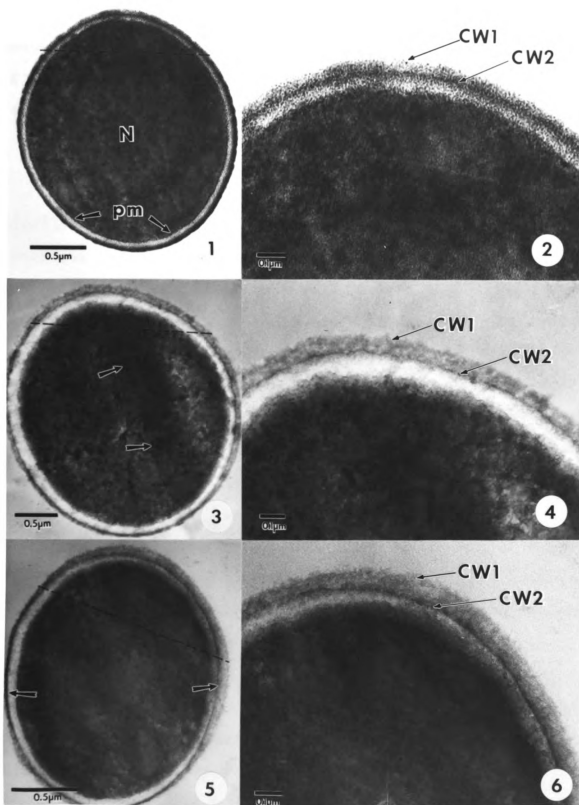
Figures 1-6. Transmission electron micrographs of Candida albicans strains susceptible and resistant to 5-fluorocytosine processed according to Method 2 - 2% glutaraldehyde, 0.5% osmium tetroxide, 0.1 M sodium cacodylate buffer, Spurr's - Mollenhauer resin.

Sections appear thick with areas of compression. Internal structures when visible are not well-delineated. Cell wall layers 1 and 2 are present and similar in both susceptible and resistant strains. Other cell wall layers are not as evident.

KEY TO LETTERING ON FIGURES 1-6.

CW1 = all wall layer 1
CW2 = all wall layer 2
N = nucleus
PM = plasma membrane

- Figure 1. MSU #1 susceptible. Whole yeast cell. Note well delineated plasma membrane.
- Figure 2. MSU #1. Enlargement of Figure 1.
- Figure 3. DA 06844, resistant. Whole yeast cell. Note area of compression at arrows.
- Figure 4. DA 06844, Enlargement of Figure 3.
- Figure 5. OHC 986, resistant. Whole yeast cell.
- Figure 6. OHC 986. Enlargement of Figure 5.



layers 1 and 2, were present and similar in both susceptible and resistant strains (Plate 2, Figs. 2,4 and 6). Other cell wall layers were not clearly discernible (Plate 2, all figures) which lead to method 3.

Method 3

Blocks embedded with Spurr's ERL medium were more difficult to trim and occasionally fractured. Ultrathin sections had very little compression and when observed in the TEM appeared uniform in thickness (Plate 3, Figs. 1 and 3). The resin was hard enough to hold the yeast cells in place at accelerating voltages of 100 kV for periods of time long enough to take several micrographs at increasing magnifications (Plate 3, Figs. 2 and 4). Staining intensity was decreased slightly as compared to methods 1 and 2 which improved the contrast between cell components (Plates 1, 2 and 3). Internal structures of the yeast cell were easily distinguished and well-defined (Plate 3, Figs. 1 and 3). Cell wall ultrastructure showed improvement in the delineation and visualization of the cell wall layers over the two previous methods (Plate 1, Fig.2; Plate 2, Figs. 2, 4 and 6; Plate 3, Figs. 2 and 4; Plate 4, both figures). All five layers as described by Djaczenco and Cassone are well-delineated (Plate 4, both figures) but it appears that cell layer 1 is scant or absent in both the

Plate 3

Figures 1-4. Transmission Electron micrographs of Candida albicans strains susceptible and resistant to 5-fluorocytosine processed according to Method 3 - 2% glutaraldehyde, 0.5% osmium tetroxide, 0.1 M sodium cacodylate buffer, Spurr's ERL Medium.

Sections are thinner and more even in thickness than Methods 1 and 2. Internal structures are observable and usually well-defined. Cell wall ultrastructure is delineated into component layers but layer 1 appears to be scant or absent.

KEY TO LETTERING ON FIGURES 1-4

CW = cell wall
ER = endoplasmic reticulum
M = mitochondria
N = nucleus
PM = plasma membrane

- Figure 1. MSU #1, susceptible. Whole yeast cell. Note the well-defined internal structures and cell wall layers. Note variations in cell wall thickness (arrows). Black granules are precipitated lead stain.
- Figure 2. MSU #1. Enlargement of Figure 1. Note delineation of cell wall layers and the plasma membrane. Cell wall layer 1 is scant.
- Figure 3. DA 06844, resistant. Whole yeast cell. Cell wall is of uniform thickness.
- Figure 4. DA06844. Enlargement of Figure 3. Cell wall layer 1 appears absent.

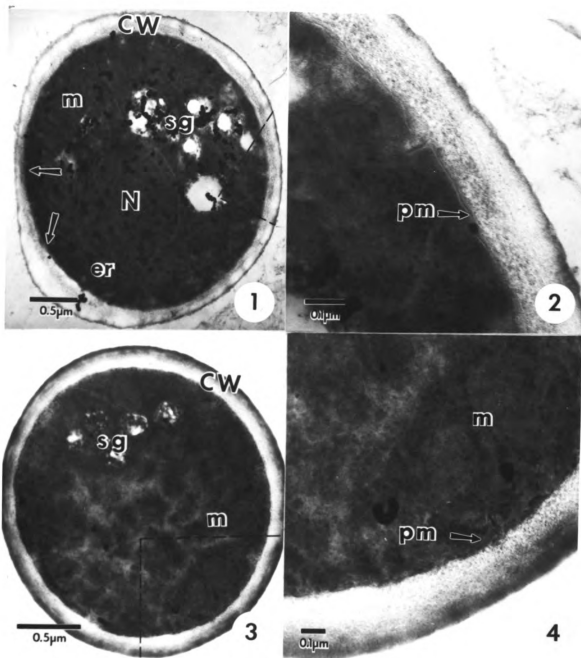
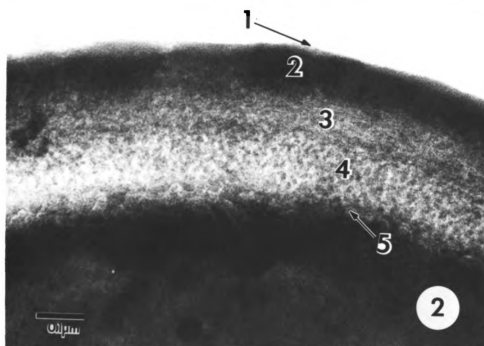
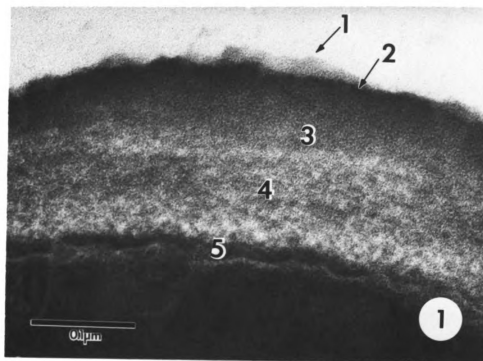


Plate 4

Figures 1-2. Enlargements of cell walls of Candida albicans strains susceptible and resistant to 5-fluorocytosine detailing the five cell wall layers described by Djaczenko and Cassone (1971). Cell layer 1 appears scant in both strains.

Figure 1. MSU #1, susceptible.

Figure 2. DA 06844, resistant.



resistant strains as compared to those cells processed using method 2 (Plate 2, Figs. 2, 4 and 6). There was no consistent observable difference in the cell wall ultrastructure of the 5 layers.

MEASUREMENT OF THE CELL WALL THICKNESS

Measurements of the cell wall thickness were compared to the diameter of the yeast cell for strains susceptible and resistant to 5-FC (Tables 3, 4 and 5). The width of the cell wall was variable in all three methods used for TEM (Plate 1, Fig. 1; Plate 2, Figs. 1, 3 and 5; Plate 3, Figs. 1 and 2). Cells processed by method 3 were used for measurement studies since this process yielded the most delineated cell wall ultrastructure. Unfortunately, cell layer 1 was not as evident as it had been in method 2. The variation in cell wall thickness did not follow any pattern.

Several of the cells measured had a very even distribution of cell wall (Table 3, OHC 910, Cells # 2 and 4; Table 4, DA 06844, Cells # 5 and 6; K 8559, Cell # 1). Many of the yeast cells had only slight irregular thicknesses of the cell wall as seen in Table 3, MSU #1, Cells # 1, 2, 4 and 6; OHC 910, Cells # 3 and 5; Table 4, DA 06844, Cells # 1, 2, 3 and 4; K 8559, Cell # 3. The remaining cells measured showed only one section of the

Table 3

Comparison of Cell Wall Thickness to Cell Diameter of
Candida albicans Strains Susceptible to
 5-Fluorocytosine Processed by Method 3

Cell Number	Direction of Measurement ^a	Cell Wall Thickness ^b		Cell Diameter CD _{A-B}	% ^c
		CT _A	CT _B		
<u>MSU #1:</u>					
1	W	17	20	180	20.6
	L	15	19	190	17.9
2	W	12	16	178	15.7
	L	16	14	196	15.3
3	W	8	7	152	9.9
	L	11	11	191	11.5
4	W	15	14	147	19.7
	L	11	12	148	15.5
5	W	6	8	153	9.2
	L	8	13	176	11.9
6	W	13	9	146	15.1
	L	10	11	160	13.1
7	W	16	16	135	23.7
	L	20	16	148	24.3
8	W	10	9	131	14.5
	L	18	12	168	17.9
9	W	8	9	154	11.0
	L	12	12	170	14.1
					X = 15.6
					Range = 9.2 - 24.3

^aMeasurements were made across the width (W) and length (L) of the yeast cell using lines intersecting perpendicularly in the center of the cell.

^bCell wall thicknesses A and B (CT_A and CT_B) refer to cell wall measurements on opposite sides of the cell diameter (CD_{A-B}).

$$c\% = \frac{CT_A + CT_B}{CD_{A-B}} \times 100$$

Table 3, Cont'd.

Comparison of Cell Wall Thickness to Cell Diameter of
Candida albicans Strains Susceptible to
 5-Fluorocytosine Processed by Method 3

Cell Number	Direction of Measurement	Cell Wall Thickness ^b		Cell Diameter CD _{A-B}	% ^c
		CT _A	CT _B		
<u>OEC 910:</u>					
1	W	10	11	171	12.3
	L	15	9	184	13.0
2	W	10	9	147	12.9
	L	10	9	169	11.2
3	W	16	13	137	21.2
	L	16	18	180	18.9
4	W	12	13	158	15.8
	L	12	12	171	14.0
5	W	16	17	162	20.4
	L	14	14	164	17.1
6	W	15	24	145	26.9
	L	17	15	176	18.2
7	W	11	10	143	14.7
	L	16	23	193	20.2
					X = 16.9
					Range = 11.2 - 26.9

^aMeasurements were made across the width (W) and length (L) of the yeast cell using lines intersecting perpendicularly in the center of the cell.

^bCell wall thicknesses A and B (CT_A and CT_B) refer to cell wall measurements on opposite sides of the cell diameter (CD_{A-B}).

$$C\% = \frac{CT_A + CT_B}{CD_{A-B}} \times 100$$

Table 4

Comparison of Cell Wall Thickness to Cell Diameter of
Candida albicans Strains Resistant to
 5-Fluorocytosine Processed by Method 3

Cell Number	Direction of Measurement ^a	Cell Wall Thickness ^b		Cell Diameter CD _{A-B}	% ^c
		CT _A	CT _B		
DA 06844:					
1	W	11	8	160	11.9
	L	10	10	163	12.3
2	W	11	12	143	16.1
	L	12	10	145	15.2
3	W	10	11	166	12.6
	L	11	13	178	13.5
4	W	14	14	139	20.1
	L	11	10	148	14.2
5	W	9	10	131	14.5
	L	10	10	141	14.2
6	W	8	8	158	9.4
	L	8	9	170	10.0
					X = 13.7
					Range =9.4 - 20.1
K 8559:					
1	W	8	7	122	12.3
	L	7	8	158	9.5
2	W	14	14	139	20.1
	L	16	22	165	23.0
3	W	7	7	135	10.4
	L	9	10	177	10.7
4	W	22	20	183	23.0
	L	27	22	197	24.9
					X = 15.6
					Range =9.2 - 24.3

^aMeasurements were made across the width (W) and length (L) of the yeast cell using lines intersecting perpendicularly in the center of the cell.

^bCell wall thicknesses A and B (CT_A and CT_B) refer to cell wall measurements on opposite sides of the cell diameter (CD_{A-B}).

$$c\% = \frac{CT_A + CT_B}{CD_{A-B}} \times 100$$

Table 5

Summary of the Comparison of Cell Wall Thickness to Cell Diameter of Candida albicans Strains Susceptible and Resistant to 5-Fluorocytosine Processed by Method 3 (From Tables 3 and 4)

Organism	Mean ($\bar{X}$)	Range
<u>5-FC Susceptible:</u>		
MSU #1	15.6	9.2 - 24.3
OHC 910	16.9	11.2 - 26.9
<u>5-FC Resistant:</u>		
DA 06844	13.7	9.4 - 20.1
K 8559	16.7	9.5 - 24.9

cell wall thicker than the rest of cell wall as seen in Table 3, MSU #1, Cells # 5, 7 and 8; OHC 910, Cells # 1, 6 and 7; Table 4, K 8559, Cells # 2 and 4.

The percentage of cell wall thickness to cell wall diameter reflected the variations described above. For those cells with fairly even cell wall thicknesses (Table 4, DA 06844, Cell # 5), the percentages computed were similar (14.5 and 14.2). If the cell width varied (Table 3, OHC 910, Cell # 6), a significant difference was seen (26.9 and 18.2) in the percent computed. The four strains tested had comparable means and ranges of percentages which did not appear to be related to susceptibility or resistance of the yeast strains to 5-FC (Table 5).

DISCUSSION

The organisms used in this study had been identified as C. albicans initially at the clinical site where it was first isolated. When received in the Medical Mycology Laboratory at Michigan State University for 5-FC studies, they were tested for germ tube and chlamydospore production and were positive for both. The strains had been transferred many times in the course of this study and before these results were presented, the identification was verified.

Strains susceptible to 5-FC, MSU #1 and OHC 910, showing no growth on the MFC50 medium and TNTC growth on MIN agar (Table 5), were categorized as Type D organisms indicating they are homozygous susceptible (Whelan and Magee, 1981). The resistant strains, however, varied in their growth patterns on MFC50 medium. DA 06844 was described as a Type C3 or homozygous resistant since all the colonies that grew on MFC50 medium were about the same size (Whelan and Magee, 1981) four out of six times it was tested. The remaining two DA 06844 sets of results which showed a slight variation in size of colonies occurring in the 5-FC testing performed before and at the time of

embedding with Method 2. The DA 06844 strain had been transferred off MIN minimal medium, instead of Sabouraud's agar, an enriched medium, which might account for the difference in colony sizes. When the 5-FC test was performed as part of the Method 3 experiment and the DA 06844 was inoculated from Sabouraud's agar, the typical Type C3 pattern of even growth on MFC50 medium was observed. Strain OHC 986 grew as a typical Type C3 on MFC50 medium. Strain K 8559-1, although initially tested as a Type C3, exhibited Type C1 growth with colonies of two distinct sizes on the MFC50 agar. Whelan and Magee (1981) stated that this type of growth indicated a heterozygous resistant organism. Thus, it is possible that a spontaneous mutation of the organism had occurred since this is rather common (Whelan and Magee, 1981).

In order to investigate the cell wall ultrastructure, it is critical that the yeast cell as it appears in the electron microscope represents the cell in its natural state as accurately as possible. To accomplish this all aspects of the preparation, fixation and embedding procedure were analyzed. Observations of yeast cells prepared according to Method 1 indicated that more cells must be incorporated into the 4% agar if sufficient numbers of cells were to be investigated. Second, a method to retain and enhance fine detail in the cell wall was

necessary. Lastly, the excessive contrast (darkness) of the cells should be eliminated (Plate 1, both figures). Method 2 showed that by growing the yeast cells in tryptic soy broth with 4% glucose (TSBG) the number of cells on the grids increased significantly from an average of one yeast cell/two grids to an average of three to five cells/grid square. The resolution of the cell wall layers was improved by changing the buffer solution from sodium phosphate to sodium cacodylate (Hayat, 1981; Bullock, 1983); by using propylene oxide instead of acetone as a transition medium (Hayat, 1981); and by decreasing the concentration of glutaraldehyde from 4% to 2% (Hayat, 1981) as seen in Plate 2, Figs. 2, 4 and 6. Cacodylate buffer does not precipitate with uranyl acetate when rinsed with dilute ethanol before staining (Hayat, 1981). Propylene oxide preserves membranes while acetone does not (Hayat, 1981). Since the yeast cells are not surrounded by agar during their first exposure to the fixative, a 2% glutaraldehyde solution should sufficiently penetrate and stabilize the cell wall. Furthermore, the lower glutaraldehyde concentration is less disruptive to the fine structures in the yeast cell wall (Hayat, 1981) (Plate 1, both figures; and Plate 2, Figs. 2, 4 and 6).

To "lighten up" the excessive contrast, the concentration of osmium tetroxide was decreased from 2% to

0.5%. Osmium acts as a post-fixative to preserve fine structures, an electron stain and as a mordant which enhances staining with lead (Hayat, 1981). Staining time with lead citrate was decreased from three minutes to 30 seconds. Contrast was improved but resolution was still poor (Plate 2, all figures).

In Method 3 when the embedding medium was changed from Spurr's-Mollenhauer to Spurr's ERL medium only, the hardness of the block was increased resulting in thinner, more even sections. Microscopically, the resolution was improved and the contrast remained good (Plate 3, all figures). The elongated embedding times may have allowed the resin to more completely penetrate the cell wall and support the delicate ultrastructure of the yeast cell (Plate 3, Figs. 2, 4 and 6; Plate 4, both figures). Cell layer 1 was not only present but thick and dense when processed by method 2 but was diminished or absent in yeast cells prepared according to method 3. This variability could be attributed to the different media on which the strains had been stored prior to preparation for TEM as reported by Poulain et.al. (1978).

Measurements of the cell wall showed that the cell wall thickness is not uniform in all cells. The variability was not restricted to cells of a particular strain or susceptibility to 5-FC. This variation could be

due to distortion during fixation and embedding, the plane at which the cell was sectioned or possible shifting of the cell layers as is seen in budding (Cassone et.al., 1973).

From this study it can be concluded that the cell wall ultrastructure depends on factors that relate to the nature of the organism and/or to the transmission electron microscopy techniques employed.

The Candida albicans strains should be identified through morphologic and biochemical testing and checked periodically to detect any possible mutation. Growth conditions should be carefully selected as was evidenced by the difference in cell layer 1 in cells fixed by methods 2 and 3 and has been mentioned by Poulain et. al. (1978).

Susceptibility to 5-FC as it relates to concentration of 5-FC was studied by Waldorf and Polak (1983). Their work proposed that minimal inhibitory concentration (MIC) of 5-FC was related to the type of resistance - homozygous sensitive (MIC ≤ 1 $\mu\text{g/ml}$), heterozygous resistant (MIC ≥ 100 $\mu\text{g/ml}$ after 48 hours) and homozygous resistant (MIC ≥ 100 $\mu\text{g/ml}$). Tube dilution or agar disc diffusion methods may be a more efficient and practical means of determining 5-FC susceptibility than Whelan's method (1981).

A more definitive study of 5-FC susceptibility would include genetic analysis to identify the mechanisms of resistance as was done for Saccharomyces cerevesiae by Jund

and Lacroute (1970). Genetic analysis methods described by Mortimer and Hawthorne (1966) were used to map loci responsible for resistance to 5-FU, 5-FC and 5-FUR (mutants of Saccharomyces). Candida albicans, although it does not have a sexual phase, is considered diploid and does exhibit mitotic crossing over which results in homozygosity (Whelan et. al., 1981). Once the mutants have been isolated that are homozygous resistant by only one mechanism, TEM to observe any possible changes in the cell wall ultra-structure may be of value.

After the organisms have been identified and characterized as to their 5-FC susceptibility, a method of preparation for TEM must be selected that will maximize the preservation and visualization of all structures to be studied. Persi and Burnham (1981) found that the cell wall thickness varied significantly depending on the fixation schedule used and that potassium permanganate did not produce well-defined regions in the cell wall. A TEM protocol may include special staining, for example, gold markers to localize mannans and chitin in the cell wall of Saccharomyces cerevisiae and Candida albicans (Horisberger and Vonlanthen, 1977). Electron cytochemistry for yeasts is still in the developmental stage and studies to detect and quantitate yeast cell enzymes have been done on cell-free extracts (Jund and Lacroute, 1970; Polak, 1974).

Measurement of cell wall thickness as compared to cell diameter (Tables 3, 4 and 5) does not appear to relate to susceptibility to 5-FC. Montplaisir et. al. (1976) attributed the presence of Layer C6 (Poulain's designation) to resistance of the yeast to 5-FC. Poulain et. al. (1978) disputed this as they found Layer C6 was found in no particular pattern in either susceptible or resistant strains.

The variations observed may be related to the age of the cell and/or the movement of proteins from layer C8 to the more outermost layers (Poulain et. al., 1978).

Other studies to support mechanisms of resistance as compared to cell wall ultrastructure could include enzyme studies on disrupted cells to quantitate amounts of cytosine permease, cytosine deaminase and uridine phosphate pyrophosphorylase. Plating strains of Candida albicans on media containing either 5-fluorocytosine, 5-fluorouracil or 5-fluorouridine could distinguish at which step of metabolism the 5-FC pathway is blocked.

The question remains - "Is TEM a useful tool in investigating the cell wall ultrastructure of Candida albicans?" Garrison (1985) states that "there is hardly any aspect of yeast cell biology that could not benefit from companion ultrastructural design." Furthermore, he suggests that combined efforts on the part of yeast

microscopists could develop improved methodologies. This study supports the need for more standardized protocols for TEM of yeast cells in order to more accurately understand and correlate data.

LITERATURE CITED

LITERATURE CITED

- Ashe, W.D., Jr., and D.E. Van Reken. 1977. 5-Fluorocytosine: A brief review. *Clinical Pediatrics*. 1:384-386.
- Beneke, E.S., and A.L. Rogers. 1980. *Medical Mycology Manual*. pp. 124-131, 4th Ed. Burgess Publishing Company. Minneapolis.
- Berkhout, C.M. 1923. *Les genres Monilia, Oidium, Oospora et Torula*. Thesis. Univ. Utrecht.
- Bullock, G.R. 1984. The current status of electron microscopy: a review. *J. Microsc.* 133:1-15.
- Cassone, A., N. Simonetti and V. Strippoli. 1972. Ultrastructural changes in the wall during germ-tube formation from blastospores of Candida albicans. *J. Gen. Microbiol.* 77:417-426.
- Chattaway, F.W., S. Shenolikar, J. O'Reilly, and A.J.E. Barlow. 1976. Changes in the cell surface of dimorphic forms of Candida albicans by treatment with hydrolytic enzymes. *J. Gen. Microbiol.* 95:335-347.
- Cooper, B. H. and Silva-Hunter, M. 1985. Yeasts of medical importance. pp. 526-541. In: E.H. Lennette (ed.), *Manual of Clinical Microbiology*, 4th Ed. Amer. Soc. Microbiol., Washington, D.C.
- Defever, K.S., W.E. Whelan, A.L. Rogers, E.S. Beneke, J.M. Veselenak, and D.R. Soll. 1982. Candida albicans resistance to 5-fluorocytosine: Frequency of partially resistant strains among clinical isolates. *Antimicrob. Agents Chemother.* 22:810-815.
- Diasio, R.B., J.E. Bennett, and C.E. Myers. 1978. The mode of action of 5-fluorocytosine. *Biochem. Pharmacol.* 27:703-707.
- Djaczenko, W. and A. Cassone. 1971. Visualization of new ultrastructural components in the cell wall of Candida albicans with fixatives containing tapo. *The J. Cell Biology*. 52:186-190.

- Drouhet, E., S. Mountplaisir, and L. Merceir-Soucy. 1974. Etude de l'incorporation des 5-fluoropyrimidines radioactives chez les Candida. Mécanismes de résistance à la 5-fluorocytosine. C.R. Acad. Sci. Ser. D 278:715-718.
- Emmons, C.W., C.H. Binford, J.P. Utz, and K.J. Kwon-Chung. 1977. Medical Mycology. 3rd Ed. 592 pp.
- Garrison, R.G. 1981. Vegetative ultrastructure. pp. 139-160 In: W.N. Arnold (eds.) Yeast Cell Envelopes: Biochemistry, Biophysics, and Ultrastructure, Vol. 1. CRC Press, Inc. Boca Raton, Florida.
- Hermans, P.E. and T.F. Keys. 1983. Antifungal agents used for deep-seated mycotic infections. Mayo Clin. Proc. 58:223-231.
- Hooper, G.R., K.K. Baker and S.L. Flegler. 1979. Exercises in Electron Microscopy. MSU Press. 143 p.
- Horisberger, M. and M. Vonlanthen. 1977. Location of mannan and chitin on thin sections of budding yeasts with gold markers. Arch. Microbiol. 115:1-7.
- Jund, R. and F. Lacroute. 1970. Genetic and physiological aspects of resistance to 5-fluoropyrimidines in Saccharomyces cerevisiae. J. Bacteriol. 102:607-615.
- Medoff, G. and G.S. Kobayashi. 1983. Mode of Action of Antifungal Drugs. pp. 325-355. In: D.H. Howard (ed.), Fungi Pathogenic for Humans and Animals - Part B. Marcel Dekker, St. Louis.
- Montplaisir, S., B. Nabarra, and E. Drouhet. 1976. Susceptibility and resistance of Candida to 5-fluorocytosine in relation to cell wall ultrastructure. Antimicrob. Agents Chemother. 9:1028-1032.
- Mortimer, R.K. and D.C. Hawthorne. 1966. Genetic mapping in Saccharomyces. Genetics 53:165-173.
- Odds, F.C. 1979. Candida and Candidosis. University Park Press. Baltimore.
- Persi, M.A. and J.C. Burnham. 1981. Use of tannic acid as a fixative-mordant to improve the ultrastructural appearance of Candida albicans blastospores. Sabouraudia. 19:1-8.

- Polak, A. 1974. Effects of 5-fluorocytosine on protein synthesis and amino acid pool in Candida albicans. Sabouraudia. 12:309-319.
- Polak, A. 1978. Synergism of polyene antibiotics with 5-fluorocytosine. Chemotherapy. 24:2-16.
- Polak, A. and H.J. Scholer. 1975. Mode of action of 5-fluorocytosine and mechanisms of resistance. Chemotherapy. 21:113-130.
- Poulain, D., V. Hopwood, and A. Vernes. 1985. Antigenic variability of Candida albicans. CRC Critical Reviews in Microbiol. 12:223-270.
- Poulain, D., G. Tronchin, J.F. Dubremetz, and J. Biguet. 1978. Ultrastructure of the cell wall of Candida albicans blastospores: Study of its constitutive layers by the use of a cytochemical technique revealing polysaccharides. Ann. Microbiol. 129:142-153.
- Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17:208-212.
- Rippon, J.W. 1982. Medical mycology. The Pathogenic Fungi and The Pathogenic Actinomycetes. W.B. Saunders Company, Philadelphia 842 pp.
- Shepherd, M.G., R.T.M. Poulter and P.A. Sullivan. 1985. Candida albicans: Biology, genetics, and pathogenicity. Ann. Rev. Microbiol. 39:579-614.
- Spurr, A.L. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
- Stamm, A.M. and W.E. Dismukes. 1983. Current therapy of pulmonary and disseminated fungal diseases. Chest. 83:911-917.
- Thiéry, J.P. 1967. Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J. Microsc. (Paris). 6:987-1018.
- Waldorf, A.R. and A. Polak. 1983. Mechanisms of action of 5-fluorocytosine. Antimicrob. Agents Chemother. 23:79-85.
- Whelan, W.L. and P.T. Magee. 1981. Natural heterozygosity in Candida albicans. J. of Bacteriol. 145:896-903.

Whelan, W.L., E.S. Beneke, A.L. Rogers, and D.R. Soll. 1981.
Segregation of 5-fluorocytosine-resistant variants by
Candida albicans. Antimicrob. Agents Chemother.,
19:1078-1081.

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