Isolation of a germination autoinhibitor produced by Candida albicans
by Kevin Clark Hazen

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in MICROBIOLOGY
Montana State University

Abstract:
Candida albicans is an important opportunistic pathogen of man. When this organism acts as a pathogen it characteristically forms yeast and mycelial cells, but as a saprophyte of the human host it forms only yeasts. Because of the possible relationship between morphology and pathogenesis, investigations were undertaken to determine how this organism regulates germination (yeast to mycelial conversion) . When attempts to induce germination were made, it was found that a low concentration of yeast cells was required. Cells produce a germination inhibitory factor which, after release into the surrounding medium, can affect yeast to mycelial conversion. A protocol to isolate the germination inhibitory factor was developed. This protocol involved charcoal adsorption, ether extraction, high performance liquid chromatography, and Sephadex LH-20 chromatography and resulted in a homogeneous compound. Preliminary identification of the germination inhibitor indicates that it may contain a heterocyclic constituent.

Other experiments to determine the effect of the germination inhibitor on cells were done. The germination inhibitor did not affect uptake of glucose or adenine by cells. However, uridine and amino acids were taken up less by cells exposed to the factor.

Various metals were tested to determine if they could affect the germination inhibitory factor. Only cobalt enhanced the germination inhibitory activity of the factor. Cobalt uptake by cells exposed to the germination inhibitory factor was increased over controls only if they were allowed to incubate in the presence of the factor for several hours before the addition of cobalt.

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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	ix
ABSTRACT	xi
INTRODUCTION	1
MATERIALS AND METHODS	27
Organisms	27
Cultural Conditions and Preparation Organisms for Germination Tests	27
Treatment of Glassware	28
Germination Media	28
Preparation of Cell Filtrates Containing a Germination Autoregulatory Substance	29
Germination Assays	31
Assessment of Morphology	33
Colorimetric Test for Free Sulfhydral Groups	35
Anion Exchange Chromatography	35
Cysteine, Tryptophol, and Phenylethyl Alcohol (PEA)	36
Determination of Cell Growth	37
Preparation of Metal Stock Solutions	37

Page

Addition of Metal to Germination Media 38

Assay for Testing Metal Enhancement of Germination Autoregulatory Activity ... 39

^{60}Co and ^{65}Zn Uptake Experiments 39

Determination of Cobalt and Zinc Levels in Media 40

Uptake of Metabolites Experiments 41

Isolation Procedure of Germination Inhibitory Substance 44

Determination of Purity 48

Proton Magnetic Resonance (PMR) Spectroscopy 49

RESULTS 51

Effect of Cell Concentration on Germination 51

Effect of Cell Growth Phase on Germination 51

Effect of a High Concentration of *C. albicans* Yeast Cells on a Low Concentration of Yeast Cells 54

Kinetics of Production of Germination Regulator 54

Effect of Cell Filtrates of Different yeasts on *C. albicans* 9938 56

Effect of Nutrition on Germination 58

Effect of Dialysis on Germination Activity 60

Page

Effect of Cysteine, Tryptophol, and PEA on Germination 60

Effect of Cell Filtrates with Germination Regulatory Activity on Growth of C. albicans 63

Effect of Metals on Germination 64

Effect of Metals on Germination Regulatory Activity 67

Effect of Cell Filtrate on ^{60}Co and ^{65}Zn Uptake 67

Zinc and Cobalt Levels in Media 74

Effect of Cell Filtrate on Uptake of Metabolites 76

Effect of Isolation Protocol on Activity of Germination Inhibitor 82

Purification Determination of Material Representing Peak C_4-2 97

Recovery of Active Material 97

UV-Visible Spectroscopy of C_4-2 97

PMR Spectroscopy of Various Fractions 102

DISCUSSION 107

CONCLUSION 124

LITERATURE CITED 125

APPENDICES 143

LITERATURE CITED IN APPENDICES 160

LIST OF TABLES

Table		Page
1.	Factors which have been reported to affect *Candida albicans* Y-->M morphogenesis	5
2.	Formulations for 10X concentrated GM-1 and GM-2	30
3.	Effect of high cell concentration on germination	55
4.	Effect of dialysis on germination activity	60
5.	Effect of cysteine of germination	62
6.	Effect of cell filtrate on growth of cells during germination assay	65
7.	Effect of cell filtrate on uptake of ^{14}C-glucose	77
8.	Effect of cell filtrate on uptake of ^{3}H-adenine	79
9.	Effect of cell filtrate on uptake of ^{3}H-uridine	80
10.	Effect of cell filtrate on uptake of amino acids	81
11.	Effect of charcoal adsorption on cell filtrate	83
12.	Effect of pyridine on elution of germination autoregulatory factor from charcoal	84
13.	Effect of ether extraction on fractionation of pyridine eluates	87

Page

14. Germination test of HPLC fractions of cell filtrate 91

15. Germination inhibitory activity of LH-20 fractions of cell filtrate 95

16. TLC of C_4-2 with five different solvents 98

17. Isolation of germination inhibitor 99

18. Effect of the components of GYEP on inhibition of germination 143

19. Effect of combining methanol insoluble material with HPLC fraction C of cell filtrate 148

20. Effect of growth temperature on sensitivity to cell filtrate 151

21. Breakage of yeast cells under various conditions 158

LIST OF FIGURES

Figure		Page
1.	Schematic diagram of the chamber used in diffusion chamber germination assay	32
2.	Different morphologies of *C. albicans*	34
3.	Flow diagram for isolation of the germination autoregulatory substance and points at which activity of the fractions were tested	45
4.	HPLC solvent program used for fractionation of ether extract	47
5.	Effect of cell concentration on germ tube formation	52
6.	Effect of growth phase on germination	53
7.	Effect of amount of incubation on production of germination inhibitor	57
8.	Effect of different concentrations of metal on germination by *C. albicans*	66
9.	Effect of metals on germination inhibitory activity	68
10.	Effect of cell filtrate on uptake of ^{60}Co by cells	69
11.	Effect of delayed addition of cobalt on uptake by cells in GM-2 and cell filtrate	70
12.	Effect of various delay periods on uptake of cobalt	72
13.	Test with different yeasts for enhanced cobalt uptake effect in cell filtrate	73

Figures

		Page
14.	Effect of delayed addition of zinc to media on uptake of zinc by cells	75
15.	HPLC eluate profiles	89
16.	Elution profile for HPLC fraction C after LH-20 chromatography	93
17.	Elution profile of material corresponding to LH-20 peak C_4	96
18.	Flow diagram for isolation of germination inhibitor	100
19.	Proton magnetic resonance spectrum of HPLC fraction C	103
20.	Proton magnetic resonance spectrum of material corresponding to peak C_3	104
21.	Proton magnetic resonance spectrum of material corresponding to peak C_4	105
22.	Proton magnetic resonance spectrum of material corresponding to peak C_4-2	106
23.	IR spectrum of MARS	153
24.	Effect of vortexing time on breakage of *C. albicans*	159

Abstract

Candida albicans is an important opportunistic pathogen of man. When this organism acts as a pathogen it characteristically forms yeast and mycelial cells, but as a saprophyte of the human host it forms only yeasts. Because of the possible relationship between morphology and pathogenesis, investigations were undertaken to determine how this organism regulates germination (yeast to mycelial conversion). When attempts to induce germination were made, it was found that a low concentration of yeast cells was required. Cells produce a germination inhibitory factor which, after release into the surrounding medium, can affect yeast to mycelial conversion. A protocol to isolate the germination inhibitory factor was developed. This protocol involved charcoal adsorption, ether extraction, high performance liquid chromatography, and Sephadex LH-20 chromatography and resulted in a homogeneous compound. Preliminary identification of the germination inhibitor indicates that it may contain a heterocyclic constituent.

Other experiments to determine the effect of the germination inhibitor on cells were done. The germination inhibitor did not affect uptake of glucose or adenine by cells. However, uridine and amino acids were taken up less by cells exposed to the factor.

Various metals were tested to determine if they could affect the germination inhibitory factor. Only cobalt enhanced the germination inhibitory activity of the factor. Cobalt uptake by cells exposed to the germination inhibitory factor was increased over controls only if they were allowed to incubate in the presence of the factor for several hours before the addition of cobalt.

INTRODUCTION

The most common disease of immunocompromised patients caused by yeasts is candidiasis (72). Species of *Candida*, most notably *Candida albicans* (Robin) Berkhout, have been reported to surpass *Cryptococcus neoformans* as the predominant agents of cerebral mycosis (118) and to be the most common yeasts to cause superficial infections in children (48).

The incidence of candidiasis, a general term to describe diseases caused by *Candida* species which range from mild and transient to life-threatening, has risen over the past three decades (64, 115). Several authors have suggested that this is because of an increased usage of antimicrobial drugs, immunosuppressive therapies, and prosthetic devices (2, 46, 64, 99, 115, 131, 169). However, because (superficial) candidiasis is also seen in apparently healthy patients, pathogenesis of the disease remains an enigma.

One aspect of candidiasis which has perhaps received the most attention concerns the dimorphic nature of *Candida albicans*, the most prevalent agent of the disease (46, 131). *C. albicans* cells can assume two *distinct* shapes: yeasts (ovoid, budding cells) and hyphae or

mycelia (tubular structures arising from either yeasts or other hyphae). Typical candidal lesions contain cells of both forms. When C. albicans is a saprophyte of the human host, it forms only yeasts. Because these observations are common, many investigators have presumed that the hyphal form of the organism is pathogenic while the yeast (Y) form is not and, thus, have not considered the importance of yeasts in development of disease (for review see 115). Indeed, although there are several studies which indicate that hyphal formation may be advantageous for the organism, vis a vis a mechanism of escape from phagocytic cells (38, 39, 84), there are also reports that only yeast form cells are required for pathogenesis (129, 166). Hence, if mycelial (M) formation is not required for pathogenesis, then it is at least a common result of host-parasite interactions.

Because elucidation of the mechanism(s) of dimorphic change of C. albicans may be potentially beneficial to man in terms of disease containment as well as providing insight into dimorphic mechanisms of other medically important fungi, such as Histoplasma capsulatum, Sporothrix schenckii, and Blastomyces dermatitidis, and because induction of morphological conversion of C.

albicans in vitro is relatively easy, a plethora of studies have been done on the phenomenon of yeast to mycelial (Y-->M) transition of C. albicans (for reviews see 12, 31, 54, 67, 96, 131, 132, 146). Most of the works prior to 1950 were concerned with the influence of the environment and nutritional factors on morphology. Physiological studies began in the late 1940s, and more recently, attempts have been made to understand the regulatory signals which dictate morphological change.

In spite of the number of studies done on Candida morphogenesis, the biochemical events important in initiation of a Y-->M transition have not been defined. In fact, evaluation of the literature in this area is a formidable task because of conflicting results and conclusions reported by many different research groups. One major problem seems to be that not all strains of C. albicans behave the same under similar conditons. The solution to this may not be simply to distribute standardized strains to various workers in the field because I have found that changes occur even within a single strain after repeated subculturing. Another problem is morphological interpretation. As alluded to earlier, Candida albicans has the ability to exist in many

morphological forms (59, 86, 115) such as budding yeasts, chains of yeasts, chains of elongated yeasts, germ tubes with a constricted base, germ tubes with a broad base and, not infrequently, a yeast cell will develop two germ tubes of which one is constricted and the other is broad based. To describe morphologically a Y-->M conversion at least five terms have been used which include hypha, germ tube, pseudohypha, pseudogerm tube, and filament--yet it is not always clear which morphology is actually observed by various investigators. Difficulty in interpreting and comparing various studies on C. albicans also stems from a lack of uniformity of growth conditions for obtaining yeast cells (see Table 1), various temperatures used to induce a Y-->M transition, a multitude of germination media used, and differences in incubation times used before cellular form is assessed.

These problems are best exemplified by studies on exogenous substances which affect morphogenesis of C. albicans. Table 1 lists some of the substances which when added to media prevent or promote M formation. Unfortunately, most of these reports have been of a phenomenological nature with little attempt at defining possible mechanisms of inhibition or stimulation of

Table 1. Factors which have been reported to affect Candida albicans Y-->M morphogenesis.

Factors tested	Temperature (°C) cells grown[a]	Temperature (°C) germination test	Resultant morphology[c]	Ref.
Carbohydrates				
glucose	37	37	M	76
glucose	37	40	NE	51
glucose	28	28	M,Y,ph	143
2-deoxyglucose	28	37	Y	142
polysaccharides	25	25	M	109
galactose	28	28	Y,ph	143
galactose	NS[d]	24	M	96
fructose	28	28	M,Y,ph	143
maltose	28	28	M,ph	143
sucrose	28	28	M,ph	143
GlcNac[e]	37	37	NE	158

Table 1 (cont'd).

Facters tested	Temperature (°C) cells grown	germinaiton test	Resultant morphology	Ref.
GlcNac	37	37	M	144
GlcNac	25	37	M	24
GlcNac	25	37	M	95
Metals				
Co	25	25	M	111
Co	28	37	NE	142
Zn	37	30	Y	168
Zn	NS	37	NE	157
Fe	37	37	M	78
Fe	NS	37	NE	157
Cu	NS	37	Y	157
Mn	37	37	M	144
Mg	NS	37	NE	157

Table 1 (cont'd).

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
Amino Acids				
cysteine	25	25	Y	101, 111
cysteine	28	28	Y	143
cysteine	30	30	NE	114
cysteine	37	37	NE	159
cysteine	NS	24	NE	96
hydroxyproline	NS	37	M	67
proline	37	37	M	35
proline	37	37	M	76
glutamate	30	30	M	114
glutamate	37	28	Y	92
methionine	37	28	M	92
arginine	37	37	M	76

Table 1 (cont'd).

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
alanine	37	37	M	76
Miscellaneous				
phenylethyl alcohol	28	37	Y	141
L-α-amino-n-butyric acid	37	37	M	93
acetate	28	28	Y,ph	143
lactate	28	28	Y,ph	143
NH_4Cl	37	37	Y	76
phosphate	37	37	Y	76
phosphate	37	37	Y	35
phosphate	NS	24	Y	96
phosphate	25	25	Y	109
phosphate	30	37	M	27
Tween 80	NS	37	M	136

Table 1 (cont'd)

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
Tween 80	28	37	NE	141
EDTA	25	25	M	104
cAMP	37	37	M	76
dibutyryl cAMP	30	37	M	29
dibutyryl cAMP	37	32-34	M	113
oxine + Co	25	25	Y	111
seminal fluid	30	37	M	28
low biotin conc.	37	30	M	167
antibody	RT[f]	37	Y	55
antibody	NS	37	M	147
Co-culture + bacteria	37	37	Y	3
Co-culture + bacteria	37	37	Y	4
Co-culture + bacteia	NS	37	Y	125

Table 1 (cont'd).

Factors tested	Temperature (°C) cells grown	Temperature (°C) germination test	Resultant morphology	Ref.
penicillin	25	25	M	109
aminopterin	25	25	M	109
5-fluorocytosine	28	37	Y	141
5-fluorocytosine	37	37	Y	121
nystatin	28	37	Y	141
2,4-dinitrophenol	28	37	Y	141
iodoacetamide	28	37	Y	141
chloramphenicol	28	37	NE	141
temperature	25	37	M	81
temperature	RT	37	M	89
temperature	NS	37	M	157
NaCl	37	37	Y	34

Table 1 (cont'd).

[a] Temperature at which cells were grown just prior to germination test.

[b] Temperature at which germination was induced.

[c] M=mycelial; Y=yeasts; ph=pseudohyphae; NE=no effect

[d] Not stated

[e] N-acetyl-D-glucoseamine

[f] Room temperature

morphogenesis. Indeed, the significance of this problem is manifested by contradictory reports concerning the effect of many different substances on Candida morphogenesis. For example, some workers obtained filamentous cells using high concentrations of glucose in an otherwise yeast inducing medium (76) but others found that glucose does not enhance germination (51, 109, 143). Reports have also indicated that glucose is important in maintaining the yeast form because if glucose levels are decreased (67) or if another carbon source such as galactose (96) or a less readily metabolizable carbohydrate is substituted for glucose (54, 109, 143) germination is enhanced.

Similar confusion exists when considering the effects of many other substances on morphogenesis. Until more definitive studies utilizing standardized conditions are done existing contradictions will not be resolved.

Regardless of the problems cited above some consistent results concerning various aspects of dimorphism of C. albicans have emerged from different laboratories.

Chemical analyses of Y and M cell walls and membranes have shown quantitative but not qualitative

differences between these morphological forms (25, 71). These findings are not unlike those obtained by others on Y and M of Mucor indicus (8) and Histoplasma capsulatum (73). Candida cell walls are composed chiefly of glucose as glucan, mannose as mannan (6), protein and a small amount of lipid (71). Whereas Y and M cell walls have a similar content of glucose and mannose, M cell walls have one third the amount of protein and three times the amount of glucosamine (presumably as chitin) as Y cell walls (25). The larger quantity of glucosamine in hyphal cell walls correlates with the suggestion (23) that an electron transparent layer which increases in width in the germ tube cell wall is composed of chitin. Increased chitin synthesis by M cells of C. albicans was also supported by others (18) who found that hyphal forming cells take up more N-acetyl-glucosamine than yeasts. In these studies, chitin deposition was found to occur at the site of budding for blastoconidial forming cells and at the apical region of hyphal forming cells.

Substantial differences in phospholipid and sterol content between cytoplasmic membranes of Y and M have been reported. Yeast cell membranes contain phosphotidyl serine, phosphotidyl inositol, sphingolipids, phosphotidyl

ethanolamine and phosphotidyl choline. However, only phosphotidyl ethanolamine and phosphotidyl choline have been detected in membranes of exponential phase M cells (94). This correlates well with other observations (5) that the major lipids synthesized from ^{14}C-acetate during Y-->M conversion are phosphotidyl choline, phosphotidyl ethanolamine, and neutral lipids. Overall lipid synthesis, however, is lower in M than in Y cells (152).

Considering the evidence that only stationary phase yeast cells are capable of germinating (24, 148), it is interesting that stationary phase yeast cell membranes more closely resemble membranes of exponential phase mycelial cells that exponential phase yeast cells (70). Taken a step further, these data may imply that changes in the chemical make-up of the cell membrane mark the initiation of morphogenesis.

At the ultrastructural level, differences between yeasts and young hyphae have been observed. Yeast cell walls seem to be composed of five layers whereas germ tubes have only four layers (23, 137). However, hyphae associated with invasion of human tissues may be composed of six distinct cell wall layers (126). There is disagreement as to which of the five layers of yeast cell

wall disappear during hyphal development. Cassone, Simonetti, and Strippoli (23) feel that dissolution the second layer of the germinating yeast cell occurs during hyphal initiation, but Scherwitz, Martin, and Ueberberg (137) believe the third layer disappears. It is interesting that initiation of bud and germ tube formation appear similar in that the major part of emerging daughter cell walls of both forms are composed of material contiguous with the fourth layer of the mother cell (137). This may indicate that the fourth cell wall layer is composed of flexible material.

In addition to fewer cell wall layers usually found in hyphal cells the wall of this form of C. albicans is about half the thickness of a yeast cell wall (23). Similar correlations between yeast and mycelial cell walls have been observed in Histoplasma capsulatum (40) and in Mucor (8).

As alluded to above, chitin synthesis rates appear to be different between Y and M cells. The key enzyme in the production of chitin from glucose, L-glutamine-D-fructose-6-phosphate aminotransferase, has been demonstrated to have a higher specific activity in germinating cells. In addition, induction of the enzyme

appears to be related to the appearance of germ tubes (30).

Further evidence that chitin synthesis is involved in germination of C. albicans cells are the observations that the last enzyme in the chitin synthesis pathway, chitin synthetase (synthase), has a higher specific activity in hyphal cells and hyphal cells incorporate ten times more N-acetyglucosamine (GlcNAc) than yeasts (18). Modeling their studies on those done on Saccharomyces cerevisiae (21, 41, 42, 69), Braun and Calderone (18) suggested that the proteolytic activator of chitin synthetase binds more strongly to the enzyme of mycelial cells than to the enzyme of yeast cells.

GlcNAc, a precursor for chitin synthesis, has been demonstrated to induce germ tube formation (144). Although the observation has been disputed (158) it is difficult to evaluate the controversy because of differences in experimental protocol. GlcNAc has also been shown to induce germination more quickly (24) and to induce Y-->M conversion in cells which normally do not germinate (i.e., early and mid-logarithmic phase cells) (95).

Exogenous GlcNAc appears to induce synthesis of a

chitin synthesis enzyme N-acetylglucosamine kinase by C. albicans (13). However, the induction kinetics are similar in both yeast and mycelial cells (142) as are the specific activities. This suggests that N-acetylglucosamine kinase is not important in Y-->M conversion (142) which is not surprising because the organism can synthesize chitin from glucose.

The possible involvement of carbohydrate utilization pathways in morphological changes of C. albicans cells has been investigated. In studies using metabolic inhibitors, Hasilik(57) reported that phosphofructokinase (PFK) and glucose-6-phosphate dehydrogenase (G6PD) are important for growth. Chattaway et al. (26) studied some enzymes of glycolysis, of the oxidative pentose pathway, and the first enzyme of chitin synthesis, viz., L-glutamine-D-fructose-6-phosphate aminotransferase. PFK activity was lowest when germination was at its peak, and cells which had germinated contained less PFK activity than yeast form cells. The investigators felt that the low PFK activity would allow more fructose-6-phosphate to be available for chitin synthesis via L-glutamine-D-fructose-6-phosphate aminotransferase. This idea correlates with their earlier findings that chitin is more abundant in mycelial form

cells than in yeasts (25). Unfortunately, the mycelial extracts used for enzyme assays were obtained from cells grown at 40°C and yeast extracts were from cells grown at 30°C. Therefore, it is impossible to know if reduced PFK levels are required for Y-->M morphogenesis or if the reduction in activity is due to an increase in incubation temperature.

Adenosine-3':5'-cyclic monophosphate (cAMP), which is well established as being involved in various metabolic events of prokaryotes (130) as well as morphogenesis of Mucor racemosus (119) has been studied for its participation in Y-->M conversion of C. albicans. Exogenous addition of cAMP to C. albicans has been shown to decrease RNA and protein synthesis (14) and to increase Y-->M conversion (77). Although others found that cAMP would not promote M development (113) Y-->M transition was enhanced by addition of the lipophilic dibutyryl derivative of cAMP (29, 113).

Attempts to correlate intracellular cAMP levels with germination reveal that the cAMP concentration increases prior to (113) and during germination (29) but these changes also correlate with a shifting of the incubation temperature from 30°C to 37°C. However, in accordance

with correlating cAMP changes with morphogenesis, substances which either increase or decrease intracellular cAMP levels increased or decreased germination, respectively (29).

Only one investigation has endeavored to integrate cAMP levels with metabolism. The addition of glucose to the C. albicans growth medium did not affect levels of cAMP or GlcNAc catabolic enzymes. Glucose, on the other hand, did repress the enzymes and cAMP levels in Saccharomyces cerevisiae. These data suggest the C. albicans catabolic repression mechanisms differ from other fungi (145). Furthermore, the mechanisms by which increased glucose may prevent mycelial formation may not involve cAMP. Reduced oxygen tension or increased atmospheric CO_2 have been shown, in other studies, to favor Y-->M conversion by Candida albicans (54, 67, 92, 96, 146, 163). Under anaerobic conditions (100% H_2 atmosphere, however, the percentage of germinating cells in serum was reduced (36). Although attempts bave been make to correlate repressed respiratory activity (mitochondrial repression) with germ tube formation (76, 77) the data obtained from using various respiratory inhibitors are not clear. A recent paper contains data

indicating that respiration rate is not different between Y cells and cells undergoing Y-->M conversion (62). However, the tubular structures observed by the investigators appear to be elongated buds and not true germ tubes.

It is interesting to note that under conditions which are considered to enhance filamentation, viz., reduced glucose concentrations (see earlier discussion), the respiratory pathway that becomes dominant is cyanide resistant. Under less limiting conditions, the major respiratory pathway is cyanide sensitive (74). Clearly, more work is needed in this area to understand how O_2 tension relates to morphogenesis.

Protein synthesis occurs at similar rates in Y and M forms of C. albicans (33). Whereas cytoplasmic protein synthesis is required for morphogenesis, mitochondrial protein synthesis seems unnecessary (141).

In an attempt to determine whether there are proteins unique to yeast or mycelial cells, Dabrowa et al.(33) used disc gel electrophoresis to analyze cell free extracts of these forms. Two protein bands were reported to be unique to yeasts and one band unique to mycelial cells. However, with two-dimensional gel electrophoresis

mycelial cells were found to have fewer proteins than yeast cells and proteins unique to mycelial forms were not detected (19, 20, 89, 90, 91). It was suggested that Y-->M conversion occurs because of post-translational protein modifications (90, 91) or differential gene expression (19).

The relationship between nucleic acid synthesis and Candida morphogenesis is not clear. Recently, evidence has been produced which indicates that germination may be a sexual characteristic of C. albicans (117). However, this view is not universally accepted (122, 135).

During a Y-->M conversion nuclear division occurs when the germ tube reaches a critical length (10, 151), whereas in budding cells nuclear division occurs when the bud attains a critical volume (10, 150). Production of M form cells was found to occur in the presence of an inhibitor of purine synthesis (109) and in adenine deficient strains of C. albicans (75). However, a Y-->M transition was blocked by using other inhibitors of RNA synthesis (141) and M form cells were reported as having more RNA than the yeast form (168). Then again, others found DNA and RNA synthesis to be similar in Y and M forms (33, 159,). It may be more informative to evaluate RNA

(33, 159,). It may be more informative to evaluate RNA synthesis in terms of which species of RNA is synthesized during initiation of morphogenesis.

All of the studies mentioned above have yielded important and, in some cases, significant insights into the nature of the mechanism of morphological change in C. albicans. However, two "classical" sets of investigations on C. albicans dimorphism which are relatively ignored by current authors but which are the original impetus for many contemporary studies are those of Walter J. Nickerson and those concerning the production of morphological regulatory substances.

Nickerson conducted extensive investigations into the the role of cysteine in Y-->M conversion of Candida albicans because of several observations. First, Trichophyton rubrum was found to release an -SH containing compound which inhibited germination of C. albicans (66, 108). Second, 10^{-2} M cysteine inhibited germination of wild type and of a filamentous mutant of C. albicans (102, 111). Third, C. albicans cells accumulated cobalt when cobalt was present in the growth medium (112), and it was known that cysteine chelates cobaltous ions (1). Fourth, the presence of a readily utilizable carbon source

such as glucose did not prevent Y-->M morphogenesis (109). From these observations, Nickerson postulated that filamentation resulted when cell growth occurs without cell division (101, 102) and cell division occurs only in the presence of a sufficient number of -SH groups (provided, for instance, by cysteine) (102). In support of this concept, C. albicans was described as having an NADH dependent cystine reductase (110, 132) of which the NADH is provided through glucose metabolism (109). Hence, carbon sources such as polysaccharides, would not yield enough NADH to power the cystine reductase and, thus, a Y-->M conversion would result.

In related studies a filamentous mutant of C. albicans, designated strain 806, was induced to grow as a yeast when incubated in the presence of a substance released from wild-type cells (105). Furthermore, strain 806 and wild-type mycelial cells, but not yeasts, released protons into the medium surrounding the colony (104). Nickerson (104) proposed that release of H^+ indicated that they were unavailable for reducing the appropriate substance(s) required for maintenance of yeast growth (103). Although the final proton acceptor molecule in yeasts was not identified, it was suggested that it may

reside in the cell wall (52, 106, 107). Furthermore, by accepting protons and subsequently forming more covalent bonds (possibly as -SH groups) the acceptor molecule in the wall would favor the prolate spheroid shape of the yeast form (53).

As in other investigations on C. albicans Y-->M conversion, however, the observations of Nickerson and co-workers suffer because the studies were done on developed yeast and mycelial cells rather than on events which occur prior to morphological evidence of a transition. Therefore, it is not possible to make definitive conclusions about the importance of cysteine in initiating Candida morphogenesis.

In 1939, Langeron and Guerra (80) reported that C. albicans cells located on apposed sides of two parallel streaks would not germinate when the distance between the streaks was sufficiently short. These workers believed that the effect might be due to production by C. albicans of a germination inhibitory factor, but some felt that this so called "Langeron effect" was merely due to nutritional depletion (88). Others, however, sought to determine the nature of the germination inhibitor of which two substances, phenylethyl alcohol (PEA) and tryptophol,

were isolated (83). Because these substances inhibited growth of C. albicans it is difficult to evaluate their effects on morphogenesis.

It is particularly interesting that other investigators have made similar observations to those of Langeron and Guerra (80), viz., that C. albicans produces substances, presumably other than tryptophol and PEA, which affect morphogenesis (66, 124, 134). The identity of these substances or how they metabolicallly affect a Y-->M conversion is unknown. The production of germination self-inhibitors by C. albicans yeast cells provides an explanation for the long observed phenomenon in the clinical laboratory. That is, germination will not occur during a germ tube test if the inoculum of the C. albicans yeast cells is too concentrated (68, 79, 143).

It is not unreasonable to expect that C. albicans does produce a germination self-inhibitor. Several other fungi such as Syncephalastrum racemosum (63), Glomerella cingulatta (82), and Mucor racemosus (100) and others (11, 15, 47, 87, 155), have been shown to produce such substances. If a germination self-inhibitor of C. albicans can be identified, then its utilization as a tool to understanding dimorphism of the organism is potentially

limitless. Furthermore, information gained from such studies could provide a much needed basis for treatment approaches to combat candidiasis.

It has been my approach to determine if, in fact, C. albicans does produce a germination self-inhibitor. Once this was established I have attempted to formulate an isolation scheme which would yield a homogeneous preparation for the autoinhibitor and allow preliminary chemical characterization to be done.

Also, in some preliminary experiments which involved using a crude preparation of the germination self-inhibitor, the metabolic effect of the inhibitor on C. albicans cells was determined.

MATERIALS AND METHODS

Organisms

Candida albicans 9938 (Mycology Unit, Tulane Medical School, New Orleans, LA) was used in all experiments unless otherwise indicated. *C. albicans* strains 2252, 3153, and 550 were obtained as gifts from T.G. Mitchell (Duke Universtiy, Durham, NC) and strains 804a and 394 from Richard R. Morrison (University of Oklahoma, Norman, OK). *Candida parapsilosis*, *Candida tropicalis*, *Crytococcus laurentii* C-16, and *Saccharomyces cerevisiae* were from the mycologic culture collection at Montana State Universtiy. All organisms were maintained on corn meal agar slants and transferred every six months.

Cultural Conditions and Preparation of Organisms for Germination Tests

Unless stated otherwise, fungi were grown in 50 ml of glucose (2.0%)-yeast extract (0.3%)-peptone(1.0%) (GYEP) broth contained in 250 ml Erlenmeyer flasks at 37°C for 48 h under constant aeration by rotation at 160 rpm (Incubator Shaker, New Brunswick Scientific, Edison, NJ). Under these conditions, over 99% of *C. albicans* remained in the yeast form. The yeasts were used immediately after incubation or within a 24 h postincubation period during

which they were refrigerated. Before use in germination experiments, the cells were washed three times with 0.15 M NaCl or sterile, deionized water.

Treatment of Glassware

All glassware was washed with 7X detergent (Flow Laboratories, Inglewood, CA) in distilled water and rinsed with deionized water. Sterilization was by autoclaving.

In some experiments glassware, washed as above, was acid cleaned by soaking for 30 min at 60°C in nitric acid (previously diluted by mixing one part concentrated nitric acid with seven parts deionized water), rinsing repetitively with deionized water, covering with aluminum foil, and sterilizing by dry heat at 400°C for 4 h. All acid cleaned glassware was used within 24 h of treatment.

Germination Media

Fetal calf serum (FCS) (Microbiological Associates, Walkersville, MD), tissue culture medium (TC199) (Microbiological Associates), and two synthetic media, derived from TC199, and designated GM-1 and GM-2 were used. When media were supplemented with TC199, one ml of a 10X concentrated tissue culture medium (10X TC199)

(Grand Island Biological, Grand Island, NY) was added per 9 ml of test medium. Otherwise, TC199 containing 1.4 g of $NaHCO_3$ / liter and 25 mM N-2-hydroxymethyl piperazine-N'-2-ethanesulfonic acid buffer (Microbiological Associates) was utilized.

GM-1 and GM-2 were prepared from 10X stock solutions (Table 2) by adding 1 part stock to 7 parts sterile, deionized water and 2 parts sterile, 0.05 M, pH 7.4 (at 37°C) tris (hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4 Tris buffer).

Preparation of Cell Filtrates Containing a Germination Autoregulatory Substance

Cell filtrates were prepared by inoculating either TC199 or GM-1 with 2×10^8 (3 times washed) C. albicans 9938 cells per ml of medium and incubating for 15 h to 18 h at 37°C in sterile, glass centrifuge tubes or 1000 ml graduated cylinders. When TC199 was used, incubation was in 5% CO_2 : 95% air. After incubation, suspensions were centrifuged and the supernatant liquid was collected, filtered through a 0.45 um porosity membrane, and adjusted to pH 7.2 by dropwise addition of 0.1 N NaOH (when TC199 was used) or to pH 7.4 by dropwise addition of tris (hyroxymethyl)aminoethane base (Trizma base, Sigma). All

Table 2. Formulations for 10X concentrated GM-1 and GM-2.

Medium	Subtance[a]	Amount(mg/l)	Molarity(M)
GM-1 and GM-2	folic acid[b]	10	2.27×10^{-5}
	L-arginine HCl	1266	6.01×10^{-3}
	L-methionine	150	1.01×10^{-3}
	L-proline	692	6.01×10^{-3}
	L-histidine HCl	420	2.00×10^{-3}
GM-1	glucose	1000	5.56×10^{-3}
GM-2	glucose	5000	2.78×10^{-2}
	d-biotin	10	4.09×10^{-5}
	NH_4SO_4	250	1.89×10^{-3}
	$NaH_2PO_4 \cdot H_2O$	413	2.99×10^{-3}
	$Na_2HPO_4 \cdot 12H_2O$	717	2.00×10^{-3}

[a] All chemicals are products of Sigma Chemical Company (St. Louis, MO) except glucose and NH_4SO_4 (J.T. Baker Chemical Co., Phillipsburg, N.J.).

[b] Components were heated into solution, and the final solution was sterilized by filtration through 0.45 um porosity membranes (HAWP, Millipore Corp., Bedford, MA)

cell filtrates were used within 48 h during which they were refrigerated.

Germination Assays

Tube germination assay. 5×10^5 washed *C. albicans* cells were resuspended in 1 ml of an appropriate medium and placed into a glass tube (12 x 75 mm) and incubated for 3 h at 37°C in air when FCS was used or in 5% CO_2 : 95% air (to prevent a drop in pH) when TC199 was used. Cell morphologies were determined at the end of the incubation period.

Diffusion chamber germination assay. To test the effect of a population of yeast cells on germination by anotner population of cells, plastic chambers consisting of an upper and a bottom well (Blind Well Chambers with a 5/16 inch [ca. 6.35 mm] bottom well, and Bio-Rad Laboratories, Richmond, CA), separated by a 0.45 um porosity, detergent free membrane (HATF, Millipore Corp., Bedford, MA) were used (Figure 1). Each well received an equal volume of yeast cell suspension. Chambers were incubated for 3 h at 37°C before the morphologies of the cells in each chamber were assessed. When the suspending medium was TC199, incubation was in 5% CO_2 : 95% air.