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Analysis of Ash1, a regulator of filamentous growth and virulence of Candida albicans

Author
Inglis, Diane O.

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Analysis of Ash1, a regulator of filamentous growth and virulence of *Candida albicans*

by

Diane O. Inglis

DISSERTATION

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DEDICATION

This thesis is dedicated to my daughter, Crystal.

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ABSTRACT

Analysis of Ash1, a regulator of filamentous growth and virulence of *Candida albicans*

Diane O. Inglis

In response to a number of distinct environmental conditions, the fungal pathogen *Candida albicans* undergoes a morphological transition from a round, yeast form to a series of elongated, filamentous forms. This transition is believed to be critical for virulence in a mouse model of disseminated candidiasis. Here we describe the characterization of *C. albicans ASH1*, a gene that encodes an asymmetrically localized transcriptional regulatory protein involved in this response. We show that *C. albicans ash1* mutants are defective in responding to some filament-inducing conditions. We also show that Ash1p is preferentially localized to daughter cell nuclei in the budding-yeast form of *C. albicans* cell growth and to the hyphal tip cells in growing filaments. Thus, Ash1p "marks" newly formed cells and presumably directs a specialized transcriptional program in these cells. Finally, we show that *ASH1* is required for full virulence of *C. albicans* in a mouse model of disseminated candidiasis.

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Asymmetric cell division and cell specialization

Introduction

One of the hallmarks of multi-cellular organisms is the presence of different cell types that are specialized to carry out particular functions. This ability is established, in part, through asymmetric cell divisions that result in the unequal distribution of cellular components between mother and daughter cells upon completion of cell division. The result is two genetically identical cells, each with distinct properties or cell fates. During the development of mammals and vertebrates, multiple rounds of asymmetric cell division occur, with asymmetrically divided cells further dividing in an asymmetric manner, ultimately contributing to an asymmetric body plan and the extraordinary diversity of cell-types and tissues present in fully developed organisms. The production of diverse cell-types that are capable of carrying out highly specialized functions is one of the characteristics that set multi-cellular organisms apart from single-celled organisms such as bacteria and yeast.

It is now known that even unicellular organisms (including yeast and bacteria) undergo asymmetric cell divisions that produce genetically identical mother and daughter cells each expressing different RNAs and proteins that promote different cell fates for mother and daughter cells. The best-studied examples of asymmetric cell division in unicellular organisms come from studies of *Caulobacter crescentus* and the budding yeast *Saccharomyces cerevisie*. In *C. crescentus*, cell division can produce two cell-types with distinct morphologies: the stalk cell and the swarmer cell. Stalk cells are non-motile reproductive cells that divide to produce non-reproductive and highly motile swarmer cells (63) (for review see (32)). Thus, the production of these two cell-types in *C.*

crescentus enables this organism to partition distinct abilities between the products of one cell division: the capacity to reproduce and the ability to move.

Other examples of asymmetric cell division occurs during development of asexual spores, called conidia, of numerous pathogenic and non-pathogenic fungi. Here, a series of asymmetric cell divisions produces specialized cells from which chains of conidia are formed. Conidia, which are usually very small and readily aerosolized, can easily enter a host through inhalation. The production of such cells is a current area of research; however, our understanding of eukaryotic cell division is much more advanced in model organisms, such as the budding yeast *S. cerevisiae*.

Factors that determine asymmetric cell division of *S. cerevisiae*

In *S. cerevisiae*, at least two mechanisms produce asymmetric cell divisions. The first is the production of a polarized mitotic spindle with proteins specifically localized to the end of the spindle pole that is destined to become part of the daughter cell (40). The cell division plane, and therefore the size asymmetry between mother and daughter cells, is determined by the precise positioning of the spindle pole. Upon cell division, the protein(s) that were attached to the spindle pole become localized to the bud tip of the daughter cells and are thereby trapped specifically in the daughter cell (40). This pattern of cell division generates the asymmetric distribution of proteins between mother and daughter cells as well as the difference in size at the time of cell division.

A second mechanism known for asymmetric cell division in *S. cerevisiae* is the directed transport of mRNAs along actin cables to the distal pole of the growing bud that is destined to become the daughter cell. Upon cell division, the mRNAs are trapped

in the daughter cells and translated into proteins that presumably carry out daughter cell specific functions. The best known example of an asymmetrically localized mRNA with a daughter-cell specific function is the GATA-like transcription factor Ash1 (4, 56). *ASH1* mRNA is localized to daughter cells through transport by a ribonucleoprotein (RNP) complex (43, 61). Upon completion of cell division, Ash1 protein represses the expression of the *HO* endonuclease that initiates mating-type switching of *S. cerevisiae* (56). Thus, the asymmetric distribution of Ash1 to daughter cells produces a mother cell that switches mating-type and daughter cell that does not.

One hypothesis about the function of repression of mating-type switching in daughter cells is that a single cell can generate a colony with both a and α cells that can mate and form diploids which may enhance the survival of isolated yeast cells in the wild (28). Another idea is that repression of mating type switching in daughter cells may reserve a subset of cells in the population that have not undergone programmed double-stranded break and repair. This process introduces either small insertions or deletions at the *HO* cleavage site about 60% of the time an *HO*-induced double stranded DNA break is repaired at the *MAT* locus of *S. cerevisiae* (44). Thus, repression of *HO* by Ash1 may also specifically enhance the survival of yeast daughter cells by reducing the risk of genome damage as a result of incorrect repair of the double-stranded DNA break at the MAT locus.

Ash1 has been described as a lineage determinant whose asymmetric localization to daughter cells generates a stem cell lineage in a dividing culture (28, 56). The idea that daughter cells are protected from mutation as a result of Ash1 localization is consistent with maintaining a lineage of stem cells that are undifferentiated. In the

wild, the localization of Ash1 in daughter cells also generates a pool of cells that switch mating type, enabling diploidization and meiosis. These cells also sustain mutations that may either enhance or reduce overall fitness. Mating between daughter cells is essentially the same as doing a backcross with the parental strain in the laboratory. Deleterious mutations can be repaired or recombined during meiosis in the diploid and advantageous mutations could then be segregated between **a** and α spores.

Factors involved in the asymmetric localization of Ash1

In *S. cerevisiae*, *ASH1* mRNA is transported to bud tips through its association with the She-complex of RNA-binding proteins (5, 41, 62). She2 binds directly to *ASH1* mRNA and is thought to function as an adaptor protein that in turn binds the She3 and She1/Myo4 complex (5, 41). She3 encodes an RNA-binding protein with no specific homology to other proteins and She1 encodes a Type V mini-myosin that binds to polarized actin cables and transports the She-*ASH1* RNP complex to the bud tip (4, 61). Mutations in any of the five SHE genes (*SHE1 - 5*) cause *ASH1* RNA and protein to be symmetrically distributed between mother and daughter cells (4, 33). She4 is required for normal actin-cytoskeleton organization and has been shown to bind directly to Myo4 and provide an essential function for both Class I and Class V myosins (64, 66). She5, also known as Bni1, is involved in budding polarity, and polarized growth (12). The precise role of Bni1 in localizing *ASH1* to daughter cells is unknown and may be indirect due to the altered actin cytoskeleton and cell polarity defects of *bni1* mutants.

In addition to the She proteins, two additional RNA binding proteins that are required for localization of *ASH1* have been identified. Khd1 encodes a KH-domain

protein that co-localizes with *ASH1* mRNA at bud tips (31). In *khd1* mutants, *ASH1* mRNA is partially delocalized. The tight anchoring of *ASH1* mRNA is thought to be coupled to its translation (7, 17) thus it was proposed that Khd1 is involved in anchoring and translation of *ASH1* at the bud tip (31). Khd1 was also named *HEK2* by another group due to its similarity to a "HEterogeneous nuclear rnp K-like gene" and also co-localizes with sub-telomeric DNA sequences (10). An exclusively nuclear RNA-binding protein, Loc1, is also required for *ASH1* asymmetry between mother cells. In the absence of Loc1, *ASH1* mRNA remains trapped in the nucleus of both mother and daughter cells (42). Loc1 was also described as a component in a complex involved in large ribosomal subunit biogenesis (27). A possible explanation for the role of Loc1 in *ASH1* localization is that like ribosomal RNA, *ASH1* RNA may require enzymatic modifications that facilitate the packaging of RNA into RNP complexes, similar to rRNA processing which is required for assembly of rRNAs with ribosomal proteins to form mature ribosomes (15).

Additional asymmetrically localized mRNAs in *S. cerevisiae*

Since the identification of *ASH1* as a transported mRNA of *S. cerevisiae* (43, 61), at least 21 additional mRNAs have been identified that are also asymmetrically localized to growing bud tips (54, 60) (the nascent daughter cell). Tagged versions of each of the three She proteins involved in *ASH1* transport have been used in genome-wide screens for RNAs physically associated with the She proteins. Surprisingly, most of the bud-tip localized She-associated transcripts identified showed asymmetric localization to daughter cells even in the absence of She2 which suggests that an additional mechanism exists to generate the asymmetric distribution of these proteins (54).

In contrast to *ASH1* localization, where the She-dependent transport from mother cells to the daughter cells serves an important role in the cell fate of mother and daughter cells, other gene products may be localized to the bud tip for some other reason. For example, some proteins may function specifically at the emerging bud site. According to this idea, mRNA localization is coupled to translation thus ensuring that the protein is produced only where it is needed (7, 17).

Pseudohyphal growth and asymmetric cell division

Diploid *S. cerevisiae* cells do not express the *HO* gene (59) and therefore do not require repression by Ash1 for regulation of *HO* expression. However, Ash1 is nonetheless asymmetrically localized to diploid daughter cells (56). In response to nitrogen starvation, *S. cerevisiae* uses an alternative growth pattern that is available to many yeast-like fungi (37). During pseudohyphal growth of *S. cerevisiae*, elongated cells bud from mother cells in a highly polarized manner and remain attached after cell division (16). This growth pattern generates filaments that extend into and along the top of solid media making filamentous colonies appear rough or wrinkled with a halo of filaments around the edge of the colony. The colony appearance of filamentous cells is dramatically different from the yeast form which produces smooth, round colonies.

Cell division during pseudohyphal growth has been described as symmetric. This is primarily due to the morphology of mother and daughter pseudohyphal cells and the synchronous timing of their cell cycles after the completion of cytokinesis (37). During yeast form growth, daughter cells are smaller than mother cells after cytokinesis and remain in G1 longer than mother cells. Mother cells initiate a new round of budding

immediately after cytokinesis whereas daughter cells are delayed in bud formation (38). During pseudohyphal growth, daughter cells are about the same size as mother cells after cytokinesis and initiate bud growth in synchrony with mother cells. The asymmetric localization of Ash1 in pseudohyphal cells (6) indicates that an asymmetry does exist between pseudohyphal mother and daughter cells despite their synchronous cell division characteristics.

Ash1 is also required for filamentous growth of diploid *S. cerevisiae* (6). In the absence of Ash1, round and smooth colonies are formed and cells remain in the yeast form. The cell-surface flocculin, *FLO11*, is an important determinant of pseudohyphal growth of *S. cerevisiae* (53) and it is regulated either directly or indirectly by Ash1. Localization studies of Flo11 showed a polarized distribution on the cell wall with brighter signal toward the pole where daughter cells formed; however, Flo11 was present on both mother and daughter pseudohyphal cells. One explanation for the presence of Flo11 on mother cells in spite of being under asymmetric regulation, is that Flo11 may be incorporated into the cell wall during daughter cell formation and become a permanent constituent of the wall. When a daughter cell becomes a mother cell, Flo11 may remain on the cell wall. Thus activation of *FLO11* expression in daughter cells by Ash1 may serve to increase the level of the *FLO11* transcript in the growing daughter at birth, when the incorporation of new cell wall material and the molecular activities that accompany morphogenesis are likely to be the most critical.

Asymmetric division and hyphal growth of *Candida albicans*

The fungal pathogen, *Candida albicans*, also undergoes asymmetric cell division. At low temperature and low pH, *C. albicans* grows as a budding yeast with asymmetric and asynchronous cell divisions that closely follow the pattern observed in *S. cerevisiae* (37). Under starvation conditions or environmental signals such as growth at 37° C, neutral pH, serum, or the presence of compounds such as N-acetyl glucosamine and proline, *C. albicans* rapidly converts to a highly elongated hyphal form of growth. During hyphal growth, *C. albicans* grows as elongated cylindrical cells that remain attached after cell division (46). Long-term growth in the hyphal form produces a mass of entangled hyphal cells known as mycelia with each mycelium comprised of chains of multiple, attached cells. Mature hyphae can give rise to new budding yeast cells that bud either from the original mother cell, sub-apical cells, or the tip cell (47). Both pseudohyphal growth and true hyphal growth are often referred to as filamentous growth of *C. albicans* although these two forms have distinct characteristics (20). True hyphal growth however, is highly polarized and asynchronous (2, 22, 24).

Growth of true hyphae occurs by polarized apical expansion of the cell wall of the growing tip cell (23, 47). During cell division of the tip cell, vacuoles are selectively inherited by sub-apical mother cells while daughter cells primarily inherit cytoplasm (2, 24). As a consequence of sub-apical mother cells of receiving the most vacuole and the least cytoplasm, sub-apical cells are delayed in initiating a new round of cell division following cytokinesis. In contrast, the newly formed daughter tip cell continues to extend and divide after cytokinesis (2). The rapid linear extension of true hyphal filaments may be driven by the forward migration of cytoplasm in tip cells with the sub-apical mother

cells acting as repositories for vacuolar organelles (24). Since this mode of growth requires very little synthesis of new cytoplasm, it is thought to enable filamentous fungi to traverse nutrient-poor environments with minimal expenditure of biosynthetic resources (2, 51).

Hyphal tip cells are specialized for secretion

Hyphal tip cells are known to be the powerhouses of secretion in filamentous fungi (8, 50, 70) and are exploited for the production of a variety of proteins in culture . The fungal secretory process is thought to be intimately associated with the process of growth at the hyphal tip (50). *C. albicans* is known to selectively secrete hydrolases and tissue degrading enzymes from the tips of hyphal cells (9, 14, 29). Secretion of aspartyl proteases enables unavailable nutrient sources, such as serum, to be hydrolyzed into oligopeptides that can be transported into the cell and utilized for growth (3, 29, 58). The secretion of phospholipases and aspartyl proteases is also known to be responsible for tissue damage during host infection and thus may enable hyphal tip cells to penetrate host tissues (9, 58). The high rate of growth at the hyphal tips require the polarized secretion of structural cell wall components and biosynthetic enzymes (20, 22). The asymmetric nature of the hyphal cell division cycle generates daughter cells at the hyphal tip that are specialized for growth and secretion and are enriched in endoplasma reticulum (ER), Golgi, and secretory organelles (8, 47). Since *C. albicans* is a commensal of humans, the secreted proteins of this organism are likely to be specialized more for survival in the host rather than for foraging in the wild where *C. albicans* is rarely found.

Hyphal tip cells are specialized to respond to touch

A discussion of cell specialization in *C. albicans* is not complete without mention of the remarkable ability of this fungus to sense and respond to physical cues in the environment. Detailed studies of hyphal tip cells behavior on scratched surfaces and on porous membranes have demonstrated that *C. albicans* has the ability to both sense physical changes in its growth surface and respond by altering its direction of growth (21, 25). This specialized activity takes place exclusively in growing tip cells of hyphal filaments and is thought to be conferred by membrane mechanosensors present at the hyphal tips (65).

Ash1 and filamentous growth and virulence of *Candida albicans*

In *C. albicans*, Ash1 protein is localized to the daughter cells of both the yeast and filamentous form cells. In growing hyphae, Ash1 is absent from all cells except for the actively dividing apical tip cell (30). Asymmetric distribution of Ash1 in *C. albicans* presumably relies on mRNA transport as it does in *S. cerevisiae* although this has not been rigorously proven for *C. albicans*. The absence of Ash1 reduces filamentous growth on solid media and attenuates virulence in a mouse model of candidiasis (26).

Other specialized cells in filamentous fungi

The production of the specialized conidial cells of fungi is the result of numerous rounds of asymmetric cell division. During conidial development, hyphal cells divide asymmetrically to produce a new type of cell, sometimes referred to as a stalk cell, which further undergoes asymmetric cell divisions to produce chains of conidia. Conidia

have several specialized properties that make them highly infectious. For example, conidia of the pathogenic fungus *Cryptococcus neoformans* produce pigments that have been shown to influence the host immune system (52), and the conidia and hyphae of several fungal species are known to contain hydrophobic proteins, called hydrophobins that are thought to play a role in breaking surface-air tension. Hydrophobin proteins self-assemble and form a layer of rod-like molecules on the surface of hyphae. This enables them to escape the surface tension of the growth substrate and produce spores, also coated with hydrophobins, that disperse easily and remain airborne for long periods of time. For reviews see (11, 36, 49, 67-69). The ability of fungi to produce infectious spores by altering their patterns of cell division enables conidiating fungi to travel large distances, perhaps to a new environmental niche, and generates a highly specialized type of cell that is resistant to environmental stresses. While many genes have been identified that regulate conidial development, particularly in the non-pathogenic organisms, *Aspergillus nidulans* and *Neurospora crassa*, the mechanisms by which asymmetric cell divisions arise during conidiation are unknown.

Other asymmetric processes in *S. cerevisiae*

Aging in yeast cells is also a property that is asymmetrically distributed between mother and daughter cells of *S. cerevisiae* (57). At least four cellular events correlate with yeast aging which is defined as the ability of mother cells to give rise to new daughter cells. The common theme between these events is that mother cells are the repository for cellular components that become damaged with age. First, mother cells preferentially accumulate extrachromosomal ribosomal DNA circles (ERCs) which are

toxic in high number and are thought to contribute to senescence of older mother cells (13, 45). The production of ERCs occurs due to hyper-recombination at the rDNA (ribosomal DNA) locus (34). A second cellular event, related to ERC production is the redistribution of the SIR silencing-complex proteins from the telomeres and the silent mating-type loci to the nucleolus in old cells (18, 35). This redistribution of Sir proteins to the nucleolus extends the life span of mother cells which typically exhibit fragmentation of the nucleolus in old age (35). Old mother cells also become sterile as Sir2 is redistributed from the silent mating type loci to the nucleolus due to derepression of silencing at HMR and HML. In the nucleolus, Sir2 plays a role in repressing rDNA transcription and represses the formation of ERCs (19). The role of the nucleolus in yeast aging remains unknown however, nucleolar changes are also associated with aging in mammalian cells (55).

The third asymmetric cellular event associated with aging is the preferential accumulation of oxidatively damaged proteins in mother cells and their relative absence in daughter cells (1). The presence of increased amounts of proteins with carbonylated proteins in mother cells was also attributed to the presence of the Sir2 protein in these studies. A fourth factor that was shown to influence age asymmetry between mother and daughter cells is the *ATP2* gene which encodes the beta-subunit of mitochondrial F(1), F(0)-ATPase. Mutation in this gene causes daughter cells to be born old and was correlated with segregation of mitochondria with reduced respiratory potential to daughter cells and eventual loss of mitochondrial transmission (39). Changes in mitochondrial function are also associated with aging (48).

Summary and Conclusion

The diversity of the cell-types present in higher eukaryotes enables them to carry out a multitude of highly specialized functions. Asymmetric cell divisions appear to drive the formation of complex patterns of development in multi-cellular organisms and also underlie the production of specialized cells in unicellular yeast and bacteria. In *C. albicans* and other filamentous fungi, asymmetric cell division during filamentous growth has the important consequence of enabling these unicellular organisms to generate an attached lineage of cells that carry out specialized functions. In spatial terms, filamentous growth distributes both the biosynthetic resources of its cells and the greatest lifespan potential at the leading edge of the growing colony, precisely poised to forage for nutrients and well equipped to secrete enzymes to convert poor nutrient sources into usable substrates. In fungi that produce conidia, the production of specialized cells through asymmetric cell division enables this fungus to generate airborne infectious propagules that facilitate the establishment of infection in a host.

The role of Ash1 in virulence of *C. albicans* is intriguing. It suggests that some asymmetric aspect of filamentous growth that also influences virulence is required specifically in daughter cells. One outstanding question is whether the asymmetric distribution of Ash1 is actually required for its function in filamentous growth. More specifically, the question is whether Ash1 is actually required in daughter cells or must Ash1 be removed from mother cells in order to exert control. From over-expression studies in *S. cerevisiae* we know that strains producing extra *ASH1* contain Ash1 protein in both mother and daughter cells and show enhanced filamentous growth. In *C. albicans*, this also appears to be true (unpublished results, S. Elson). It seems more likely then that

Ash1 function is required for filamentous growth specifically in daughter cells rather than its absence being required in mother cells. If so, the active transport of *ASH1* out of mother cells may serve to pool the transcripts from both cells into the daughter cell to increase the amount of Ash1 present in daughters. High concentrations of Ash1 in daughter cells may be important to activate low-affinity target genes or may amplify the signal in daughter cells to promote development as a filamentous cell.

Furthers studies of Ash1 could provide a better understanding of the molecular and cellular events that generate cell asymmetry during hyphal cell division and daughter cell specialization at hyphal tips. The differences between Ash1 function in *C. albicans* and *S. cervisiae* (such as target genes) could also distinguish between the asymmetric aspects of filamentous growth that are conserved and those that have diverged for specialization in these organisms. Thus, Ash1 provides a unique experimental tool for investigating daughter cell function and an opportunity for insight into asymmetric cell division and the events that contribute to cell fate and specialization in a unicellular fungal pathogen.

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Chapter 2

Ash1 Protein, an Asymmetrically Localized Transcriptional Regulator, Controls Filamentous Growth and Virulence of *Candida albicans*

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Ash1 Protein, an Asymmetrically Localized Transcriptional Regulator, Controls Filamentous Growth and Virulence of *Candida albicans*

Diane O. Inglis and Alexander D. Johnson*

Department of Biochemistry and Biophysics and Department of Microbiology and Immunology, University of California—San Francisco, San Francisco, California 94143

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In response to a number of distinct environmental conditions, the fungal pathogen *Candida albicans* undergoes a morphological transition from a round, yeast form to a series of elongated, filamentous forms. This transition is believed to be critical for virulence in a mouse model of disseminated candidiasis. Here we describe the characterization of *C. albicans ASH1*, a gene that encodes an asymmetrically localized transcriptional regulatory protein involved in this response. We show that *C. albicans ash1* mutants are defective in responding to some filament-inducing conditions. We also show that Ash1p is preferentially localized to daughter cell nuclei in the budding-yeast form of *C. albicans* cell growth and to the hyphal tip cells in growing filaments. Thus, Ash1p "marks" newly formed cells and presumably directs a specialized transcriptional program in these cells. Finally, we show that *ASH1* is required for full virulence of *C. albicans* in a mouse model of disseminated candidiasis.

Candida albicans is a common fungal pathogen that causes mucosal infections in healthy individuals and can cause life-threatening disseminated infections in immunocompromised patients. *C. albicans* is capable of colonizing most tissue types in humans and is therefore able to adapt and thrive in the diverse microenvironments encountered in a host (7, 36). *C. albicans* responds to changes in its environment by altering its patterns of gene expression, and these responses appear critical to the survival and virulence of this opportunistic pathogen. In the laboratory, *C. albicans* responds to changes in growth conditions, including starvation, 37°C temperature, neutral pH, exposure to serum, contact with animal cells, or the presence of compounds such as proline and *N*-acetylglucosamine by switching from a round, single-celled budding-yeast form to elongated filamentous forms. These filamentous forms include a spectrum of morphologies that range from pseudohyphae (chains of elongated cells that remain attached after cell division) to true hyphae (long cylindrical cells separated by septal walls that lack constrictions at sites of cell division) (35). Current evidence is consistent with the idea that the interconversion of these forms is critical for virulence of *C. albicans* (for reviews, see references 6, 10, 11, 20, 22, 33, 44, and 47). For example, all of the morphological forms are found in infected tissues, and mutations that lock *C. albicans* into either the yeast or filamentous form produce mutants with significantly reduced virulence when tested in mouse models of disseminated candidiasis. Additional links between filamentous growth and virulence come from studies showing the differential expression of certain cell surface and secreted proteins in filamentous cells compared to budding yeast cells; for example, newly formed filaments adhere better to mammalian cells than do yeast-form cells. It may be that increased adherence and invasion are important for early stages of *C. albicans* infection; the ability to form yeast cells that bud off from adherent hyphae may subsequently promote the colonization of diverse tissues during disseminated infection (36).

Filamentous growth is common to many species of fungi. For example, *Saccharomyces cerevisiae* undergoes a process of pseudohyphal growth that is similar in some respects to filamentous growth of *C. albicans* (17; for reviews, see references 28 and 31). In particular, diploid *S. cerevisiae* cells grow, in response to nitrogen starvation, as elongated chains of pseudohyphal cells that extend as filaments into solid media. Although *S. cerevisiae* and *C. albicans* are closely related evolutionarily, there are important differences between the types of filamentous growth in these two organisms. For example, *C. albicans* filaments are induced in response to several growth conditions (such as 37°C temperature and exposure to serum) that do not critically affect filamentous growth of *S. cerevisiae*. Moreover, both *S. cerevisiae* and *C. albicans* produce pseudohyphae, yet only *C. albicans* can make true hyphae, defined by the absence of constrictions at the sites of cell division and by a particular pattern of mitosis and cell division (45). Despite these differences, many of the components that control filamentous growth—particularly those in signaling pathways—are conserved between *S. cerevisiae* and *C. albicans* (for example, see references 2, 9, 12, 16, 25, 26, 29, 30, and 40).

The *S. cerevisiae* Ash1 protein (Ash1p) was originally isolated in screens designed to identify proteins involved in mating-type switching in haploid yeast cells (1, 43). Later, it was discovered that Ash1p is also required for pseudohyphal growth in *S. cerevisiae* (8). With regards to pseudohyphal growth, Ash1p has been linked to a transcriptional regulatory cascade that, in response to nitrogen starvation, activates expression of the *FLO11* gene (37); *FLO11* encodes a cell surface protein that is required for pseudohyphal growth. For these reasons, we hypothesized that an *ASH1*-related gene in *C. albicans* has a role in filamentous growth, and in this paper, we test this idea.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of California—San Francisco, 513 Parnassus Ave., S-410, San Francisco, CA 94143-0414. Phone: (415) 476-8783. Fax: (415) 476-8201. E-mail: ajohnson@cgl.ucsf.edu.

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
C. albicans[a]		
CAF2-1	*URA3/ura3::imm434*	13
CAI4	*ura3Δ/ura3Δ*	13
RM1000	*ura3Δ/ura3Δ his1::HisG/his1::HisG*	34
YDI-1	*ura3Δ/ura3Δ ASH1/ash1::HisG-URA3-HisG*	This study
YDI-7	*ura3Δ/ura3Δ ash1::HisG/ash1::HisG-URA3-HisG*	This study
YDI-11	*ura3Δ/ura3Δ ash1::HisG/ash1::HisG*	This study
YDI-129	*ura3Δ/ura3Δ cph1::HisG/cph1::HisG ash1::HisG/ash1::HisG-URA3-HisG*	This study
YDI-154	*ura3Δ/ura3Δ ash1::HisG/ash1::HisG::ASH1-URA3 (2d)*	This study
YDI-157	*ura3Δ/ura3Δ ash1::HisG/ash1::HisG::ASH1-URA3 (6d)*	This study
YDI-199	*ura3Δ/ura3Δ ash1::HisG/ash1::HisG-URA3-HisG::myc6-ASH1*	This study
JKC19	*ura3Δ/ura3Δ cph1::HisG/cph1::HisG-URA3-HisG*	29
S. cerevisiae[b]		
CGX69	*MATa/MATα ura3/ura3*	17
YDI-56	*MATa/MATα ura3/ura3 ash1::HisG/ash1::HisG*	This study
YAS242-10B	*MATα ASH1 can1::HO-CAN1 ho::HO-ADE2 ade2-1 his3-11,15 trp1-1 ura3 leu2-3,112*	Sil, unpublished
YAS234-1A	*MATα ash1::TRP1 can1::HO-CAN1 ho::HO-ADE2 ade2-1 his3-11,15 trp1-1 ura3-leu2-3,112*	Sil, unpublished

[a] All *C. albicans* strains listed as *ura3Δ/ura3Δ* are homozygous for the *ura3::imm434* mutation, except for CAF2-1, which is heterozygous for the mutation.
[b] CGX69 and YDI-56 are of the Σ1278b strain background; YAS242-10B and YAS243-1A are of the W303 strain background.

First, we describe the isolation and characterization of a *C. albicans* gene that is homologous to the Ash1p gene of *S. cerevisiae*. *C. albicans* Ash1p can complement an *S. cerevisiae ash1* mutant, indicating that *C. albicans* Ash1p has biochemical activities (for example, its ability to function as a transcriptional repressor) (32) similar to those of *S. cerevisiae* Ash1p. *C. albicans* strains with *ASH1* deleted show defects in filamentous growth in vitro and have reduced virulence in a mouse model of systemic candidiasis. We also show than when *C. albicans* proliferates in the budding-yeast form, Ash1p is asymmetrically localized to the daughter cell nuclei following each cell division, a pattern previously observed for Ash1p from *S. cerevisiae* (1, 43). When *C. albicans* enters the hyphal form of growth, Ash1p is localized preferentially to the nuclei of the hyphal tip cells: that is, to the nuclei of only the most recently formed hyphal cells. Given that Ash1p is a transcriptional regulator, it seems likely that its presence in hyphal tip cells endows them with a specialized transcriptional program. Indeed, a number of observations in the literature indicate that *C. albicans* hyphal tip cells have properties distinct from those cells that make up the internal (subapical) portions of the hyphae. For example, hyphal tip cells are known to selectively secrete phospholipase B (reviewed in references 14 and 21); it has been proposed that the secretion of this and other hydrolytic enzymes destroys the material in front of the hyphal tip cell and thereby enables it to invade host tissues, as reviewed in reference 18. The hyphal tip cells of the filamentous fungus *Aspergillus niger* are also known to have specialized properties; for example, the enzyme glucomylase is selectively secreted from the tips of mycelia (46). In *C. albicans*, we propose that Ash1p directs, at least in part, the specialization of the hyphal tip cells.

MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. G. Fink and colleagues generously provided the *cph1/cph1* strain, JKC19 (29). The *S. cerevisiae* strains YAS242-10B, YAS243-1A, and YAS204 were obtained from A. Sil and colleagues (University of California—San Francisco [UCSF]; unpublished data). Strains YAS242-10B and YAS243-1A have the *ADE2* gene integrated at the *HO* locus and the *HO* promoter integrated upstream of the *CAN1* locus. Both of these promoter-gene fusions cause reporter gene expression to be controlled by the *HO* promoter. Deletion of *S. cerevisiae ASH1* has been described previously (43). The strains CGX69, YAS204, and YDI-56 are of the *S. cerevisiae* Σ1278b background (16). YDI-56 is a derivative of YAS204 (*ash1::HisG-URA3-HisG/ash1::HisG-URA3-HisG*, *ura3Δ/ura3Δ*), which was selected for Ura auxotrophy on plates containing 5-fluoroorotic acid (5-FOA).

S. cerevisiae strains were transformed by lithium acetate transformation (15) with selection on SD −Leu or SD −Ura medium. *C. albicans* transformations were performed by the lithium acetate method described in reference 4. Strains were routinely grown on YPD plates and liquid medium at 30°C or on SD −Ura medium unless otherwise noted. Counterselections against the *C. albicans URA3* gene were performed by overnight growth at 30°C in YPD liquid medium followed by selection on 5-FOA and uridine.

Phenotype testing and induction of *C. albicans* filamentous growth were done with solid Spider medium as described previously (29); with Lee's medium as described previously (27), at pH 6.8 with the addition of 2% agar; and with YPD with 10% fetal calf serum. Either cells were streaked onto plates, or overnight liquid cultures were diluted in water or YEP medium and plated for single colonies. Plates were incubated for 5 to 6 days at 30°C in plastic sleeves and photographed at a ×7.5 magnification under a Nikon SMZ-U microscope (Fig. 3A) or a Leica M420 microscope (Fig. 3B and C). Sabouraud dextrose agar (Difco), used for tissue fungal burden assays, was prepared according to the manufacturer's directions.

Cloning the *C. albicans ASH1* gene. The *S. cerevisiae* Ash1p sequence (GenBank accession no. CAA82028.1) was used in a tBlastn search of the *Candida albicans* sequence database (http://alces.med.umn.edu/gbsearch/ybc.html), which yielded three overlapping sequence tags with 63% identity to the C terminus of *S. cerevisiae* Ash1p. With these sequence data, the oligonucleotides DI-1FW (5′-TCTACACACAAATTCATTCC-3′) and DI-2RV (5′-ATTTAGGAAGTA CTTCAA CT-3′) (Operon) were designed and used to amplify a 357-bp fragment from *C. albicans* SC5314 genomic DNA. The resulting PCR fragment was gel purified (Qiaquick gel extraction kit), labeled with ^{32}P in a random prime reaction (Amersham), and used to probe a size-selected *C. albicans* genomic library made in the Lambda ZapII vector (kindly provided by B. Braun; www.sacs.ucsf.edu/home/JohnsonLab/). Eleven strongly hybridizing clones were obtained. Eight clones were sequenced through the open reading frame (ORF)-containing region and through the vector junctions. Six of these clones contained a full-length *ASH1* ORF, and two were N-terminal truncations. One full-length clone, pDI-16, was sequenced to completion and found to contain 1.3 kb upstream and 335 bp downstream, including part of another ORF, *DSK2*, which begins 185 bp after the stop codon of *ASH1*. Alignment of the Ash1p proteins was done with GCG Pileup program (Wisconsin Package, version 8.0; Genetics Computer Group, Madison, Wis.) and presented for viewing with Seqvu 1.1 (Garvan Institute of Medical Research, Sydney, Australia).

Expression of *C. albicans ASH1* in *S. cerevisiae*. For expression studies, the *C. albicans ASH1* ORF was amplified by PCR with *Pfu* Turbo polymerase (Strat-

agene) by using primers DI-5FW (5′-CAAAACTACCGTGATACAC-3′) (Operon) and DI-25RV (5′-GGATCCGAGCTCATTGTTGATTATTCGGTA TAGAG-3′) (UCSF BRC) with plasmid pDI-16 as the template. A 2.6-kb fragment that includes sequence 1 kb upstream and 185 bp downstream of *ASH1* was cloned into the vector pRS425 (42) as an *Xho*I-*Sac*I fragment to construct the *LEU2*-marked plasmid pDI-24. This PCR-generated insert was sequenced to verify its accuracy. The *Xho*I-*Sac*I fragment was digested from pDI-24 and cloned into pRS426 to create the *URA3*-marked plasmid pDI-25. The *S. cerevisiae ASH1* expression plasmid, pAS174, was described previously (43). pAS199 (A. Sil, unpublished observations) contains the same *S. cerevisiae ASH1* fragment as pAS174, except pRS425 (*LEU2*) is the vector backbone. For the *HO* repression assays, strains were streaked onto SD −Leu plates containing 10 mg of adenine per ml and 0.03% canavanine (1), incubated for 5 days at 30°C, and then transferred to 4°C for 3 days to enhance color differences between Ade$^+$ and Ade$^-$ strains before photographing. Pseudohyphal growth assays were performed by incubating cells at 30°C for 5 days on SLAD medium containing 50 μM ammonium sulfate (17). Photographs were taken at a magnification of ×38 on a Nikon SMZ-U microscope.

Disruption of the *C. albicans ASH1* gene. To disrupt the *C. albicans ASH1* gene, a modified Ura blaster method was used (5, 13). Heterozygous strains were first constructed by replacing *ASH1* coding sequences with the *URA3* selectable marker flanked by *HisG* repeats. Ura$^+$ strains that were deleted for one copy of *ASH1* were grown on nonselective medium and plated on 5-FOA and uridine medium to select for loss of the *URA3* gene. Two independent Ura− *ASH1* heterozygous strains were used to disrupt the second allele of *ASH1* with another *HisG-URA3-HisG* cassette, pDI-23. Sequences from the 5′ and 3′ regions of *ASH1* were amplified by PCR, digested with restriction enzymes, and cloned into the disruption vector pBB510, a derivative of pMB7 (13) described in reference 5, to create the disruption plasmid pDI-03. The 5′ flank was amplified with primers 5′ KO-HIII (5′-TACATTAAGCTTCGTGCTGGTCATTACAGCC-3′) and 3′ KO-*Pst*I (5′-ATGTAACTGCAGTTCGGAGTTTGGTTGTAGG-3′), digested with *Hin*dIII and *Pst*I, and then cloned into the *Hin*dIII-*Pst*I sites of pBB510 to create the cloning intermediate p510-5′. The 3′ flank was amplified with the primers 5′ KO2-*Nsi*I (5′-ATTGAAAATGCATAGCTAAATAACCAT CATCATCACGCACC-3′) and 3′ KO2-*Kpn*I (5′-AATACTGGTACCAACTC AAGATTTAGGAAGT-3′), digested with *Nsi*I and *Kpn*I, and cloned into the *Nsi*I-*Kpn*I sites of p510-5′. The resulting plasmid, pDI-03, contains the *HisG-URA3-HisG* disruption cassette flanked by sequences in the *ASH1* promoter and within the 3′ end of *ASH1*. To avoid disruption of *DSK2* promoter sequences at the 3′ untranslated region (UTR) of *ASH1*, the 3′ flank contains ORF sequence from the 3′ end of *ASH1*. A second disruption plasmid, pDI-23, was made by digesting pDI-03 with *Bam*HI and *Bgl*II to release the *HisG-URA3-HisG* cassette, which was then gel purified and cloned back into the pDI-03 backbone in the reverse orientation.

Strains with *ASH1* deleted were created by homologous integration of a *Hin*dIII-*Xmn*I fragment from pDI-03 (to disrupt the first allele) or pDI-23 (to disrupt the second allele). Isolates were screened by PCR and by Southern blotting for the presence of both disruption constructs at the *ASH1* locus and for the absence of the disrupted ORF sequence (data not shown). Four independent isolates homozygous for deletions in *ASH1* were obtained by this method. Because *ash1* mutants obtained by the Ura blaster method retained 93 codons (although no in-frame start codons) at the C terminus, a fifth isolate with the complete *ASH1* coding sequence deleted was obtained by the PCR product method (48, 49) in the Ura$^-$ His$^-$ strain RM1000 (34). The primers DI-55DR (5′-CCGAAGAACCTAAAAAAAAAAAGTAGTCAACATTGTCGAAGCT ACCAAATAACCACAGTAGTTTTCCCAGTCACGACGTT-3′) and DI-56DR (5′-TAACAGATATCTAATCCTATATAAATGAAGCTTCCTTTACA ATACTTTTCTAAACTCAAGTGTGGAATTGTGAGCGGATA-3′) (vector hybridizing sequence is underlined) were used in separate PCRs with the templates pGEM-HIS1 and pDDB57 to generate the PCR disruption products. Whole-cell PCR, using primers internal to the *HIS1* (5′HIS1-RV, 5′-TTGACT ATACCTTCGCTGTC-3′; 3′ HIS1-FW, 5′-GCAATAAACCCCTTGTGGAC-3′) or *URA3* (5′URA3-RV, 5′-TGGTGAGGCATGAGTTTC-3′; 3′URA3-FW, 5′-GAGATGCTGGTTGGAATGC-3′) selectable markers and outside the flanking region of homology to the *ASH1* locus (upstream DI-8-FW, 5′-TCAA GACAAATCACAATTCC-3′; downstream DI-9RV, 5′-ATCCTTCAACACCT TTCC-3′), was used to identify an *ash1*::*URA3*/*ash1*::*HIS1* isolate. The phenotype of this strain, with a complete deletion of the *ASH1* ORF, was identical to those of *ash1*::*HisG*/*ash1*::His-*URA3-HisG* strains obtained by the URA blaster method.

Reintroduction of the wild-type *ASH1* gene into *C. albicans ash1/ash1* mutants. A *Kpn*I-*Sac*I fragment from pDI-25 was cloned into the *Kpn*I-*Sac*I sites of pDI-26 (D. Inglis, unpublished), a derivative of the maltose-inducible expression plasmid pAU15 (kindly provided by M. A. Uhl), to create the reintegration construct, pDI-29, which expresses *ASH1* under the control of its own promoter. pDI-29 was linearized with *Pac*I and transformed into strain YDI-11 (*ura3Δ*/*ura3Δ*, *ash1*::*HisG*/*ash1*::*HisG*). Ura$^+$ transformants were screened by whole-cell PCR with primers DI-20FW (5′-CTGATTTAGTCTACACTACCCAC-3′) and DI-46RV (5′-AGATCTAGTGTGTGAAGGG-3′) (Operon) to verify correct insertion of *ASH1* at the 5′ region of *ash1*::*HisG*. Forty-five Ura$^+$ transformants that yielded positive results by PCR were tested on Spider plates to compare the filamentous growth of these strains with that of the *ash1/ash1* parent strain and the *ASH1/ash1* heterozygote, which also carries one copy of *ASH1*. Surprisingly, the integration of wild-type *ASH1* with plasmid pDI-29 failed to restore filamentous growth to all but 2 of the 45 transformants that screened positive by PCR for correct integration of the plasmid. Of these two transformant strains, one (YDI-154) was slightly more filamentous than the wild-type CAF2-1 strain on Spider medium (data not shown). The other transformant (YDI-157) produced filaments similar to those of the *ASH1/ash1* heterozygote (YDI-1) and was also restored for virulence comparable to that of YDI-1.

Localization studies of Ash1p. A PCR fragment generated by using *Pfu* Turbo polymerase (Stratagene) and containing a multimerized epitope of myc (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn), flanked by *Bgl*II restriction sites, was cloned into the *Bgl*II site 8 amino acids downstream of the third potential start methionine at the N terminus of the *ASH1* coding sequence of the 2μm Ca*ASH1* plasmid, pDI-25. The insertion was sequenced for accuracy, and the plasmid complemented the pseudohyphal growth defects of the *S. cerevisiae ash1/ash1* mutant YDI-56. An *Xho*I-*Hin*dIII fragment containing myc-*ASH1* and 1 kb of the *C. albicans ASH1* promoter sequence from pDI-25 was cloned into the *Xho*I-*Hin*dIII sites of pDI-29 to generate the plasmid pDI-30, which is identical to the reintegration construct pDI-29, except for the sequence encoding the myc epitope. pDI-30 was digested with *Pac*I and integrated into the *ASH1* promoter region of YDI-11 to generate the myc-tagged *ASH1* strain YDI-199.

For cells in the budding-yeast form of growth, cultures were grown to mid-log phase (optical density at 600 nm [OD_{600}] of ~ 6) in M199 culture medium (pH 4.5) at 23, 25, and 30°C or in YPD at 25 or 30°C. Chains of connected budding yeast cells (pseudohyphae) were produced by growth in M199 (pH 7.0) at 23°C. Hyphal forms of *C. albicans* were induced by growing late-log (OD_{600} of 20) to stationary-phase cultures (OD_{600} of 40) and diluting cells to OD_{600} of 0.8 to 1.0 in prewarmed YPD with 10% serum at 30, 35, and 37°C or in YPD at 35°C with 20% serum in a shaking water bath. Cells were harvested by sterile pouring or by transfer with a wide-bore pipette tip into a 15-ml conical tube and fixed at room temperature in 4.5% formaldehyde for 1 h. Cells were prepared for antibody hybridization and microscopy by methods described in reference 43 with modifications for *C. albicans* based on reference 45. Cells were resuspended in a total volume of 0.5 ml in SP (1.2 M sorbitol, 0.1 M potassium phosphate) buffer with 1 to 2 μl of β-mercaptoethanol and 40 μl of Zymolase-20T (ICN Pharmaceuticals) and incubated at 37°C with gentle shaking for a total of 10 to 15 min for yeast-form cells and 15 to 20 min for hyphal cells. Sixteen microliters of cells was transferred to a polylysine-coated slide well, and cell wall digestion was continued for 10 min at room temperature, while the cells settled onto the slide wells. Excess solution was gently aspirated from the wells, and the cells were allowed to dry. To flatten cells, slides were submerged in −20°C methanol for 5 min, followed by −20°C acetone for 30 s, and then dried completely. The 9E10 myc monoclonal antibody (a gift from Joachim Li, UCSF) was used at a 1:6,000 dilution for the yeast and hyphal experiments shown. Previous batches of 9E10 from the same source have been used at 1:300 for yeast-form cells and at 1:800 for hyphal cells. A Cy3-conjugated goat anti-mouse antibody (obtained from Jackson ImmunoResearch Laboratories, West Grove, Pa.) was used at a 1:200 dilution to detect the myc epitope. A rabbit polyclonal antibody (38) raised against *S. cerevisiae* Tup1p cross-reacts with *C. albicans* Tup1p and produces a staining pattern that is tightly associated with the nucleus in both mother and daughter cells of *C. albicans* yeast and filamentous forms. A purified version of this antibody (S. Green, unpublished observations) was used at 1:400 as a positive control for nuclear stain and detected with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (SC2012; Santa Cruz Biotechnology). Cells were blocked at room temperature for 1 to 2 h in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% Tween 20. Sixteen microliters of the 9E10 and anti-Tup1p primary antibodies (diluted in PBS with 1% BSA) was hybridized to cells for 1 h at room temperature. Sixteen microliters of secondary antibodies was hybridized to cells in the dark for 1 h. Excess primary and secondary antibodies were removed by washing the slide wells four times with PBS. To visualize cell nuclei, cells were stained after the first secondary antibody wash by applying 16 μl of 1-μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) (diluted in PBS) to slide wells for 2 min. Slide wells were then washed four times with PBS and aspirated before the slides were

treated with Fluormount-G (Southern Biotechnology Associates, Inc.). Digital images were captured by a charge-coupled device (CCD) camera on a Leica DMLB microscope through either a ×100 oil objective lens for yeast cell images or through a ×40 objective lens for hyphal cell images.

Virulence studies and histological sections. Groups of six female (18 to 20 g) BALB/c mice (Charles Rivers) were each injected by tail vein with *C. albicans* cells and monitored daily for survival and other signs of infection. Cells were prepared for injection by diluting overnight cultures (grown at 30°C in liquid YPD) into fresh YPD to an OD_{600} of 0.1 to 0.2, growing the cells for approximately 4 h, and then washing and resuspending the cells in sterile saline (0.9% $NaCl_2$) and counting them with a hemacytometer. A total of 10^6 cells per mouse (0.5 ml of 2 × 10^6 cells per ml of solution) were injected by tail vein. Animals were sacrificed by CO_2 and handled according to UCSF Committee on Animal Research (CAR) guidelines.

Whole kidneys were dissected from infected mice and prepared for histological staining (41) by being fixed overnight in 4% neutral buffered formalin (NBF), followed by serial dehydration in alcohol and then toluene. Samples were embedded in paraffin, cut into 5-μm sections, and stained with a periodic acid-Schiff base (Sigma Diagnostics), followed by a light green counterstain (Harleco). Images were photographed through a Leica DMLB microscope. Colony counts were obtained by removing kidneys from infected mice, weighing them, and homogenizing the tissue in 1 to 3 ml of sterile distilled water. Serial dilutions were plated onto Sabouraud dextrose agar, and plates were incubated at 30°C for 1 to 2 days before CFU were counted.

Nucleotide sequence accession number. The complete *C. albicans ASH1* sequence has been submitted to the GenBank database under accession no. AF237674.

RESULTS

Identification and sequence of *C. albicans ASH1*. To identify an *ASH1* homolog in *C. albicans*, we searched the partial *C. albicans* sequence database available in July 1998 (http://alces.med.umn.edu/gbsearch/ybc.html) for sequence traces similar to those of *S. cerevisiae* Ash1p. A 577-nucleotide sequence was identified that, when translated, matched the zinc finger region located in the C terminus of *S. cerevisiae* Ash1p. Oligonucleotides were designed to PCR amplify a 358-nucleotide-pair fragment from genomic DNA, and this PCR product was used to probe a lambda library containing *C. albicans* genomic fragments (5). Several full-length clones were obtained and sequenced, and the complete *C. albicans ASH1* sequence was submitted to the GenBank database (accession no. AF237674). This sequence is identical to that now found in assembly 6 of the *C. albicans* genome sequence provided by the Stanford DNA Sequencing and Technology web site (http://www-sequence.stanford.edu/group/candida/).

C. albicans ASH1 is predicted to encode a 449-amino-acid protein with 36% identity overall to the *S. cerevisiae* protein (Fig. 1). The zinc finger region is 81% identical over 34 amino acids. The N-terminal regions are much less similar, with only 23% identity over 359 amino acids. Like the *S. cerevisiae* protein, *C albicans* Ash1p has three potential consensus sites for phosphorylation by Cdc28/CDK (1) and two consensus sites in the zinc finger for acetylation, a modification that alters the activity of the related GATA-1 transcriptional regulator in mammals (3).

***C. albicans* Ash1p can regulate expression of the *HO* promoter and promote pseudohyphal growth in *S. cerevisiae*.** As reviewed in the introduction, *S. cerevisiae* Ash1p regulates both expression of the *HO* gene (which encodes the endonuclease that initiates mating-type switching) (1, 43) and pseudohyphal growth under conditions of nitrogen starvation (8). To determine whether *C. albicans* Ash1p is a functional homolog of the *S. cerevisiae* protein in both respects, we asked whether *C. albicans* Ash1p could regulate (in this case, repress) expression of *HO* and promote pseudohyphal growth in *S. cerevisiae ash1* mutant strains. For these experiments, *C. albicans ASH1* and *S. cerevisiae ASH1* were each cloned into a high-copy-number 2μm vector and transformed into *S. cerevisiae ash1* mutant strains.

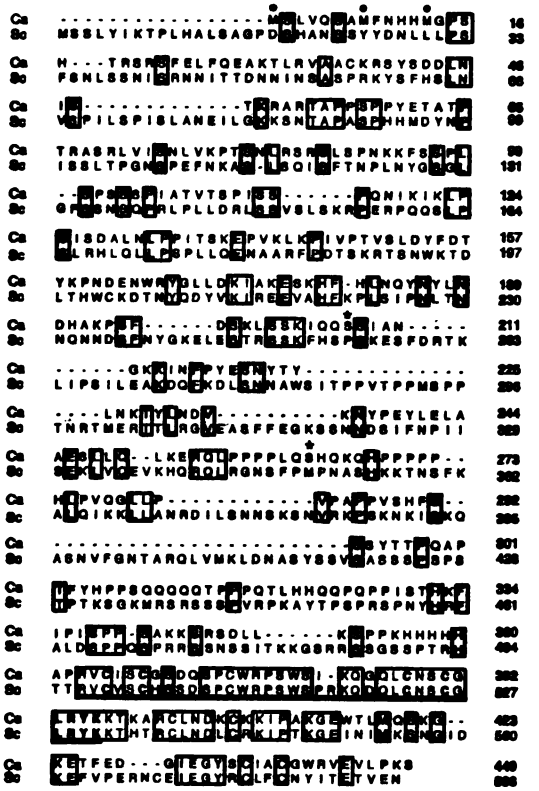

FIG. 1. Sequence alignment of the predicted amino acid sequences from *C. albicans* and *S. cerevisiae* Ash1p. Boxes indicate identical residues. Three possible start codons for *C. albicans* Ash1p are indicated by dots. Two predicted serine residues encoded by nonstandard CUG codons of *C. albicans* (39) are indicated by asterisks. The highly conserved GATA-like zinc finger region is underlined. Amino acid numbers are indicated on the right.

To detect *HO* repression, we used a reporter strain with *ASH1* deleted and which carries the *CAN1* and *ADE2* reporter genes, each under the control of the *HO* promoter (Fig. 2A, B, and D). A control strain (Fig. 2C) carries both reporter constructs, the wild-type genomic copy of *ASH1*, and a vector control plasmid. These strains allow growth of colonies on medium that contains canavanine only when *HO* is repressed, because expression of *CAN1* is lethal in the presence of canavanine. When *ASH1* is deleted from this strain, colonies fail to grow in the presence of canavanine, indicating that *HO-CAN1* is expressed (Fig. 2D). The *HO-ADE2* gene provides a second test of *HO* expression: expression of *HO-ADE2* produces white colonies in low-adenine medium, whereas repression of *HO-*

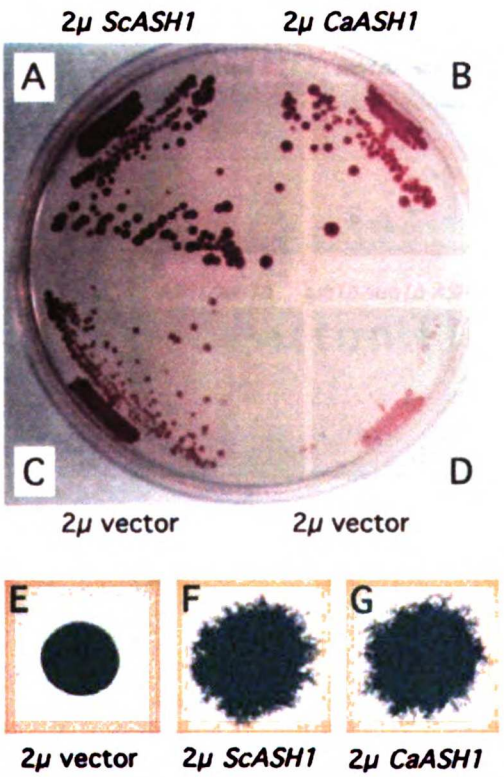

FIG. 2. *C. albicans* Ash1p represses *HO* and promotes pseudohyphal growth in *S. cerevisiae ash1* mutant strains. The *HO-ADE2 HO-CAN1 ash1* reporter strain produces red colonies on 0.03% canavanine and 10 mg of adenine per ml of SD −Leu medium when transformed with a high-copy-number plasmid that carries either *S. cerevisiae* (A) or *C. albicans* (B) *ASH1*. The same strain transformed with vector (D) fails to grow on this medium; the few surviving colonies are white, indicating *HO-ADE2* is not repressed. The *HO-ADE2 HO-CAN1 ASH1* control strain (C) has the genomic copy of *ASH1* intact and contains the vector control plasmid. Strains were incubated for 5 days at 30°C. For the experiments shown in panels E to G, diploid *ash1/ash1* strains of the Σ1278b background were transformed with the indicated plasmids and then incubated on nitrogen-limiting SLAD medium for 5 days at 30°C. Colonies were photographed at a magnification of ×38.

ADE2 results in red colonies. When the *C. albicans ASH1* plasmid was introduced into *S. cerevisiae ash1* strains, healthy red colonies grew on low-adenine medium containing canavanine (Fig. 2A and B), indicating that *C. albicans ASH1* can repress both the *HO-CAN1* and *HO-ADE2* reporter genes.

To test whether *C. albicans ASH1* can stimulate pseudohyphal growth in *S. cerevisiae*, *ash1/ash1* strains of the *S. cerevisiae* Σ1278b background (17) were transformed with plasmids expressing either the *C. albicans* or the *S. cerevisiae ASH1* gene and grown on low-nitrogen medium. We found that *C. albicans ASH1* restored pseudohyphal growth as efficiently to the *S. cerevisiae ash1/ash1* strain as did the *S. cerevisiae ASH1* (Fig. 2F and G). Based on these results, we believe that *C. albicans* Ash1p is a functional homolog of *S. cerevisiae* Ash1p in that it can replace the function of Ash1p in *S. cerevisiae* both for repression of *HO* and for promotion of pseudohyphal growth.

***C. albicans ash1/ash1* mutants have defects in filamentous growth.** *C. albicans* is diploid, and to determine whether *ASH1* has a role in regulating filamentous growth in *C. albicans*, strains lacking both copies of *ASH1* were constructed in the SC5314 background by replacing coding sequences with the *URA3* selectable marker (13). *ASH1/ASH1* (wild type), *ASH1/ash1*, and *ash1/ash1* strains were tested on several types of solid media that induce filament formation. Both *ash1* heterozygous and homozygous mutants show significantly reduced filamentous growth on Spider medium (Fig. 3A). The reintroduction of one copy of wild-type *ASH1* to the *ash1/ash1* strain partially restores filamentous growth (Fig. 3A), indicating that the original filamentous growth defects are attributable to the loss of *ASH1*. On YPD with 10% serum at 30°C, *ash1/ash1* homozygous mutants show a slight reduction in hyphal growth compared with the parental strain (Fig. 3B). *ash1/ash1* mutants showed no obvious defects in forming germ tubes, regarded as precursors to hyphal growth, in response to serum in liquid media (data not shown). On solid Lee's medium at neutral pH, *ash1/ash1* strains were severely reduced for filamentous growth (Fig. 3C). These results show that *ASH1* is important for filamentous growth under several specific environmental conditions.

Analysis of *ash1 cph1* double mutants. In *S. cerevisiae*, Ash1p and Ste12p regulate parallel but independent pathways of filamentous growth in response to Ras2p signaling. To determine the relationship between *C. albicans ASH1* and the *C. albicans* Ste12p homolog, *CPH1*, *C. albicans* strains lacking both *ASH1* and *CPH1* were constructed by the method described above. On Spider medium, the *ash1/ash1 cph1/cph1* double-mutant strain exhibited less filamentous growth overall than did either of the single-mutant strains, although the double mutant did produce low levels of peripheral hyphae (Fig. 3A). On YPD with 10% serum at 30°C, each of the single mutants formed filamentous colonies, whereas the *ash1/ash1 cph1/cph1* strain formed round, smooth afilamentous colonies under the same conditions (Fig. 3B). These data suggest that Ash1p and Cph1p contribute additively to filamentous growth in *C. albicans*.

Ash1p is asymmetrically localized to daughter cells. When *S. cerevisiae* proliferates by budding, Ash1p is asymmetrically localized to daughter cell nuclei, where it represses transcription of the *HO* gene (1, 43). To test whether this asymmetric localization also occurs in *C. albicans*, Ash1p was tagged at its N-terminal end with six tandem copies of the 11-amino-acid myc epitope (23). This altered form of Ash1p complements the filamentous growth defect when reintegrated into the *C. albicans ash1/ash1* strain, YDI-11; moreover, *C. albicans* myc6-Ash1p successfully promotes pseudohyphal growth when introduced into the *S. cerevisiae ash1* mutant strain, YDI-56 (data not shown).

To localize Ash1p, a strain containing one copy of myc6-*ASH1* as the sole source of Ash1p was stained with monoclonal antibodies against the myc epitope and visualized by indirect immunofluorescence (23). When *C. albicans* was grown as a budding-yeast form (in M199 [pH 4.5] and YPD media at 23 and 30°C, respectively), Ash1p was specifically localized to daughter cell nuclei: of 125 postanaphase cell pairs stained for Ash1, 79% showed localization of Ash1p to the nucleus of the

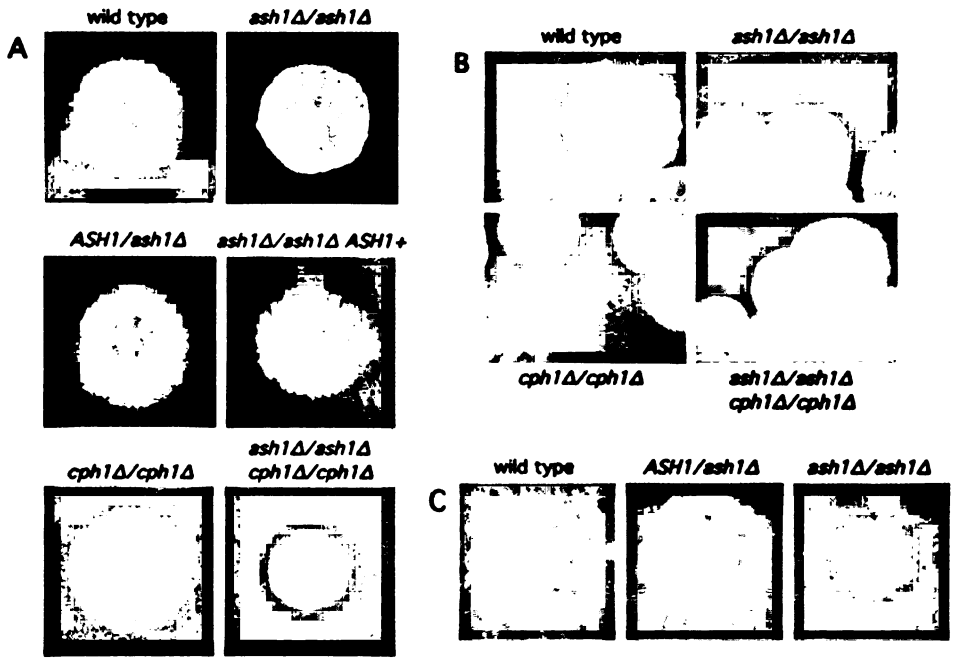

FIG. 3. *ASH1* is required for filamentous growth of *C. albicans*. Heterozygous or homozygous strains of the indicated genotypes were grown on various types of filament-inducing solid media: Spider medium (A), YPD medium plus 10% serum (B), and Lee's medium (pH 6.8) with 2% agar (C). Plates were incubated for 5 to 6 days at 30°C. In panel A, the strain depicted in the panel in the second row of the second column is an *ash1/ash1* strain into which an intact copy of *ASH1* has been introduced.

daughter cell, but not to the nucleus of the mother cell (Fig. 4 and 5A). *C. albicans* Ash1p was not observed in mother-daughter cell pairs undergoing mitosis (not shown), as is also the case for *S. cerevisiae* (1, 43). *C. albicans* yeast-form cells that are exposed to mild filament-inducing conditions bud in a unipolar fashion, similar to pseudohyphal cells of *S. cerevisiae*, and produce chains of cells. Under these conditions, *C. albicans* Ash1p is also localized to daughter cells, seen as cells that bud from the growing chain (Fig. 5B). This pattern of Ash1p localization is similar to that seen in *S. cerevisiae* pseudohyphal cells (8).

Ash1p is localized to hyphal tip cells. When *C. albicans* cells are grown for short periods of time in medium that strongly induces hyphal growth (YPD plus 20% serum at 35°C, for example), the mother cells appear rounded, and the daughter cells appear highly elongated, because the latter are beginning to form hyphae. In such mother-daughter pairs, Ash1p is localized specifically in the daughter cell nuclei (Fig. 4 and Fig. 6). Of the stained mother-daughter pairs observed in this study, 92% showed Ash1p in the daughter cell nucleus and not in the mother cell nucleus. If these mother-daughter pairs are incubated for longer times (YPD plus 20% serum at 35°C for 6 to 8 h), mature hyphae form in which multiple cells are joined end to end. In such hyphae, Ash1p is observed in the nuclei of apical hyphal cells (hyphal tip cells), but not in any of the other cells in the hyphae (Fig. 6). The apical cell is the site of active hyphal growth and constitutes the newest cell of the growing hypha.

Upon longer exposures to YPD plus 20% serum at 35°C, hyphae begin to generate blastospores, which bud off from multiple positions along the hyphae. It also appears that the nuclei of these newly released blastospores stain positively for Ash1p (Fig. 7), but in this experiment, it is difficult to rigorously distinguish cells that have recently budded from hyphae from those that have undergone additional cell divisions.

An important control for all of the localization experiments discussed in this and the previous section is the demonstration that an antibody directed against a different nuclear protein effectively stains all of the nuclei in the cell population. This is particularly important for growing hyphae, because the susceptibility of cells to the staining procedure could vary along the hypha. For this control, we used antibodies against the nuclear protein Tup1 (Fig. 5 to 7). Tup1p is observed in all nuclei (compare with the DAPI-stained images), and this observation rules out the possibility that the daughter cell and hyphal tip-cell-specific staining of Myc-Ash1p is due to the greater susceptibility of these cells to antibody.

***ash1* mutant strains are reduced for virulence.** A mouse model of systemic candidiasis is a sensitive assay for determining differences in virulence between *C. albicans* strains, and in this way, we tested whether Ash1p is important for virulence in

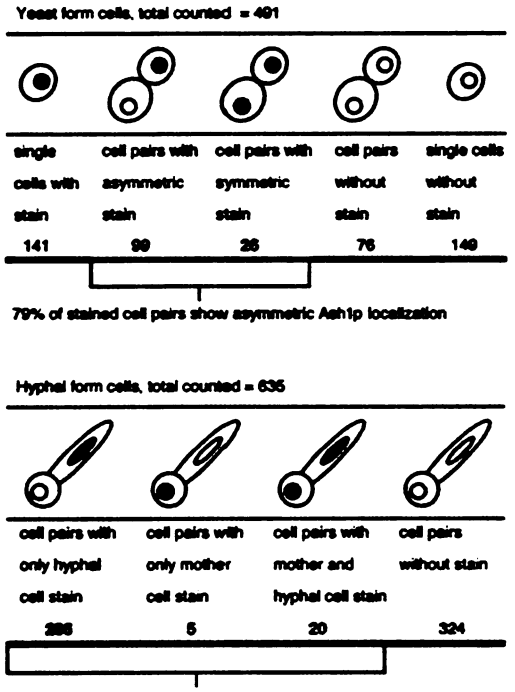

FIG. 4. Summary of the characteristics of the yeast- and hyphal-form cells examined in this study.

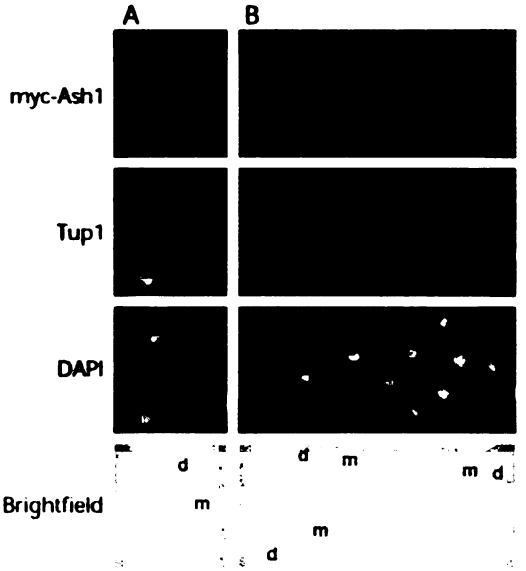

FIG. 5. Ash1p is localized to daughter cells of *C. albicans* growing in the yeast form. (A) Cells expressing myc-Ash1p (YDI-199) were grown in M199 (pH 4.5) at 23°C (conditions that favor the budding-yeast form) and processed for indirect immunofluorescence (see Materials and Methods). Cells were stained for myc-Ash1p with 9E10 mouse antibodies and Cy3-conjugated secondary antibodies that recognize the mouse 9E10 antibody. Tup1p was stained with rabbit polyclonal antibodies and FITC-conjugated secondary antibodies. Cell nuclei were visualized with DAPI stain, and whole cells were examined by bright-field imaging. Cells are stained as described in panel A. Yeast cells grown in M199 (pH 7.0) at 23°C appear as chains of attached budding cells. In panels A and B, selected mother (m) and daughter (d) cells are labeled.

vivo. For this analysis, 10^6 wild-type or mutant *C. albicans* cells were injected by tail vein into groups of six mice. We performed two separate experiments with two independently derived *ash1/ash1* mutant strains.

In the first experiment, we compared four strains: the *ASH1/ASH1* parental strain (CAF2-1), an *ASH1/ash1* heterozygous strain (YD-01), an *ash1/ash1* (YDI-7) fully mutant strain, and a homozygous mutant strain (YDI-157) that carries an intact *ASH1* gene reintegrated into the genome. As shown in Fig. 8A, disruption of one copy of *ASH1* results in decreased virulence, and disruption of both copies reduces virulence even further. When an intact copy of the *ASH1* gene is reintroduced into the *ash1/ash1* strain, virulence is increased to approximately that of the *ash1/ASH1* heterozygote, a result consistent with the fact that these two strains each have a single intact copy of *ASH1*. This result confirms that the virulence defects of the *ash1/ash1* mutant strain are due to the absence of *ASH1* and not to some other change resulting from transformation or other manipulations used in the construction of mutant strains. The doubling times of *ash1/ash1* mutant strains grown at 30 and 37°C in YPD medium were comparable to that of the wild type when measured in vitro (data not shown), suggesting that its defect in virulence is not due simply to a nonspecific growth defect.

In a second virulence experiment (Fig. 8B), we compared an independently constructed *ash1/ash1* mutant strain (YDI-27), the wild-type strain (CAF2-1), a *cph1/cph1* strain (JKC19), and an *ash1/ash1 cph1/cph1* strain (YDI-129). The *cph1* mutants are included in this experiment, because, as described above, the *ash1 cph1* double mutant shows a greater defect in filamentous growth than do the single mutants. As in the first virulence experiment, we found that deletion of *ASH1* leads to a marked reduction in virulence. In contrast, the *cph1/cph1* mutant strain appears fully virulent, as reported by Lo et al. (30). The additional deletion of *CPH1* from the *ash1/ash1* strain led to only small differences in survival of mice infected with these two strains. One notable difference between the *ash1/ash1* strain and the *ash1/ash1 cph1/cph1* double-mutant strain was that animals injected with the double-mutant strain showed fewer symptoms of infection, such as weight loss, reduced activity, or roughened coat appearance, compared to the *ash1/ash1* single-mutant strain, even though the survival times were only slightly different.

***ash1* mutants colonize tissues and produce hyphae in vivo.** To observe the morphology of *C. albicans* cells in vivo, kidneys of mice systemically infected with 10^6 *C. albicans* cells were removed, sectioned, and stained with periodic acid-Schiff base for the presence of *C. albicans*. Mice injected with 10^6 wild-type *C. albicans* cells of the SC5314 strain background succumb to infection within 2 to 4 days (Fig. 8) and at 2 days show a

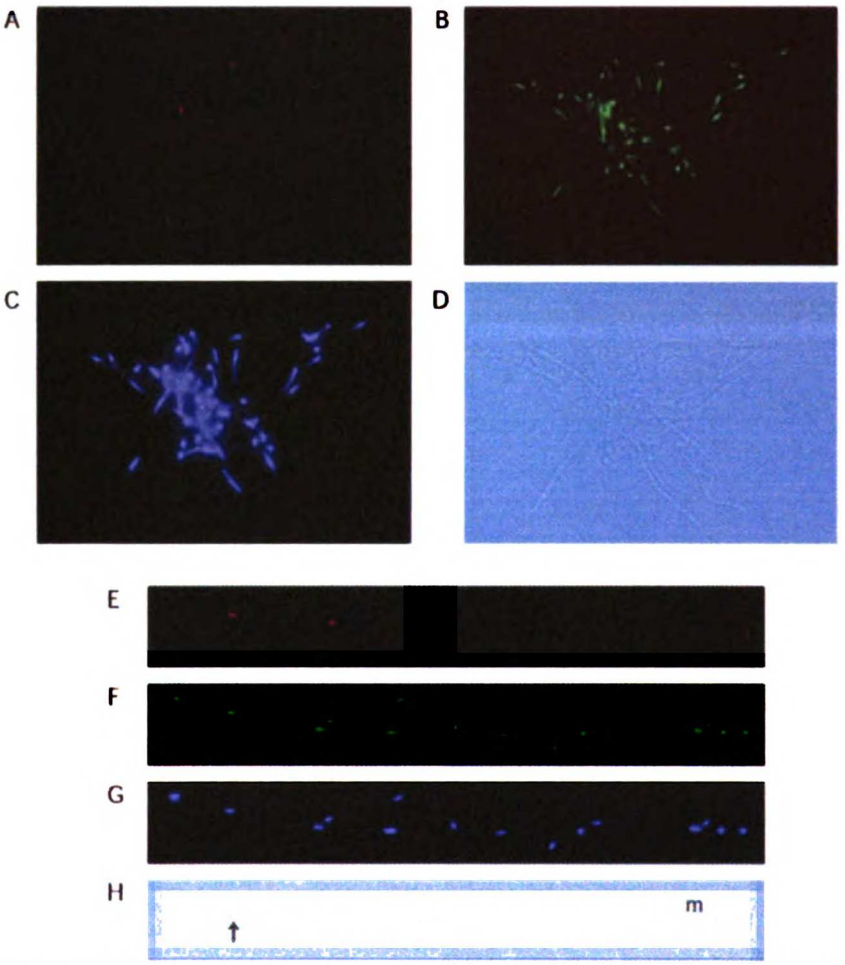

FIG. 6. Ash1p localizes to hyphal tip cells. (A to D) Ash1p is observed in the hyphal daughter cell nucleus of mother-daughter hyphal cell pairs grown for 2 h at 35°C in YPD plus 20% serum (A, anti-myc stain; B, anti-Tup1p stain; C, DAPI stain; D, bright-field image). (E to H) Hyphal cells grown for 4 to 6 h show chains of cells in a hyphal filament with Ash1p located only in the nucleus of the hyphal tip (apical) cell (indicated by the arrow). The original mother cell (m) has also produced budding cells. The images are as described for panels A to D.

large number of *Candida* cells in the kidneys (Fig. 9A and B). Both hyphae and blastospores are visible at the sites of infection. At the same time point, very few *ash1/ash1* cells are observed in the kidney (Fig. 9C and D). At later time points (>7 days), *ash1/ash1* cells could easily be detected in the kidney, and both blastospores and hyphae were present (Fig. 9E and F). Thus, although most mice infected with 10^6 *ash1/ash1* cells survive past 18 days, *ash1/ash1* strains were nonetheless observed in the kidney as yeast and filamentous forms after 7 days and even after 30 days (the longest time point at which kidneys infected with the *ash1/ash1* strain were examined; Fig. 9E and F). The tissue fungal burden of animals that succumbed to infection was quantified by homogenizing the kidneys and brains and plating on Sabouraud agar. Both *ash1/ash1* and *ash1/ash1 cph1/cph1* mutant strains achieved similar levels of colonization (3×10^7 CFU/g of kidney) to those reached by the wild type. However, the wild-type strains achieved this density within a day or 2, while the *ash1/ash1* mutant strains required several weeks. These observations indicate that the virulence defect of the *ash1/ash1* mutants is not simply due to the inability to survive or to grow as filaments in the mouse. It is possible, for example, that the attenuated virulence of *ash1/ash1* mutants is due to defects in the regulation of filamentous growth or in the proper specialization of hyphal tip cells.

DISCUSSION

Asymmetric cell division is critical for the development of nearly all organisms, ranging from unicellular bacteria and

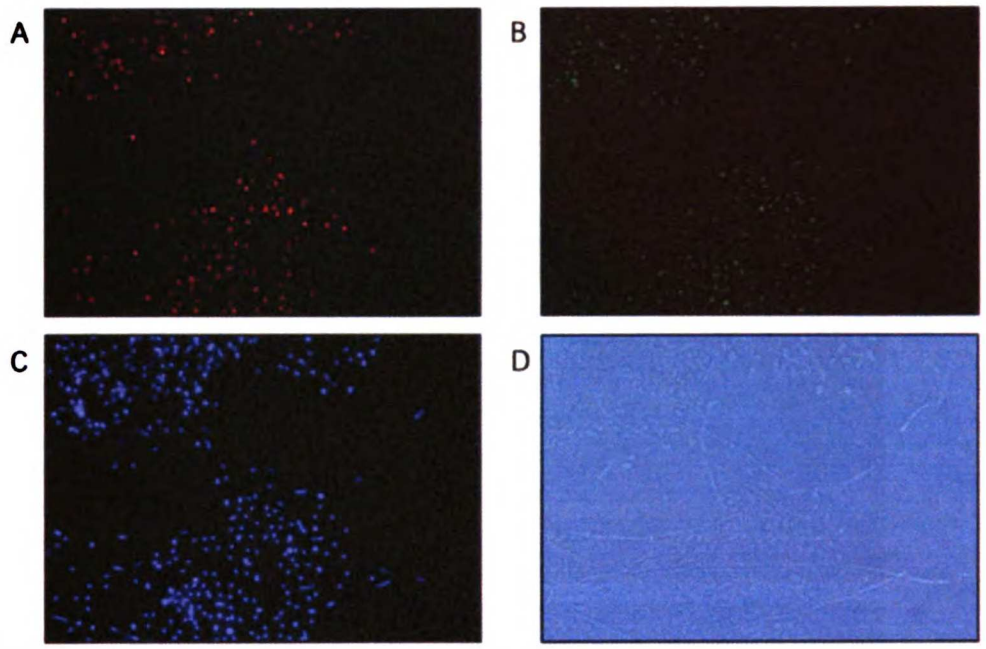

FIG. 7. Mature hyphae produce budding daughter cells that express Ash1p. Cells were grown overnight at 35°C in YPD plus 20% serum and stained as described in the legend to Fig. 6.

yeasts to multicellular plants and animals. The asymmetric localization of gene products to one of the two daughter cells formed during cell division leads to genetically identical cells with the potential for dramatically different cell fates.

In this paper, we show, by staining the transcriptional regulator Ash1p, that the human fungal pathogen *C. albicans* undergoes asymmetric cell division in all three of its morphological forms—budding-yeast-form cells, pseudohyphae, and hyphae. All three forms are found in infected tissues, and the ability of *C. albicans* to switch between them is thought to be crucial for its pathogenesis (for a recent review, see reference 19). In budding-yeast-form cells, *C. albicans* Ash1p is observed in daughter cell nuclei, but not in mother cell nuclei. It is also observed only in daughter cell nuclei in chains of pseudohyphal cells. Finally, in mature hyphae, Ash1p is observed only in the nuclei of hyphal tip cells—that is, to the cells active in hyphal growth. Thus, as far as can be seen by immunofluorescence, hyphae consist of long, branched chains of elongated cells, with Ash1p absent from all but the growing tip cells.

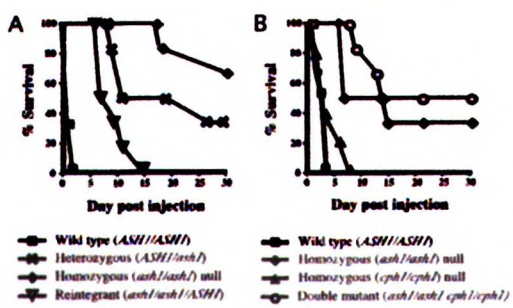

FIG. 8. *ASH1* is important for virulence in a mouse model of systemic candidiasis. Shown are survival curves of mice systemically infected with *URA3*$^+$ *C. albicans* strains of the genotypes *ASH1/ASH1* (CAF2-1), *ASH1/ash1* (YDI-1), *ash1/ash1* (YDI-7), and *ash1/ash1*::*ASH1* (YDI-157) (A) or *ASH1/ASH1* (CAF2-1), *ash1/ash1* (YDI-27), *ash1/ash1 cph1/cph1* (YDI-129), and *cph1/cph1* (JKC19) (B).

It has long been appreciated that the hyphal tip (apical) cells of *C. albicans* differ from internally positioned (subapical) hyphal cells. For example, much of the metabolism and growth of hyphae is concentrated in these cells. Moreover, the organelle composition of the hyphal tip cell appears different from that of other hyphal cells; in particular, hyphal tip cells are vacuole poor and cytosol rich compared to the rest of the hyphal cells (for review, see reference 18). Finally, secretion of at least some hydrolytic enzymes occurs selectively at the hyphal tip cell (reviewed in references 14 and 20). Many studies indicate that the penetration of host epithelial surfaces by *C. albicans* is carried out by the hyphal tip cells, and it is reasonable to believe that the tip cells specifically secrete hydrolytic enzymes that damage host tissues, providing sites of penetration. (For recent reviews, see references 19 and 21.) Because Ash1p is localized to the nuclei of hyphal tip cells and because it is a transcriptional regulator, it seems likely that Ash1p regulates

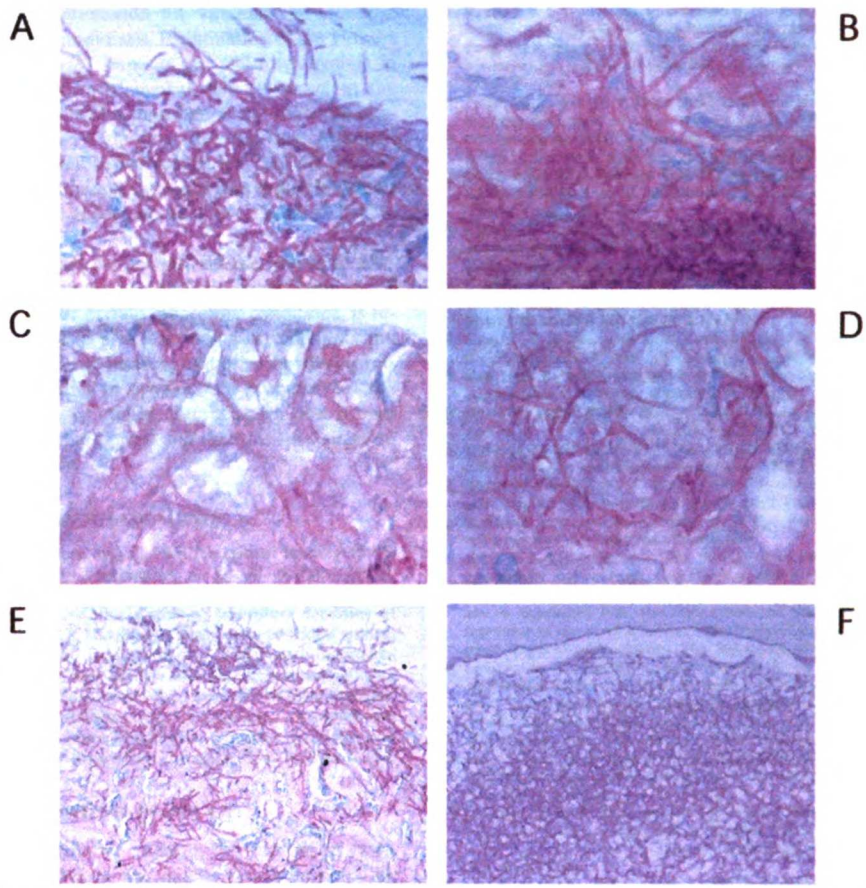

FIG. 9. *ash1/ash1* mutants colonize kidneys much more slowly than do *ash1/ash1 ASH1*+ reintegrants, although they do produce hyphae in vivo. Histological sections of mouse kidneys were prepared as described in Materials and Methods. (A, B) The *ash1/ash1 ASH1*+ strain 24 h after infection. Panel A shows a locus of infection on the edge of the kidney and a large colony inside the kidney (B). (C to F) Shown are the *ash1/ash1* mutant strains after 2 (C and D), 15 (E), and 30 (F) days. Panels A to D were photographed at ×40, and panels E and F were photographed at ×20.

at least a portion of the specializations that take place in hyphal tip cells.

In addition to specifically "marking" daughter cells, Ash1p is also required for filamentous growth on some types of filament-inducing medium. However, the severity of the defect caused by deleting *ASH1* varies, depending on the nature of the medium. These results suggest that one function of Ash1p in daughter cells is to establish or maintain filamentous growth on certain types of media. For example, *ash1/ash1* mutant cells show a pronounced defect of filamentous growth on Spider (low-nutrient) medium. It has been proposed that, in response to this medium, *C. albicans* grows filamentously in a "foraging mode," seeking out new locations of greater nutritional richness (24). It is possible Ash1p in the hyphal tip cells regulates a process that is required for hyphae to sense or to respond to low-nutrient medium, thus causing the filamentation defect of *ash1/ash1* mutants on this medium.

In *S. cerevisiae*, the best-understood function of Ash1p takes place in budding cells. In daughter cells, it represses transcription of the *HO* endonuclease gene, which carries out the first step in mating-type interconversion. Because Ash1p is specifically localized to daughter cell nuclei, only mother cells are able to switch mating types. As far as is known, *C. albicans* is unlikely to carry out mating-type interconversion; its genome lacks silent mating cassettes and a gene closely related to *HO*. It is possible that the asymmetric localization of Ash1p has a deeply conserved function in *S. cerevisiae* and *C. albicans* (perhaps involving filamentous growth) and that its regulation of *HO* was a relatively recent evolutionary add-on in *S. cerevisiae*.

Finally, our work shows that *ash1/ash1* mutants of *C. albi-*

cans are significantly attenuated for virulence in a mouse model of disseminated candidiasis. Examination of the kidneys revealed that the *ash1/ash1* mutants can still form hyphae in vivo; however the number of hyphal cells observed in the kidney was significantly smaller than that of the wild-type control strain observed at the same time after infection. Although it is premature to make a firm conclusion, it is possible that the virulence defect in the *ash1/ash1* strain is due to subtle defects in the regulation of hyphal formation or to a defect in hyphal tip cell specialization.

In conclusion, this study has focused on the role of *C. albicans* Ash1 protein. We found that this protein is required for filamentous growth under certain laboratory conditions, is required for full virulence in a mouse model of infection, and marks daughter cell nuclei in budding and pseudohyphal forms and tip (apical) cell nuclei in hyphae. We propose that the asymmetric localization of Ash1p is crucial for the proper specialization of the hyphal tip cell and that this specialization is important for virulence.

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ADDENDUM IN PROOF

While this paper was under revision, the expression of *C. albicans ASH1* in *S. cerevisiae* was independently reported by Munchow et al. (S. Munchow, D. Ferring, K. Kahlina, and R. P. Jansen, Curr. Genet. **41**:73–81, 2002).

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Chapter 3.

Genome-wide expression profiling of *Candida albicans ash1* mutants

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Introduction

Candida albicans is an opportunistic pathogen of humans that grows as a yeast form and as a range of elongated filamentous (hyphal) forms. The ability to switch between these morphologies is considered a virulence trait of *C. albicans*. It is thought that this range of morphological forms, which are found in host tissues, contribute to the ability of *C. albicans* to thrive and cause disease in a host. The yeast form is small and can easily disseminate to new sites of infection whereas the hyphal form appears to be specialized for the invasion of tissues. The tips of growing hyphae are known to selectively secrete lipases and other hydrolytic enzymes (12, 14) capable of digesting infected tissues.

Previously I showed that Ash1 is localized preferentially to daughter cells of *C. albicans* yeast cells and is localized to hyphal tip cells when *C. albicans* is induced to form filaments (17). *ash1* mutants are attenuated both in virulence and filamentous growth which implies that a daughter cell specific function is important for virulence and filamentous growth of *C. albicans*. The localization of Ash1, a transcriptional regulator, to daughter cells and to hyphal tips cells implies that Ash1 regulates the expression of target genes in these cells.

Genome-wide expression profiling is a sensitive and powerful method of identifying differences in gene expression between two strains. This technology (also referred to as microarray analysis) measures the relative abundance of every mRNA expressed in two different strains. To identify genes that may be regulated by Ash1, I used genome-wide expression profiling of wild-type and *ash1* mutant strains to identify genes whose expression was either reduced or increased in *ash1* mutants when compared

to wild-type strains. Since *ash1* mutant strains lack the Ash1 protein, genes that are activated by Ash1 should show reduced expression in *ash1* mutants when compared to wild-type (*ASH1*+) strains. Conversely, genes that are repressed by Ash1 are expected to show increased levels of expression in *ash1* mutants by microarray analysis.

The identification of hyphal tip cell- and daughter cell-specific genes is the first step in the identification of novel functions of these cells and will hopefully contribute to our understanding of how *C. albicans* uses asymmetric localization as a mechanism for generating daughter cells and hyphal tip cells that are specialized for filamentous growth and for full virulence in a host.

Experimental Design

Growth conditions

To identify genes whose expression is altered in *ash1* mutants during filamentous growth, I grew three different wild-type (*ASH1*+) strains and three *ash1* mutant strains under two different types of hyphal growth conditions, Lee's pH 6.8 at 37° C and YPD with 20% serum at 35° C. Lee's medium is a defined synthetic growth medium for *C. albicans* that induces filamentous growth at pH 6.8 and maintains the yeast form of growth at low pH (4.5) (19). Serum is a powerful inducer of filamentous growth. The addition of late log or stationary phase *C. albicans* cells to YPD containing 10 – 20% serum rapidly induces filament formation within about 45 minutes. After about 2 hours in either medium, the germ tubes that initially emerged from the mother cells have generally

completed one round of hyphal-cell division and each hyphal tip cell contains its own nucleus. It was at this stage that the hyphal cells were harvested for microarray analysis.

For the yeast form of growth, I performed a time course of expression (2, 4, 6, and 8 hours) after dilution into fresh 30° C YPD medium. YPD is a rich growth medium and under these conditions, *C. albicans* grows logarithmically as a round single-cell budding yeast. By approximately 2 hours after dilution into YPD, yeast form cells begin to enter logarithmic growth phase (log phase) from stationary phase. Cells continue in log phase through 4 hours after dilution into YPD and enter mid log-phase after 6 hours of growth in YPD. At the 24-hour time point, cells have reached stationary phase and have saturated the culture (OD_{600} 25-40). From these yeast-form experiments, the expression profiles of yeast form cells in log phase, mid-to-late log phase, and stationary phase culture were examined.

Mother and daughter cells in culture

The presence of Ash1 in only a subset of cells (daughter cells) in a culture population presents a challenge for detecting differentially regulated transcripts. The presence of non-Ash1 expressing mother cells in a mixed(mother and daughter cell)-culture dilutes the transcripts produced by daughter cells. To address this difficulty, I examined the expression profiles of wild-type and *ash1* mutant cells at the two-cell stage of hyphal development, when 50% of the cells are mother cells and 50% are hyphal daughter cells that remain attached to the mother cells after cell division. At this stage of hyphal growth, the maximum percentage of daughter cells that can be obtained in culture is achieved (Figure 3.1A).

Hyphal growth proceeds in a linear fashion with new daughter cells forming at the tips of the growing filaments. At later stages of hyphal growth, the 3-cell or 4-cell stage for example, the percentage of daughter cells in the population is 33% and 25% respectively (Figure 3.1A). In other words, as hyphal cell cultures grow, the percentage of daughter/tip cells in the population decreases. By growing the cells for 2 –2.5 hours in filament-inducing medium, I obtained cultures with the maximum fraction of daughter cells possible in a hyphal culture and thus the greatest difference in transcript abundance between the wild-type and *ash1* mutant daughter cells.

In contrast to the hyphal form of growth, yeast cell cultures grow logarithmically. Each mother and daughter cell pair separates and then each of the mothers and daughters are capable of forming new daughter cells. This pattern of cell division in log phase cultures maintains an average of half mother cells and half daughter cells (Figure 3.1B) during all phases of logarithmic growth in asynchronous culture. To examine the expression profile of *ash1* mutants in the yeast form, a time course of expression was monitored at 2, 4, 6, and 24 hours after dilution into 30° YPD medium.

Expected patterns of gene expression in mother and daughter cells

Under the most optimal of growth conditions, Ash1-expressing daughter cells are present in only about half of the wild-type cell population. These experiments measure the relative mRNA abundance between all of the cells in wild-type and *ash1* mutant strains. Daughter cell specific genes that are either activated or repressed by Ash1 are predicted to show relatively small changes in gene expression when the mRNA is isolated from in mixed populations of wild-type and *ash1* mutant mother and daughter

cells. Figure 3.2 shows the expected pattern of gene expression in wild-type and *ash1* mutant cells of genes that are activated by Ash1 and of those that are repressed by Ash1.

Genes that are activated by Ash1 in wild-type daughter cells are expressed in 50% of the cells. In the absence of Ash1, these genes may not be expressed to detectable levels unless other transcription factors are capable of activating expression of these genes independent of Ash1. The fold-change in gene expression for genes activated by Ash1 is likely to vary depending upon the level of activation of the individual genes. A gene activated 10-fold by Ash1 in wild-type daughter cells is predicted to have approximately 5-fold increase in expression in the wild-type vs. *ash1* mutant comparison. A gene whose expression is enriched in daughter cells by only 2-fold, may be more difficult to detect by this analysis as the expression ratios of *ash1* mutant vs. wild-type cells is likely to be close to 1.5.

Repressed genes, in contrast, are presumed to be expressed only in mother cells of wild-type strains and should be expressed in both mothers and daughters of the *ash1* mutant strains. Thus, in wild-type cells, 50% of the cells will express the de-repressed transcript and 50% of the cells will not. In the *ash1* mutant strains, 100% of the cells are expected to produce the transcript. Therefore, the level of expression of repressed transcripts is expected to be, at most, two-fold in *ash1* mutants when compared to wild-type strains.

A control strain that expresses an additional copy of Ash1 (myc6-*ASH1*), YDI-203, was used in the Lee's pH 6.8 medium experiment to compare the expression profile of *ash1* mutants with a strain expressing 3 copies of *ASH1* (Ash1+/+/+). In this strain, the presence of a third copy of *ASH1* is expected to further increase the expression of

activated target-genes above that of wild-type levels. The comparison of this strain to *ash1* mutant strains is expected increase the magnitude of the fold-change expected for genes whose expression is controlled by Ash1, particularly for those genes activated by Ash1.

The expression of repressed target genes is not expected to change significantly between the standard wild-type strain, CAF2-1, and the YDI-203 (Ash1+/+/+) strain since these genes are expected to be off in daughter cells of both the CAF2-1 and YDI-203 strains. Therefore, the fold-changes between *ash1* mutants and CAF2-1 should also be similar to the fold-changes observed between *ash1* mutants and the YDI-203 strain for genes repressed by Ash1.

Control for strain background differences

Microarray analysis is a sensitive method for identifying genes that are differentially regulated between two strains. In addition to specific gene deletions, differences in gene expression due to auxotrophies and strain background differences will also be detected. The common laboratory strain for constructing gene knockouts in *C. albicans*, CAI4, is known to differ from its parental strain, CAF2-1, in at least one genetic locus. In addition to *URA3*, the gene used to select for transformation of CAI4, the *IRO1* gene, thought to encode a transcription factor involved in regulation of iron utilization (13), was deleted during the construction of CAI4. The *IRO1* gene resides next to *URA3* in the genome of *C. albicans*. Thus, CAF2-1 is heterozygous for both the *URA3* and *IRO1* loci whereas CAI4 is homozygous null for both loci (Table 3.1).

Two of the *ash1* mutant strains analyzed in this study were generated in the CAI4 strain and were compared to the standard CAF2-1 wild-type strain for microarray analysis. As a control for changes in gene expression due to *IRO1* and any additional, as of yet, unknown difference between CAF2-1 and CAI4, I also compared a control strain, YDI-203 which is derived from CAI4, to the CAF2-1 strain. Since YDI-203 (described above and see Materials and Methods) carries a myc6-*ASH1* plasmid containing the *URA3* selectable marker integrated in CAI4 in addition to the two normal copies of *ASH1*, this strain serves as a control for both the presence of *ASH1* and as a means to rule out genes that are differentially expressed due to strain background differences between CAF2-1 and its Ura- derivative CAI4.

RM1000, is a second strain used for constructing gene knockouts; it allows one to use two selectable genetic markers, *URA3* and *HIS1* (23, 29). The parent strain of RM1000 is RM1. Both were derived from CAI4; therefore the *URA3* and *IRO1* loci are deleted in both strains. One of the *ash1/ash1* strains used in the hyphal growth experiment was constructed in RM1000. This strain was independently derived from the other *ash1* mutant strains used in this study and its comparison to RM1 further aided in the elimination of genes from the analysis with expression changes due to differences in strain backgrounds rather than to the presence of Ash1.

Universal mRNA reference

In order to directly compare multiple experiments performed at different times and to potentially compare my data with independent experiments carried out by others, I used a pool of mRNAs (the universal reference mRNA) obtained from growing *C.*

albicans under over dozens of growth conditions, to competitively hybridize to each wild-type and *ash1* mutant mRNA sample. For a complete description of the universal reference mRNA, see Appendix A. The experimental ratios of gene expression from each microarray hybridization reflect the abundance of sample mRNA to universal reference mRNA. Since the reference mRNA for all experiments is the same, the values for the reference mRNA drop out of the equation when the data for the *ash1* mutants is divided by the data for the wild-type hybridizations. For example, the value of *ash1*/Ref divided by the value of WT/Ref equals the expression ratios of *ash1*/WT.

Materials and Methods

Strains and Media

All strains were routinely cultured at 30°C in YPD medium or on YPD plates. Strains induced to form filaments in Lee's pH 6.8 medium were pre-grown overnight at 30°C in Lee's medium, pH 4.5 prior to filament induction. YPD + serum medium was prepared to a 1X final YPD concentration with 20% fetal calf serum (UCSF Cell Culture Facility). Lee's pH 6.8 medium was made as previously described (17). The strains used in this study are listed in Table 3.1. Strain YDI-203 was constructed by digesting a myc6-*ASH1* integrating plasmid (PDI-30) (17) with PacI and transforming into the Ura- wild-type strain CAI4.

Yeast Cell Experiment Growth Conditions

100 mL cultures of each strain were grown overnight at 30°C until saturated (OD_{600} = 20 - 40). Cells were diluted in a 2L culture flask into 800 mL YPD to OD_{600} = 1 and incubated with shaking at 30°C. 200 mL of cells were harvested at 2 hrs, 4 hrs, 6 hrs, and 24 hrs after dilution into 30°C YPD medium. Two strains, *ash1*Δ/Δ (YDI-7) and CAF2-1 were compared in this experiment.

Hyphal Cell Experiment Growth Conditions

Two filamentous growth-inducing conditions were used in these experiments, YPD with serum at 35° C and Lee's pH 6.8 medium at 37° C. For the serum condition, yeast cells were grown in YPD at 30°C until saturated (OD_{600} = 20-40) and were then diluted into 800 mL pre-warmed 35°C YPD with 20% serum to an OD_{600} of 0.8-1. The cells were grown for 2 hrs with shaking and were harvested. For the Lee's medium experiment, yeast cell cultures were grown at 30° C in Lee's pH 4.5 medium (which maintains the yeast form of growth) to an OD_{600} of approximately 12-15. The cells were diluted into pre-warmed 37°C Lee's medium, pH 6.8 to OD_{600} 1 and were grown with shaking for 2.5 hours before harvesting the cells. Three *ash1*Δ/Δ strains (YDI-7, YDI-8, and YDI-16) and two wild-type strains (CAF2-1 and RM1) were compared in the serum experiment. Two *ash1*Δ/Δ strains (YDI-7, YDI-8) and two wild-type strains (CAF2-1 and YDI-203) were compared in the Lee's medium experiment.

Harvesting of cells and RNA sample preparation

Cells were collected by vacuum filtration onto filters and snap frozen with liquid nitrogen in 50 mL Falcon tubes for storage. Total RNA preps were performed as follows: Filters were washed in buffer and pellets were resuspended in 65°C acid phenol solution (TAE equilibrated crystalline phenol, %SDS, %TAE) and incubated for 30 mins at 65°C. The cell debris was pelleted and the supernatants were extracted a second time with acid phenol. The supernatants were transferred to new Falcon tubes and extracted once with 15 mL chloroform in 50 mL PhaseLock tubes (Fisher Scientific). Sodium acetate (to 0.3M) and 2 volumes (~25 mL) 100% ethanol were added to the supernatants which were stored at –20°C overnight to precipitate the RNA. The pellets were washed in 70% ethanol (resuspended completely), pelleted again and then air-dried.

Poly (A) mRNA Selection

Poly(A)-modified mRNAs from harvested cells were selected from the total RNA using an Oligotex PolyA Selection Kit (Qiagen). 1mg. total RNA was used as the input for each sample. The typical yields per sample were 8 to 10 μg mRNA per mg RNA.

cDNA Synthesis and Probe Labeling

First strand cDNA was synthesized with Stratascript reverse transcriptase (Stratagene) using 4 μg Poly(A)-selected mRNA as the template. Amino-alyl dUTP (Sigma, #A0410) was incorporated into the cDNA and the reactions were quenched and cleaned up according to established protocols (4). The amino-alyl labeled cDNA was

coupled to Cy3 and Cy5 fluorescent dyes (Amersham Biosciences) and the probes were cleaned up and vacuum-dried as described (4).

Microarray Hybridizations

Labeled cDNA probes were resuspended in 3X SSC, 50% formamide, polyA in a volume of ~40 μL. The probes were heated to 100°C for 3 minutes and applied between the glass slide and a specially designed cover-slip (Lifter-Slip). The microarray slides were incubated at 44°C in metal hybridization chambers for 16 – 18 hours. Prior to hybridization, the surface of the glass slide microarrays were treated by incubation in 1% BSA, 3X SSC solution to block non-specific binding of the probe to the surface of the array. Following hybridization, the slides were washed first in 2X SSC with 0.5% SDS, then in 0.5X SSC with 0.5% SDS, then in 0.1X SSC followed by a final rinse in water. The slides were dried for 5 minutes at 6300 rpm at room temperature in a table-top centrifuge.

Microarray Scanning and Data Processing

The hybridized microarrays were scanned with an Axon 4000B scanner and GenePix Pro 3.0 scanning software (Axon Instruments, Foster City, CA). The microarray images were gridded and results files were generated for each array with GenePix Pro 3.0 software. The results files were submitted to the Nomad Microarray Database (1) for storage of multiple array experiments and for normalization and extraction of the data with annotation from the database.

Normalized data and annotation for all experiments was extracted as tab-delimited text files from the Nomad database. The normalization was based on the bulk-scale method that calculates the sum of the intensities in the Cy5 and Cy3 channels and assumes the sums of each channel should be equal. The extracted data files contained the ratio of the median intensity of Cy5 (sample) to Cy3 (reference) for each spot in every experiment. The tab-delimited text files were processed in Excel to transform the ratios from the *ash1* strains over the CAF2-1 sample.

Data filtering and Gene Clustering

Filtering of the dataset was carried out in Filemaker Pro and Excel. The data was filtered for genes that showed a 15% or more difference in relative expression between *ash1* mutants and wild type strains in 5 out of 6 hyphal growth experiments and either had no change (for activated and repressed target genes) or changed in the opposite direction (for activated target genes) in the myc6-*ASH1*+ wild-type control strain. Filtered data was organized using the hierarchical clustering program, Gene Cluster (9). The clustered gene data was visualized with Treeview (9) for Mac OSX.

Classification of gene function

The classification of gene function was assigned based on homology of the *C. albicans* gene products to *S. cerevisiae* (3) and other genomes (2). The gene functions in the dataset were annotated largely through literature searches and by examining the homology to proteins of known function in *S. cerevisiae* and, to a lesser extent, by homology to other organisms.

Results

Genome-wide expression profiling of *ash1* mutants

A total of three *ash1* mutant strains were compared to two different wild-type strains under two different filament-inducing conditions, 35° C YPD with 20% serum (3 experiments) and 37° C Lee's pH 6.8 medium (4 experiments) in a total of 7 experiments. A control strain carrying a third copy of *ASH1* (myc6-*ASH1)* was also grown in Lee's medium. This strain also aided in the identification of genes whose expression is increased beyond the wild-type (2X) levels of Ash1 by the presence of a third copy of *ASH1*. Finally, I compared one *ash1* mutant and one wild-type strain in a time course of expression (4 time points) in 30° C YPD medium, a condition that favors the budding yeast form of growth.

To identify small but reproducible changes in gene expression between the daughter cells of wild-type and *ash1* mutant strains, the data was filtered for genes with a minimum of 15% change in expression in at least 5 out of the 6 hyphal growth experiments. This corresponds to an expression ratio of 0.85 or less for reduced transcripts and 1.15 or more for transcripts with increased expression. In most cases, the ratios in the 5 experiments were much lower (for the reduced genes) or higher (for the genes with increased abundance) than the 15% cutoff. Genes that met this criterion were further filtered by comparison of the YDI-203 strain to the wild-type CAF2-1 strain to ensure the result was specific to the presence or absence of *ASH1*. Five genes that showed reduced expression in *ash1* mutants (*IRO1, CHT2, CAORF27/orf6.604*, *CAORF151/* orf6.604, and *CAORF625/orf6.370*) were ruled out of the analysis by comparison of this

strain with CAF2-1. In other words, differences in the expression level of these genes were due to strain background differences rather than the presence or absence of Ash1.

Genes potentially activated by Ash1

Figure 3.3 shows the hierarchical clustering of the genes in eleven experiments with significant differences in expression between wild-type and *ash1* mutant strains. At least 80 genes show reduced expression in the absence of Ash1 during both serum- and pH-induced hyphal growth. Table 3.2 lists the top 39 genes with the most significant decreases in expression and includes the data for 3 *ASH1* spots for comparison. Approximately 15 of these genes show reduced expression in *ash1* mutants in every experiment under both yeast and filamentous growth conditions. Surprisingly, over half of the genes with reduced expression in the *ash1* mutants are required for ribosomal RNA processing and ribosome subunit biogenesis or have a nucleolar localization or function. The majority of these proteins function as RNA-binding proteins in the cell.

At least five genes involved in ER (endoplasmic reticulum)-Golgi transport, protein mannosylation, and secretion showed decreased expression in *ash1* mutants when compared to the wild-type strains. One of these genes, *F1M20.2*, encoding a dolichyl-phosphate mannosyltransferase of the Golgi apparatus, shows the highest increase in expression (3.8-fold) in the 3X *ASH1*-expressing strain, YDI-203, second only to *ASH1* itself (Table 3.2, column 1). This gene has no direct match in the *S. cerevisiae* genome and there is no match in the Stanford assembly 6 orf set, therefore this gene is currently listed with a non-conventional *C. albicans* name after an *Arabidopsis* protein with which it shares homology. Both *MNN11* and *MNN2* encode Golgi mannosyltransferases with

homologs in *S. cerevisiae*. These enzymes transfer mannose residues to proteins, such as cell wall and secreted proteins, which require these modifications for their normal function. The fourth gene in this class, *GOT1*, encodes a Golgi membrane protein involved in ER-Golgi and ER-endosome transport. ARF2-2 encodes a GTP-binding protein of the ARF (ADP-ribosylation factor) family involved in ER-Golgi and intra-Golgi vesicle transport.

Of the remaining genes that are activated with increased Ash1 expression and are reduced in the absence of Ash1, three are regulators of gene expression, *USV1*, *SFL1-3*, *PLP2*. *USV1* encodes a C_2H_2-zinc finger transcription factor named for its pattern of expression ("Up in StarVation 1"). *PLP1*, or "phosducin-like protein", encodes a GTP-ase inhibitor that activates gene expression in response to pheromones in *S. cerevisiae*. *SFL1-3* encodes for one of two *C. albicans* genes with homology to *S. cerevisiae SFL1* and to heat shock transcription factors in several organisms. The protein with the greatest homology to Sfl1-3 is *Candida tropicalis* Hsr1, a protein that confers salt tolerance when expressed in *S. cerevisiae* (5). In *S. cerevisiae*, *SFL1* is a negative transcriptional regulator that is directly regulated by PKA (24) and is required for normal cell-surface assembly, filamentous growth, and flocculation (26).

Among the genes down-regulated in the *ash1* strains is the *ASH1* transcript itself. This serves as a useful control since the *ASH1* gene is missing in these strains. The data for three *ASH1* spots on the microarray are shown in the top three rows of Table 3.2. On average, the levels of *ASH1* mRNA detected were 1.7- to 2.0-fold greater in wild-type CAF2-1 than in *ash1* mutant strains. The 3X-*ASH1*-expressing strain showed an increase of between 1.1- and 1.4-fold higher expression of the *ASH1* transcript than CAF2-1

(which bears 2 copies of *ASH1*) and between 6.5- to 20-fold higher than the Ash1 deletion strains.

Genes potentially repressed by Ash1

Approximately 50 genes show increased expression in *ash1* mutant strains in the majority of the microarray experiments. Table 3.3 lists the top 40 of these genes, according to function, whose transcripts are both up-regulated in the absence of Ash1 and repressed by an additional copy of Ash1. Nine of these genes encode proteins required for mitochondrial function or for regulation of nutrient metabolism and energy production in the cell. Mitochondrial genes that are up-regulated in the absence of Ash1 include *ALT1*, a mitochondrial alanine aminotransferase, *DLD1*, which encodes a D-lactate dehydrogenase, *HEM15*, the final enzyme in the heme biosynthesis pathway, and *RNA12*, a gene required for the proper timing of pre-rRNA maturation and for maintenance of the mitochondrial genome.

Two genes, *SRA1-1* and *SRA1-2*, which encode homologs of the cAMP-dependent protein kinase A (PKA) regulatory subunit (also known as *BCY1* in *S. cerevisiae*) are up-regulated in the absence of Ash1. The PKA pathway is known to activate filamentous growth of both *S. cerevisiae* (24) and *C. albicans* (6, 8). An increase in both of the *SRA1* transcript levels is predicted to cause a decrease in PKA signaling which is consistent with the reduced filamentous growth of *ash1* mutants.

At least 4 genes involved in ER-Golgi function are up-regulated in *ash1* mutant strains. One of these genes, *MNN2-2/orf6.1866*, encodes a mannosyltransferase that adds mannose residues to branches on the outer chain N-linked mannans of cell wall proteins.

CYP5 is an ER protein that is transcriptionally up-regulated in response to unfolded proteins in the ER, is consistently up-regulated in these microarray experiments. *SEC17*, which encodes a secretory vesicle protein responsible for fusion of vesicles with target membranes, and *YML059C*, which encodes an ER-localized protein of unknown function (15), are also reproducibly up-regulated in the absence of Ash1 under all conditions tested.

Genes that encode the cell wall proteins *ECM4*, *HYR1-14*, and *YBR056W* are strongly up-regulated in *ash1* mutants compared to wild-type strains. The products of these genes may serve as substrates for the activity of the mannosyltranferases of the ER and secretion system. *ECM4* encodes a protein involved in cell wall biosynthesis and *HYR1-14* is predicted to encode one of several members of a gene family with weak homology to the repetitive sequence of the hyphal-specific *C. albicans* cell wall protein, Hyr1. This protein also shares weak sequence similarity (BLAST score 46, e-value 6e-06) with *S. cerevisiae* Yol155c, a protein with glucosidase activity that is involved in cell wall organization and biosynthesis. *YBR056W* also encodes a protein with similarity to a glucan 1,3-beta-glucosidase of *Schizosaccharomyces pombe* that is likely to function in cell wall biosynthesis or remodeling.

Several genes involved in a wide-assortment of cellular functions were consistently up-regulated in the absence of Ash1. A few of the genes worth noting include *RAD51*, *HOS3*, and *MHP1*. *RAD51* encodes an *E. coli* RecA homolog involved in the recombinational repair of double-strand breaks in DNA, chromatin modeling and telomere maintenance (3). This protein is thought to the first of several Rad-proteins that bind DNA adjacent to sites of double-stranded breaks (30) and therefore may be rate-

limiting for the initiation of double-stranded break repair in the cell. The up-regulation of *RAD51* in *ash1* mutants may indicate higher frequency of double-stranded DNA breaks are occurring in cells that lack Ash1.

HOS3, encodes a histone deacetylase that has been localized to the bud-neck in *S. cerevisiae*. Hos3 was recently shown to preferentially associate with and repress the expression of ribosomal DNA (27). *MHP1*, encodes a microtubule-interacting protein that was identified in *C. albicans* by transposon insertional mutagenesis (28) as a gene that influences filamentous growth on solid media. The *MHP1/mhp1* heterozygous transposon mutant showed increased filamentation on Spider plates (28) and therefore, has a negative effect on filamentous growth. The increase in *MHP1* in *ash1* mutants is consistent with this role and the reduced filamentous growth of *ash1* mutants.

Finally, fourteen genes of unknown function showed increased expression in *ash1* mutant strains. Seven of these genes have no BLAST homology to any proteins in the available databases and three of these genes are homologous to *S. cerevisiae* proteins of unknown function. One unknown protein, *FGR13*, encodes a protein with homology to eukaryotic single-stranded DNA and double-stranded RNA binding proteins and to retroviral Gag proteins. This gene was also identified in the transposon screen for mutants with altered filamentous growth phenotypes, however, the molecular function of this protein is unknown.

Discussion

Ribosome processing and biogenesis

The experiments presented in this chapter identified a large number of genes with reduced expression in the absence of Ash1. Many of these genes (seventeen of the top thirty-nine genes or 44%) encode proteins involved in ribosome biogenesis and nucleolar functions. The nucleolus is the site at which rDNA, rRNA, rRNA processing, and ribosome subunit assembly occur. The coordinated down-regulation of nucleolar genes implies a down-regulation of ribosome production, and presumably, of protein synthesis. The reduced expression of such a large number of nucleolar genes overall suggests that the nucleolus in *ash1* mutants may have differences in function or structure. Moreover, the results suggest that the nucleolus may be different in mother and daughter cells. In Appendix II this issue is addressed.

ER-Golgi transport and secretion

The second largest group of genes with reduced expression in *ash1* mutants are involved in aspects of ER-Golgi transport, mannosylation (which occurs in the Golgi apparatus), or secretion. Genes involved in ER-Golgi function or secretion were identified as both activated and repressed genes of the top-regulated genes in the data set. One intriguing result as that two genes, both homologous to the mannosyltransferase *MNN2*, are affected by the deletion of Ash1, but the expression of one is increased and the expression of the other is reduced in *ash1* mutants. *MNN2* (*orf19.1995*) expression is reduced in *ash1* mutants by approximately two-fold relative to CAF2-1 and the

expression of *MNN2-2* (*orf19.3803*) is increased by 1.3-1.5-fold in *ash1* mutants. Both of these genes shares similar BLAST homologies to *S. cerevisiae MNN2* with a BLAST expect value (e-value) of about 1e-67, and to each other by an e-value of only 2e-50 (indicating these are not alleles of the same gene). It is possible that *MNN2-2* may be up-regulated as a compensation for the decrease in *MNN2* expression in *ash1* mutants. The down-regulation of components of the glycosylation and secretion pathway in *ash1* mutants may contribute to defects in secretion of tip-cell specific enzymes or glycosylated cell wall proteins in *ash1* mutant daughter cells.

Cell wall defects and *ash1* mutants

Three genes involved in cell wall biogenesis and cell-surface remodeling (*ECM4*, *HYR1-14*, *YBR056W*) showed increased expression in the absence of Ash1 and several other cell wall genes (*SLN1*, *PKH2*, *SFL1-3*) showed reduced expression. Sln1 functions as both subunits of a two-component system histidine kinase system required for growth in high osmolarity conditions in *C. albicans* (22). In response to cell wall defects, Pkh2 activates the bud-neck localized protein-kinase C, Pkc1, which regulates cell wall biosynthesis in *S. cerevisiae* (16). *PKH2* is also required for endocytosis in *S. cerevisiae* (11). The reduced expression of *SLN1* and *PKH2* together with the increased expression of genes involved in cell wall biogenesis and modeling suggests that *ash1* mutants may have defects in the cell wall structure or contain improperly modified cell wall proteins.

Link between cell wall defects and ribosome biogenesis

The coordinated down-regulation of both ribosome and tRNA synthesis in cells with defects in secretion and cell wall function has been reported in the literature (20, 31). Specifically, perturbations of the yeast cell wall have been shown to trigger a repair mechanism that reconfigures its molecular structure to preserve cell integrity (18). Through genome-wide profiling of *S. cerevisiae* cell wall mutants, a substantial subset of genes induced in these mutants were involved in cell wall construction. They also observed an enrichment of metabolic, energy generation, and cell defense, while genes involved in transcription, protein synthesis, and cellular growth were underrepresented in response to cell wall defects. These trends are similar to those observed in *ash1* mutants. Not only are cell wall biosynthesis genes up-regulated in *ash1* mutants but, twelve of the forty most-induced genes (30%) are involved in energy production (mitochondrial function, metabolism, nucleotide biosynthesis). The link between defects in cell wall integrity and the global down-regulation of ribosomal RNA and ribosomal protein gene expression in *S. cerevisiae*, suggests that Ash1 may directly regulate cell wall synthesis but that its effects on ribosome biogenesis are indirect. The localization of Ash1 to daughter cells and hyphal tip cells is consistent with this view as these are the sites where cell wall synthesis is greatest.

Interaction between the cAMP pathway and Ash1

Ash1 has been linked to the cAMP pathway in two different studies in *S. cerevisiae*. Both Ash1 and another transcriptional regulator, Phd1, were required for enhanced filamentous growth stimulated by the *RASv19* allele (7). One conclusion from

this study was that Ash1 acted downstream of the Ras-cAMP signaling pathway (21). A second study (25) used over-expression of the PKA pathway catalytic subunit, Tpk2, to show that Phd1 and Ash1 were both required and contributed independently to cAMP-PKA pathway-activated filamentous growth and to activation of *FLO11* gene expression.

The microarray studies of this chapter show that *C. albicans* Ash1 represses the expression of the two genes (*SRA1*) that encode the regulatory subunits of PKA, and this suggests a mechanism by which Ash1 functions in cAMP/PKA pathway signaling. By repressing the expression of the regulatory subunits, Ash1 could "free up" the catalytic subunits (Tpk1 and Tpk2 in *C. albicans)* and allow them to activate filamentous growth. In the absence of Ash1, the increased expression of *SRA1* would negatively regulate filamentous growth by inactivating the catalytic PKA subunits. According to this model, wild-type mother cells, which lack Ash1, express *SRA1* at high levels and are relatively insensitive to activation by PKA. Filamentous growth requires only that hyphal tip cells assume the filamentous form, as hyphal tip cells become mother cells and may no longer require PKA signaling for cell morphology since they are already elongated. In other words, once a daughter cell is born with hyphal morphology, that cellular morphology persists upon becoming a mother cell; therefore, PKA signaling may not be required once a hyphal cell becomes a mother cell. This idea suggests that the activation of filamentous growth may only be required in daughter cells, and that Ash1 carries out this function in part by repressing expression of *SRA1*. Consistent with this idea is the observation that *ash1* mutant cells show defects in filamentous growth, especially in filaments at the periphery of colonies (presented in Chapter 2).

Unknown genes

Of the genes up- and down-regulated by Ash1, several encode proteins of unknown function. Four genes of unknown function show reduced expression in *ash1* mutants while fourteen unknown genes are de-repressed in the absence of Ash1. The unknown genes identified in these experiments are a potential source for discovering asymmetrically localized proteins of novel function. One of the most intriguing of the unknown genes is *FGR13*. This gene was identified in a large-scale transposon mutagenesis screen to identify genes that affected filamentous growth on plates (28). The *FGR13/fgr13* heterozygous mutant showed an increase in filamentous growth and my experiments show that Ash1 negatively regulates *FGR13* expression. This result supports a model where Ash1 represses the expression of *FGR13* a repressor of filamentous growth in hyphal-tip cells. The overall effect would then be to activate filamentous growth in hyphal tip cells. We further suggest that this mechanism works in conjunction with the regulation of *SRA1* (discussed above) to ensure that filamentous growth takes place exclusively in hyphal tip cells.

Summary and Conclusions

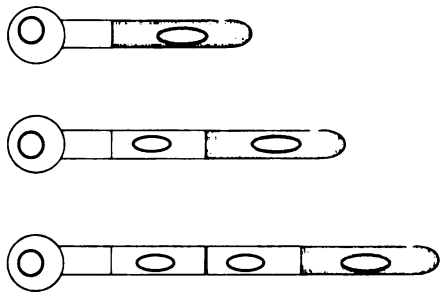
Genome-wide expression profiling provides a powerful tool to identify not only the genes regulated in response to environmental conditions but, can also identify entire biological pathways that respond to changes in conditions. Using glass-slide DNA microarrays constructed in the Johnson laboratory, I examined the expression of about 6300 genes of *C. albicans* in response to the deletion of Ash1. This analysis identified approximately 50 genes that are potentially activated by Ash1 and 35 genes that are potentially repressed by Ash1. Strikingly, almost half of the genes with reduced levels of gene expression in *ash1* mutants encode proteins involved in ribosomal subunit biogenesis, RNA processing, or have nucleolar functions. Genes involved in secretion and cell wall biosynthesis, a process linked to ribosome biogenesis in *S. cerevisiae* (20), show both increased and decreased expression in *ash1* mutants. The finding that the two genes encoding the regulatory subunits of the cAMP-dependent PKA pathway are repressed by Ash1 suggests that Ash1 activates the PKA pathway by repressing the expression of the regulatory subunit. This would ensure that hyphal growth is restricted to the tip cell.

I expect that some of the genes identified in this analysis will be hyphal tip cell specific and thus, may contribute to the specialized properties of hyphal filaments. Further experiments that determine the localization of these proteins are needed to distinguish between the genes affected in both mother and daughter cells and the those regulated specifically in daughter cells. Some of these genes could be used as daughter or tip cell markers in future studies of asymmetric cell division during filamentous growth. Daughter cell wall markers driven by conditional promoters would be particularly useful

for directly following the process of new daughter cell formation during filamentous growth and the fate of these cells upon becoming mother cells. The identification of daughter-cell specific proteins that localize to organelles would allow the visualization of the underlying cellular asymmetries and the specialization of daughter cells during filamentous growth of *C. albicans*. Thus, knowledge of the function of these genes may lead to a better understanding of how this unicellular fungus generates cell-types with specialized functions that enable it to thrive and cause disease in susceptible hosts.

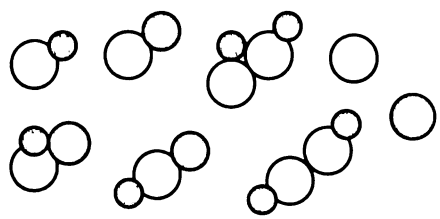
Figure 3.1. Cell growth patterns of *C. albicans* mother and daughter cells in the hyphal form and yeast form of growth. A. The percentage of mother and daughter cells in a culture growing as attached hyphal cells that are produced in a linear fashion from mother cells and subsequent sub-apical cells. B. A logarithmically growing culture showing approximately equal proportions of mother and daughter cells. Logarithmic growth for *C. albicans* continues until ~OD_{600} 10.

A. Linear growth of hyphal filaments



	Mother cells	Daughter cells
2-cell stage	50%	50%
3-cell stage	66%	33%
4-cell stage	75%	25%

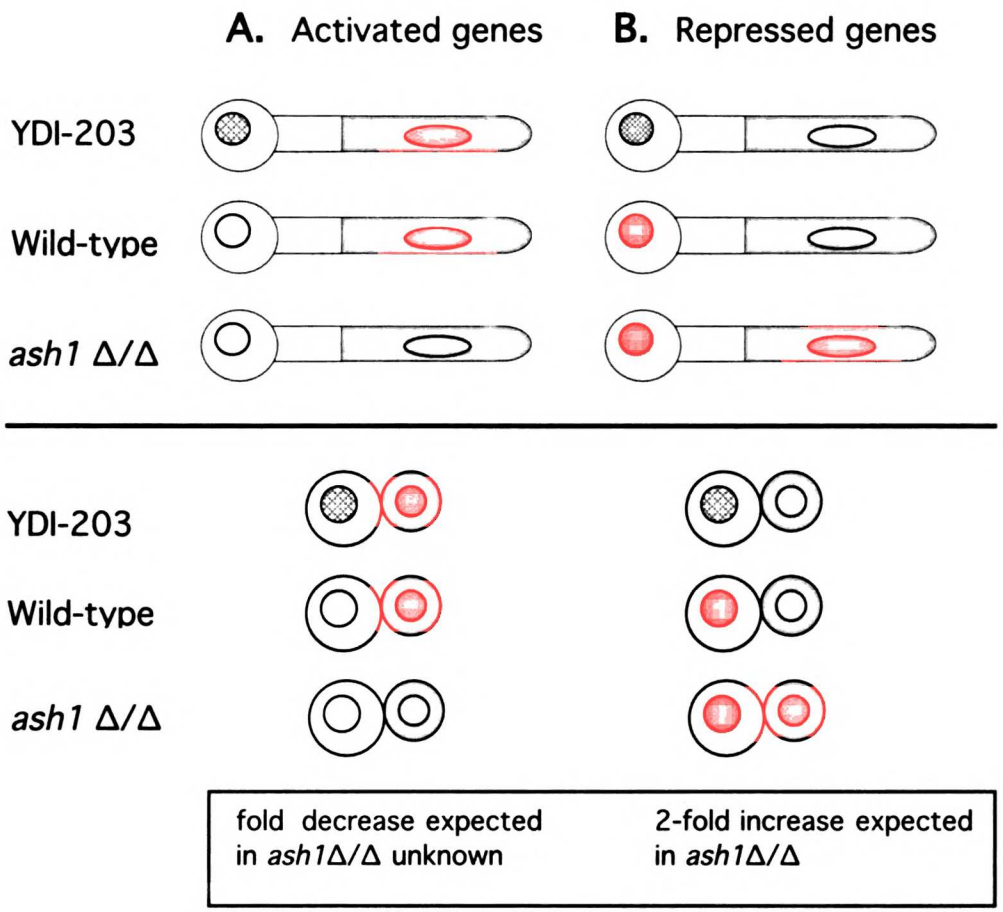
B. Logarithmic growth of yeast cells



~ 50% mother cells
~ 50% daughters cells
(or more)

Figure 3.1. Cell growth patterns of *C. albicans* mother and daughter cells in the hyphal form and yeast forms of growth.

Figure 3.2. Expected patterns of expression of daughter-cell specific target genes in wild-type and *ash1* mutant cells (A-B) and the relative changes in expression predicted for each strain comparison (C). The three types of strains used in these experiments are diagramed in both the yeast and filamentous form. A. The expression pattern of genes activated by Ash1. B. The expression pattern of genes repressed by Ash1. The daughter cells of both forms are shaded in gray. The cell nuclei are shown and filled in red to indicate the presence of Ash1 or are blank to indicate its absence. The hatched red nuclei of mother cells in strain YDI-203 indicates possible "spill-over" of Ash1 into mother cells by the presence of a third copy of *ASH1* in these strains (A and B). C. The relative changes in expression for each of the strain comparisons made in these experiments.



C.

Strain Comparison	Activated Genes	Repressed Genes
*ash1*Δ/Δ / WT	down	up
*ash1*Δ/Δ / *ASH1*+/+/+	very down	very up
ASH1+/+/+ / *ash1*Δ/Δ	very up	very down
ASH1+/+/+ / WT	up or possibly no change	no change or slightly up

Figure 3.2. Expected pattern of gene expression of Ash1-regulated target genes in mother and daughter cells and the relative changes expected in four types of strain comparisons.

Figure 3.3. Cluster of eleven microarray experiments showing all genes significantly up- and down-regulated in *ash1* mutant strains. A - C. Genes with increased expression in *ash1* mutants. D – G. Genes with reduced expression in *ash1* mutants. (A) Genes increased in Lee's medium and YPD with serum with either no change or slightly reduced expression in 30° YPD. (B) Cluster of genes with increased expression in all three experimental conditions. (C) Genes with increased expression in YPD and YPD with serum and either no change or reduced expression in Lee's medium. (D) Genes with increased expression in YPD and decreased expression in Lee's medium. (E) Genes with reduced expression in all conditions in *ash1* mutants and increased expression in YDI-203. (F) Genes with reduced expression in all three conditions with no change in expression in the YDI-203 strain. (G) Cluster of genes with consistently reduced expression in all conditions with particularly decreased expression in the stationary phase time point (experiment 11). The spot for *ASH1* is included in this cluster and is indicated by an asterisk*.

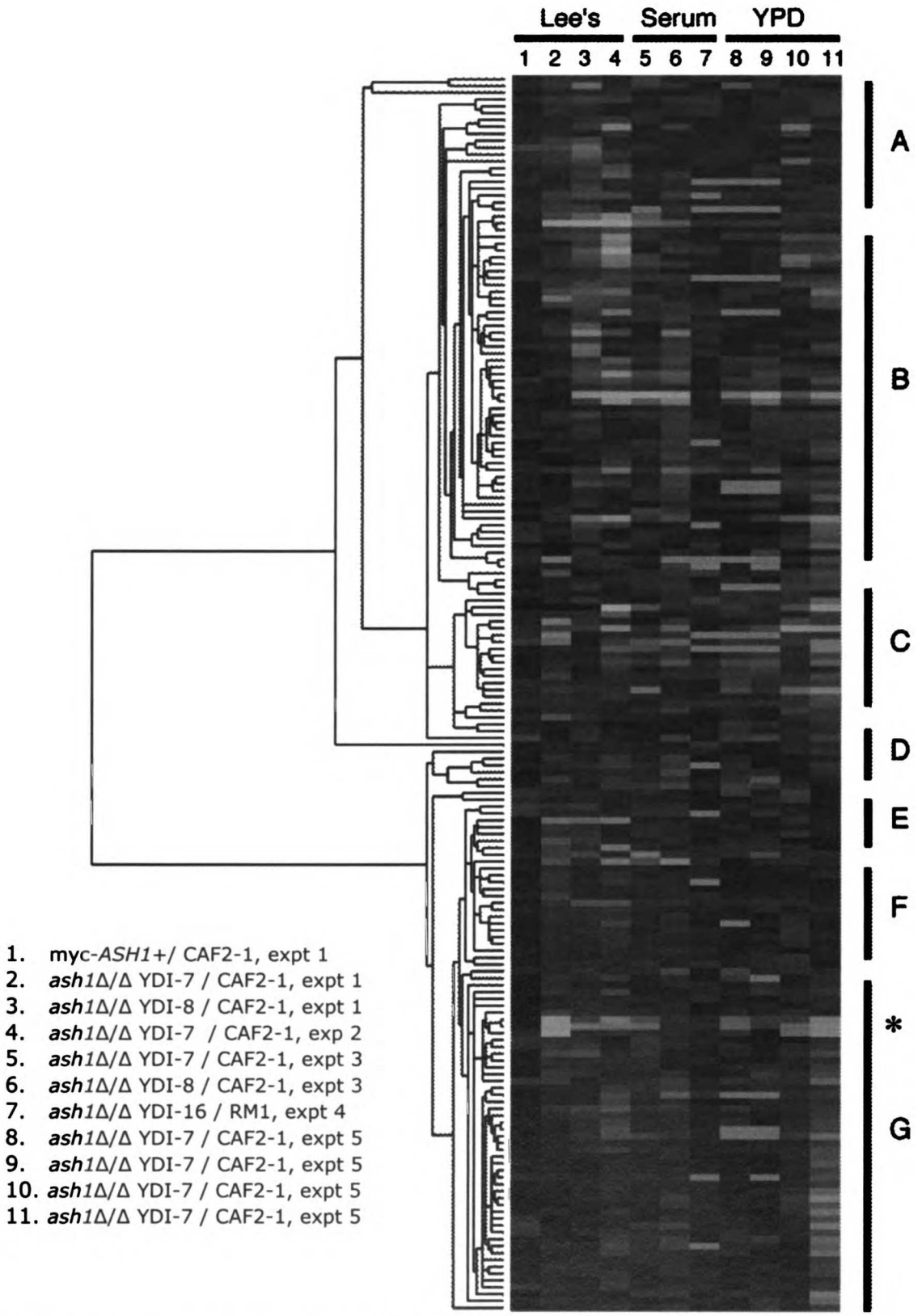


Figure 3.3. Cluster analysis and Treeview visualization of the log base 2 ratios of the median intensities for genes with altered expression in *ash1*

Table 3.1. C. *albicans* strains used in this study. The genotype of each strain is listed. A pedigree chart of the common laboratory strains *C. albicans* and derivative strains that were used in these experiments.

Strain	Genotype	Reference
CAF2-1	*URA3 IRO1/ura3 iro1*::imm434	(10)
CAI4	*ura3 iro1*::imm434/*ura3 iro1*::imm434	(10)
RM1	*ura3 iro1*::imm434/*ura3 iro1*::imm434, *HIS1/his1*::HisG	(23)
RM1000	*ura3 iro1*::imm434/*ura3 iro1*::imm434, *his1*::HisG/*his1*::HisG	(23)
YDI-7	CAI4 with *ash1*::HisG/*ash1*::HisG-*URA3*-HisG	(17)
YDI-8	CAI4 with *ash1*::HisG/*ash1*::HisG-*URA3*-HisG	This study
YDI-16	RM1000 with *ash1*::*URA3*/*ash1*::*HIS1*	(17)
YDI-203	CAI4 with *ASH1/ASH1*::myc6-*ASH1-URA3*	This study

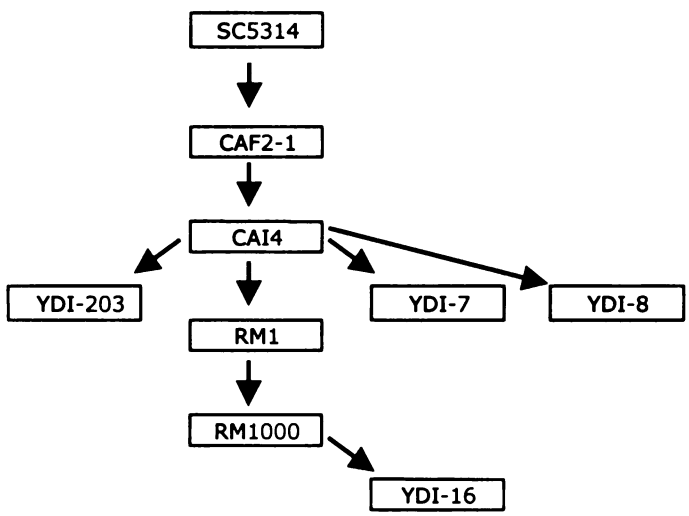


Table 3.1. *C. albicans* strains used in this study and pedigree diagram of these and other common laboratory strains.

Table 3.2. The expression ratios for genes with reduced expression in *ash1* mutants and increased expression in a 3X *ASH1* expressing strain, YDI-203. (A) The genes most consistently down-regulated in *ash1* mutants in all experiments and up-regulated in the presence of increased levels of Ash1. (B) Genes with increased expression in the presence of additional Ash1 and reduced expression in five out of six hyphal growth experiments with either no significant change or a trend toward down-regulation in the 30° YPD experiments (columns 12 – 13). The genes are grouped according to function except for the *ASH1* control spots. Boxes are color coded as follows: expression ratios ≤ 0.8 are filled in green, ratios between 0.8 – 1.1 are considered neutral (no significant change) and are unfilled, ratios between 1.2 – 1.9 are filled in red, ratios ≥ 2.0 are filled in orange. The strain comparisons and growth conditions are listed at the top.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A			203/Δ*	203/CAF	CAF/203	Δ7/203	Δ8/203	Δ7/CAF	Δ8/CAF	Δ7/CAF	Δ7/CAF	Δ8/CAF	Δ16/RM1	Δ7/CAF			
orf6 name	Name	Function	#1 Lee's		#1 Lee's			#1 Lee's		#2 Lee's	serum			2H YPD	4H YPD	6H YPD	24H YPD
orf6.8332	ASH1	gene expression	20.5	1.2	0.8	0.0		0.1		0.3	0.3	0.6	0.7	0.3	0.5	0.3	0.2
orf6.8332	ASH1	gene expression	6.6	1.1	0.9	0.1	0.4	0.1	0.4	0.2	0.3	0.5	0.8	0.3	0.5	0.4	0.2
orf6.8332	ASH1	gene expression	8.5	1.4	0.7	0.1	0.4	0.1	0.6	0.5	0.6	0.6	0.9	0.5	1.0	0.3	0.2
orf6.4984	CAORF2065	unknown function	2.1	0.8	1.2	0.5	0.4	0.4	0.4	0.6		0.6	0.8	0.6	0.4	0.7	0.7
orf6.6704	CAORF91	unknown function	3.1	1.7	0.6	0.3	0.4	0.4	0.8	0.6	0.6	0.4	0.9	0.5	0.6	0.6	0.3
orf6.9046	DBP10	nucleolus-ribosome	2.0	1.8	0.6	0.5	0.5	1.0	0.9	0.7	0.5	0.4	0.7	0.7	0.9	0.8	0.2
orf6.6554	KRR1	nucleolus-ribosome	2.1	1.6	0.6	0.6	0.4	0.9	0.7	0.5	0.6	0.6	0.8	0.6	0.8	0.8	0.4
orf6.1563	MNN11	ER-Golgi-secretion	1.7	1.3	0.8	0.6	0.6	0.7	0.7	0.5	0.5	0.5	0.8	0.9	0.7	0.8	0.6
orf6.2946	NOP58	nucleolus-ribosome	1.7	1.1	1.0	0.5	0.7	0.6	0.7	0.4	0.9	0.8	0.7	0.8	0.5	0.7	0.5
orf6.4844	URA3	nucleotide biosynthesis	2.3	1.1	0.9	0.5	0.4	0.5	0.4	0.3	0.5	0.5	0.8	0.6	0.8	0.8	0.5
orf6.1888	RPL11B	nucleolus-ribosome	1.8	0.9	1.1	0.5	0.6	0.4	0.6		0.6	0.8	0.7	0.6	0.6	0.7	1.0
orf6.2951	RPL17B	nucleolus-ribosome	3.2	1.5	0.7	0.3	0.3	0.5	0.5	0.8	0.5	0.6	0.9	0.7	1.0	0.7	0.5
orf6.4711	RPS6B	nucleolus-ribosome	3.0	1.1	0.9	0.3	0.3	0.4	0.4	0.6	0.5	0.8	1.0	0.7	1.0	0.7	0.4
orf6.8917	SIK1	nucleolus-ribosome	2.2	1.7	0.6	0.4	0.5	0.7	0.8	0.5	0.8	0.7	0.8	1.0	0.7	0.8	0.5
orf6.4994	RAD50-2	DNA repair (41 4e-04)	2.0	1.2	0.8	0.5	0.5	0.6	0.7	0.7	0.6	0.9	0.9	0.7	0.7	0.8	0.4
orf6.8258	SLN1	osmosensing	2.7	1.7	0.6	0.4	0.4	0.6	0.6	0.7	0.7	0.6	0.9	1.0	0.7	0.9	0.9
orf6.86	KIP2a	microtubule-kinesin	1.8	1.2	0.8	0.5	0.7	0.6	0.8	0.5	0.7	0.8		1.1	1.0	1.0	1.0
B																	
orf6.9073	ARF2-2	ER-Golgi-secretion	1.7	1.2	0.8	0.5	0.6	0.6	0.8	0.8	0.7	1.0	0.8	1.0	1.1	0.9	0.9
orf6.6095	CAORF3089	unknown function	1.6	1.1	0.9	0.6	0.6	0.7	0.7	0.6	0.8	0.6	0.9	1.1	1.1	0.8	0.9
orf6.7999	DIM1	nucleolus-ribosome	2.2	1.5	0.7	0.4	0.5	0.6	0.7	0.5	0.5	0.6		0.9	0.4	1.5	
no orf6	F1M20.2	ER-Golgi-secretion	3.8	1.1	0.9	0.3	0.3	0.3	0.3		0.8	0.8	0.9	0.9	1.2	0.6	0.9
orf6.4378	GAP1-8	amino acid permease	1.7	1.0	1.0	0.6	0.6	0.6	0.6	0.5	0.8	0.7	1.1	0.5		0.9	0.7
orf6.5648	GOT1	ER-Golgi-secretion	1.8	1.2	0.9	0.6	0.5	0.7	0.6	0.5	0.6	0.5		0.6	1.0	1.2	1.7
orf6.6624	IDH2	energy-metabolism	1.8	1.3	0.8	0.5	0.6	0.7	0.7	0.8	1.0	0.8	0.8	1.1	0.6	0.8	0.3
orf6.4657	MAK16	nucleolus-ribosome	2.1	0.9	1.1	0.4	0.7	0.7	0.7	0.8	0.6	0.8	0.6	1.3	0.7	0.6	1.0
orf6.6364	MNN2	ER-Golgi-secretion	2.1	1.1	0.9	0.5	0.5	0.5	0.5	0.8	0.6	0.7	0.9	0.9	0.8	0.5	1.7
orf6.6121	NUP60	nuclear pore	1.9	1.4	0.7	0.6	0.5	0.8	0.7	0.8	0.8	0.8	1.0	0.9	0.7	0.5	1.1
orf6.3685	PKH2-1	cell wall biogenesis	1.7	1.1	0.9	0.7	0.5	0.7	0.6	0.7	0.8	0.7	1.0	0.9	0.8	0.9	0.7
orf6.2868	PLP2	gene expression	2.3	1.3	0.8	0.3	0.6	0.4	0.8	0.8	0.8	0.8	0.7	1.0	0.9	0.8	1.1
orf6.2368	PWP1	nucleolus-ribosome	1.6	1.3	0.7	0.6	0.6	0.8	0.9	0.6	0.7	0.7	0.8	1.0	1.0	0.8	0.3
orf6.1842	RPL20B	nucleolus-ribosome	3.0	1.7	0.6	0.3	0.4	0.5	0.6	0.9	0.5	0.7	0.8	0.9	1.0	1.0	0.6
orf6.8846	RPS12	nucleolus-ribosome	3.7	1.9	0.5	0.3	0.3	0.5	0.5	0.8	0.6	0.6	1.0	0.7	0.8	0.9	1.1
orf6.9110	RPS15	nucleolus-ribosome	2.1	1.0	1.0	0.4	0.5	0.4	0.6	0.6	0.6	0.7	1.1	0.9	0.6	0.8	0.9
orf6.6772	RPS25	nucleolus-ribosome	2.1	1.1	0.9	0.4	0.5	0.5	0.6	0.8	0.6	0.7	1.1	0.7	1.1	0.9	1.3
orf6.1675	RPS5	nucleolus-ribosome	2.2	1.3	0.8	0.5	0.5	0.6	0.6	0.6	0.6	0.8	0.8	1.0	1.0	0.8	0.6
orf6.2746	SFL1-3	gene expression	1.6	1.2	0.8	0.6	0.6	0.8	0.7	0.7	0.7	0.6	0.9	1.1	1.0	0.7	0.4
orf6.6367	SIR2	nucleolus-ribosome	2.9	1.2	0.9	0.3	0.4	0.3	0.5	0.7	0.8	0.7	1.0	0.8	0.7	1.0	1.0
orf6.9152	SNU13	nucleolus-ribosome	2.5	1.7	0.6	0.4	0.4	0.6	0.7	0.7	0.6	0.8	1.0	0.4	0.9	0.4	1.0
orf6.7565	TPO1-4	polyamine transport	2.2	1.1	0.9	0.4	0.5	0.5	0.6	0.7	0.7	0.5	1.0	0.9	0.5	0.7	0.4
orf6.3315	TRM3a	t-RNA modification	1.8	1.3	0.8	0.5	0.7	0.6	0.9	0.5	0.5	0.4	0.8	0.4	1.9	0.6	1.0
orf6.6020	USV1	gene expression	1.9	1.3	0.8	0.6	0.5	0.7	0.6	0.7	0.7	0.4	1.1	0.4	0.7	0.7	0.8
orf6.2791	YDR266C	unknown function	2.3	1.0	1.0	0.6	0.4	0.6	0.4	0.4	0.6	0.6	0.8	1.4	0.6	0.7	0.9

Table 3.2. Expression ratios of genes activated by increased Ash1 expression and reduced in the absence of Ash1.

Table 3.3. Expression ratios of genes with increased expression in *ash1* mutants. The genes are grouped and listed by function. The color-coding and order of experiments listed is identical to Table 2.2.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
			203/Δ*	203/CAF	CAF/203	Δ7/203	Δ8/203	Δ7/CAF	Δ8/CAF	Δ7/CAF	Δ7/CAF	Δ8/CAF	Δ16/RM1	Δ7/CAF			
orf6 name	Name	Function	#1 Lee's		#1 Lee's			#1 Lee's		#2 Lee's	serum			2H YPD	4H YPD	6H YPD	24H YPD
orf6.2117	SRA1-1	cAMP signalling	0.8	0.8	1.3	2.4	2.2	1.8	1.7	1.1	1.2	1.5	1.0	1.3	1.3	1.1	1.0
orf6.2193	SRA1-2*	cAMP signalling	0.7	0.9	1.1	1.2	1.8	1.1	1.6	1.0	1.4	1.5	1.1	1.2	1.3	1.4	2.0
orf6.4272	ECM4	cell wall biogenesis	0.4	0.7	1.5	1.9	3.1	1.3	2.1	1.9	1.1	1.3		1.1	1.1	1.2	3.0
orf6.8114	YBR056W	cell wall biogenesis	0.4	0.8	1.3	2.0	4.8	1.5	3.6	1.4	1.4	2.1	1.3	1.0	1.5	1.0	2.2
orf6.3407	HYR1-14	cell wall biogenesis	0.1	0.4	2.4	5.6	8.5	2.3	3.5	1.3	1.4	1.1	0.9	1.4	1.0	1.5	1.3
orf6.4214	RAD51	chromatin remodeling	0.7	0.9	1.1	1.2	2.0	1.0	1.8	1.5	1.3	2.1	1.3	0.9	1.1	1.6	1.5
orf6.6518	MHP1	cytoskeleton	0.6	0.9	1.1	1.4	1.7	1.3	1.5	1.5	1.5	2.0	1.2	0.9	1.3	1.1	1.2
orf6.7040	HEM15	energy-metabolism	0.6	0.8	1.2	1.8	1.8	1.5	1.5	1.3	1.3	2.0	1.5	1.2	1.7	1.6	1.7
orf6.6650	YDL086W	energy-metabolism	0.4	0.7	1.5	2.1	2.5	1.4	1.6	1.2	1.1	1.8	1.4	1.5	1.4	1.6	1.5
orf6.8960	CTM1	energy-metabolism	0.4	0.5	2.0	2.2	2.4	1.1	1.2	1.9	1.5	2.2	1.1	1.9	2.1	1.3	1.6
orf6.6639	DLD1	energy-metabolism	0.4	1.0	1.0	2.4	2.8	2.4	2.8	3.4	1.3	1.3	1.0	1.6	1.5	1.0	3.6
orf6.6463	EBP1-3	energy-metabolism	0.6	0.8	1.2	1.3	2.8	1.1	2.4	2.0	1.3	2.1	1.1			2.0	2.4
orf6.1017	GLK1-2	energy-metabolism	0.4	0.6	1.8	2.0	3.8	1.1	2.1	2.6	1.8	2.7	1.0	1.8	2.0	1.2	2.6
orf6.6754	HXK2	energy-metabolism	0.7	0.9	1.1	1.3	1.7	1.3	1.6	2.9	1.5	2.3	1.1	1.7	1.9	1.1	1.0
orf6.6985	YIR035C	energy-metabolism	0.4	0.8	1.2	1.8	3.5	1.5	3.0	1.2	1.0	1.5	1.5	1.3	1.1	1.0	1.4
orf6.7230	ALT1	energy-metabolism	0.7	1.0	1.0	1.4	1.6	1.4	1.6	1.3	1.1	1.5	1.4	1.5	1.8	1.0	1.5
orf6.7736	CYP5-1	ER-Golgi-secretion	0.6	0.8	1.2	1.5	1.6	1.2	1.3	1.1	1.3	1.6	1.3	1.2	1.4	1.1	1.2
orf6.1866	MNN2-2	ER-Golgi-secretion	0.6	0.8	1.2	1.3	2.2	1.1	1.8	4.2	1.2	1.6	1.3	1.0	1.0	1.6	1.8
orf6.7858	YML059C	ER-Golgi-secretion	0.6	0.9	1.2	1.5	1.8	1.2	1.5	1.8	1.5	1.4	1.0	1.5	1.7	1.1	1.5
orf6.1399	SEC17	ER-Golgi-secretion	0.6	1.0	1.0	1.3	1.8	1.3	1.8	1.1	1.1	1.7	1.4	1.0	1.1	1.1	1.3
orf6.6580	HOS3	gene expression	0.6	0.9	1.1	1.5	2.3	1.4	2.2	2.0	1.3	1.9	1.0	1.4	1.6	1.6	1.4
orf6.7463	STR3	methionine biosynthesis	0.5	1.1	0.9	1.8	2.0	2.0	2.3	1.8	1.5	1.8	1.4	1.3	1.3	1.4	2.3
orf6.2807	RNA12**	mitochondria/rRNA	0.4	0.8	1.4	2.3	2.9	1.7	2.3	1.5	1.7	1.9	1.2	1.2	1.9	1.8	2.1
orf6.8467	KAP114	nuclear pore	0.5	0.8	1.2	2.3	1.9	1.9	1.6	1.1	1.2	1.1	1.1	1.2	1.4	1.1	1.0
orf6.8405	THI13	nucleotide biosynthesis	0.4	0.8	1.3	2.5	3.1	2.0	2.5	1.2	1.0	0.8	1.2	1.3	1.0	1.0	1.3
orf6.4806	THI6	nucleotide biosynthesis	0.4	0.9	1.2	2.2	2.4	1.8	2.1	1.3		1.6				1.4	1.4
orf6.9161	CAORF2698	unknown function	0.5	0.8	1.2	1.5	2.2	1.2	1.8	1.3	1.2	2.1	1.5	1.1	1.5	1.7	1.8
orf6.2096	CAORF316	unknown function	0.5	1.0	1.1	1.7	2.7	1.6	2.5	5.5	2.5	3.0	1.1	1.3	2.2	1.8	1.7
orf6.7879	YJR012C?	unknown function	0.6	0.8	1.2	1.7	1.7	1.4	1.4	1.5	1.2	2.0		1.3	3.8	1.1	1.8
orf6.1776	orf6.1776	unknown function	0.5	0.9	1.1	3.2	1.5	2.9	1.3	2.1	2.1	2.9				1.5	2.7
orf6.6766	CAORF763	unknown function	0.4	0.6	1.6	2.5	2.6	1.5	1.6	2.1	1.7	1.6				1.7	1.9
orf6.2575	CAORF1525	unknown function	0.6	1.1	0.9	1.3	2.7	1.4	2.9	7.3	1.1	2.0	1.8	1.2	1.5	1.9	1.9
orf6.4356	CAORF2713	unknown function	0.8	1.1	0.9	1.4	1.3	1.5	1.3	1.5	1.4	1.8	1.4	1.5	1.3	1.5	2.4
orf6.3295	CAORF581	unknown function	0.6	0.9	1.2	1.8	1.4	1.5	1.2	1.3	2.2	1.9	1.0	1.1	1.2	1.1	1.6
orf6.8245	CAORF60	unknown function	0.7	0.8	1.3	1.3	1.6	1.0	1.3	2.0	1.3	1.7	1.5	1.3	1.7	1.0	1.3
orf6.2094	CAORF94	unknown function	0.6	1.1	0.9	1.4	2.3	1.5	2.5	3.9	2.1	1.1	1.0	1.1	1.4	3.7	2.7
orf6.2141	FGR13	unknown function	0.8	1.0	1.0	1.2	1.3	1.3	1.3	1.4	1.7	2.0		1.2	1.6	1.4	2.1
orf6.7858	YML059C	unknown function	0.6	0.9	1.2	1.5	1.8	1.2	1.5	1.8	1.5	1.4	1.0	1.5	1.7	1.1	1.5
orf6.5260	YOR220W	unknown function	0.6	0.8	1.3	1.6	1.8	1.3	1.4	1.7	1.4	1.1	1.1	1.0	1.3	1.2	1.2
orf6.4011	YSC83?	unknown function	0.7	0.8	1.2	1.4	1.6	1.1	1.3	1.1	1.1	1.8	1.4	1.1	1.7	1.3	1.4

Table 3.3. Expression ratios of genes up-regulated in the absence of Ash1.

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Chapter 4.

Construction and annotation of a DNA microarray for *Candida albicans*

Acknowledgements

It is a privilege to write about the construction of a DNA microarray for *C. albicans*. The goal of this project was to generate a tool for genome-wide expression profiling of *C. albicans* in the Johnson laboratory. This goal has been achieved through the guidance and support of Alexander Johnson and through the energies and talents of several members of the Johnson laboratory. This effort was also achieved through a productive collaboration with Michael Lorenz and Gerry Fink of the Fink laboratory at MIT. It was with great pleasure that I managed the Johnson laboratory efforts to construct these arrays. This effort would not have been possible without the help of many dedicated individuals.

The key initial stages of this project were carried out by Dr. Burkhard Braun, a former post-doctoral fellow of the Johnson laboratory, now a part-time consultant for the lab. Dr. Braun wrote all of the computer programs to process the *C. albicans* genome sequence, identify ORFs, design primers, and he assembled the output files to construct the early versions of the Johnson *Candida* Gene Database. From Burk, I have learned much about computers and of handling large amounts of genome data and have experience working with the data in Filemaker Pro relational databases. This knowledge enabled me to manage the large amounts of data associated with microarray production and analysis and provided me with the information necessary to annotate the microarray.

The preliminary annotation of the *C. albicans* ORFs required a large number of hours over a period of several months to complete. The annotation of the Johnson *Candida* Gene Database was carried out by B. Braun, M. Andrew Uhl, and me. Andrew Uhl and I selected and organized the genes into plate files for primer synthesis. Several

individuals contributed custom primers and gene sequences to the microarray including Christina Hull, Mathew Miller, Andrew Uhl, Ryan Raisner, Michael Lorenz and me. Many of these custom sequences proved to be important in the subsequent analysis of microarray experiments in our laboratory.

The construction and printing of microarrays is a laborious process with multiple steps, each having to be done correctly for the arrays to be used in expression analyses. The success of this project required the coordinated efforts of several members of the Johnson laboratory including Burk Braun, Andrew Uhl, David Kadosh, Mathew Miller, Bethann Hromotka, Josie Huadong, Annie Tsong and Richard Bennett. Almost every lab member has had a hand in PCR amplification of plates, poly-lysine coated slide preparation, or in an actual print run for these arrays. I am grateful for the hard work contributed by all.

This ORF predictions and sequence analysis would not have been possible without the *C. albicans* genome sequence provided by the Stanford Genome Technology Center. The *C. albicans* genome resource website hosted by Andre Nantel and colleagues at the BRC-CRCS has been particularly useful for obtaining *C. albicans* BLAST results, sequences and annotation data. The *CandidaDB* Webserver, hosted by Christophe D'Enfert at the Institut Pasteur, has also been an excellent resource for gene annotation and graphical viewing of sequence maps. The online sequence and genomics resources provided by Genbank/NCBI and SwissProt were also extremely valuable. We also owe thanks to SACS, the genomic sequence analysis resource at UCSF (http://www.sacs.ucsf.edu/) that dedicated large amounts of hard drive space and cpu time on the Socrates file server for much of the *C. albicans* sequence processing.

The success of this project was also greatly influenced by the advice of Joe DeRisi, Holly Bennett and Anita Sil, experts in microarray technology at UCSF. Special acknowledgement is due to Joe DeRisi who has paved the way for making microarray technology readily accessible to academic laboratories. Joe provided training and, most importantly, built the arrayer that was used to construct our microarrays. Joe is also responsible for the Nomad database at UCSF that is used to store and extract our data.

This project brought me the opportunity to gain extensive experience with fungal genome resources and sequence analysis. As a result of this work, I had the opportunity to participate and make an impact in the *C. albicans* Community Annotation Group, a group of genome-wise individuals that have worked towards an annotation of a universal set of genes for *C. albicans* that is freely available to all members of the research community.

I have been fortunate in being able, as a graduate student, to manage such a large project that required expertise in bioinformatics, genomics and traditional bench work. The experience I have gained is from this project is certain to be valuable for my future scientific endeavors.

Introduction

Genome-wide expression profiling is a powerful new method for discovering the genes that are responsible for growth or development of an organism or for its response to changes in its environment. This technology is currently being employed, by many scientific groups in both academic and private settings, to examine the expression profiles of a growing number of important species including humans, mice, *C. elegans*, *D. melanogaster*, *S. cerevisiae*, *H. capsulatum*, *H. pylori* and *P. falciparum* (23, 25-27, 30, 31, 36).

Genome-wide expression profiling can be achieved with glass-slide DNA microarrays, a recently developed technology that uses complementary base pair hybridization between DNA spots on a microarray and the RNA or DNA extracted from a cell to measure the relative differences between two strains. DNA microarrays typically consists of PCR-amplified DNA fragments that represent open-reading frames (ORFs) that are spotted onto coated glass slides. The slides are hybridized with equal amounts of Cy5- and Cy3-labeled probes and competitively hybridized to the array. Typically the probes are DNA copies of the spectra of RNAs produced by a population of cells grown under a specific set of conditions.

The hybridized slides are then washed to remove excess probe and the microarray data is collected with an array scanner that is able to detect the Cy5 and Cy3 emmision signals of each spot. The ratios of the median Cy5 and Cy3 intensities for each spot is calculated with microarray analysis software. These ratios indicate the relative level of expression of every gene between the two samples. Spots with greater signal intensity in the Cy3 channel indicate enrichment of the corresponding ORF in the Cy3-labeled

sample. Spots with greater signal intensity in the Cy5 channel indicate genes whose expression is enriched in the Cy5-labeled sample. By comparing the expression profiles of a wild-type and a mutant strain for example, the genes whose expression change due to the mutation can be identified. Alternatively, genes regulated under a particular environmental condition, such as starvation, can be identified by comparing a strain grown in nutrient rich and a nutrient poor growth media. The experimental data can be organized by Cluster analysis and visualized with Treeview (16) microarray data imaging software. These programs allow the visualization of patterns of gene expression from microarray experiments such as time course experiments conducted over a wide range of experimental conditions and thus enable us to gain a global view of gene expression patterns and a better perspective on gene function in our organism of interest.

Construction of a DNA Microarray for *Candida albicans*

As a tool for genome-wide expression profiling of the fungal pathogen *C. albicans*, a whole-genome microarray representing approximately 6300 *C. albicans* genes was constructed in the Johnson laboratory. Using the available *C. albicans* genome sequence, potential open-reading frames were identified and annotated by analyzing the BLAST (9) homology to *S. cerevisiae* proteins and to protein sequences available in the NCBI non-redundant protein database (8). A Johnson *Candida* Gene Database (12) was constructed in Filemaker Pro that contains the *C. albicans* ORF sequences, size, protein translations, BLAST data and other sequence information. Based on these ORF sequences, primers were designed and synthesized for PCR amplification of approximately 5200 ORFs. An

additional 6400 primers for amplification of *C. albicans* ORFs were obtained through a collaboration with the Fink Laboratory at The Whitehead Institute (MIT). A total of 11,689 ORF DNA fragments representing approximately 6300 *C. albicans* genes were successfully amplified by PCR using the combined Johnson and Fink laboratories primer sets. The PCR products were analyzed to determine whether a single-band of the correct size was amplified for each ORF. A total of 10, 092 PCR products were considered a "success" and 1671 PCR reactions were determined to be "fails" with either multiple bands or low yields. The PCR products were transferred to 384-well microtiter plates and spotted onto poly-lysine coated glass slides using a custom-built Derisi-style "arrayer" (J. Derisi, UCSF). These glass-slide DNA microarrays have been used successfully in the Johnson laboratory as a tool for examining the expression profile of *C. albicans* under a variety of conditions (11, 14, 33) (D. Kadosh, UCSF, B. Hromatka, UCSF).

Open reading frame identification

The 16 megabase (Mb) genome of *C. albicans* has currently been sequenced to 10.5X coverage by the Stanford Genome Technology Center (6). At the time the array construction began, the available Contig Assembly 4 sequence (July 1999) was estimated to represent 5.4X coverage of the *C. albicans* genome. Soon after, Contig Assembly 5 (October 1999), representing 7X coverage of the genome, was made available. The genome sequences contained first in Contig Assembly 4 and later in Contig Assembly 5 were downloaded from the Stanford ftp site (1). These sequences exclude the repeats of the ribosomal DNA sequences that are normally present in multiple copies and the mitochondrial DNA sequence that uses a different genetic code for translation (2, 29).

To identify potential genes for spotting on a *C. albicans* microarray, the Perl computer programming language was used to generate an ORF-finding program to mine the contig assembly sequence for potential genes (B. Braun, unpublished). This program was designed to identify sequences that begin with a methionine codon and are capable of encoding proteins of 100 amino acids or greater in length. Because some genes are interrupted by the beginning or end of a contig, we also identified ORFs at the ends of contigs that were not required to start with a methionine codon. A total of 8311 ORFs were predicted in the assembly 4 sequence with these criteria. Six additional genes predicted to be less than 100 amino acids in length were identified by BLAST homology to *S. cerevisiae* proteins that contain less than 100 amino acids (34). Ten additional genes that were either published *Candida* genes present in Genbank or that had been cloned and sequenced in the Johnson laboratory but were not present in the available contig sequence were added to the gene list. Thus, a total of 8317 potential genes were identified from the Contig Assembly 4 sequence with 10 additional *Candida* genes added from outside sources. This is likely to be an over-estimate of the number of real genes thought to be present in *C. albicans* since approximately 1000 predicted ORFs represent a second or adjacent fragment of a single gene and over 700 ORFs overlap a named gene.

Similarly, the Contig Assembly 5 sequence was processed to extract ORF sequences of 100 amino acids or greater using the Perl-based ORF-finding algorithm written by B. Braun. Our experience with the Assembly 4 sequence suggested that frameshifts due to sequencing and assembly errors occurred frequently in the unfinished genome sequence. In order to maximize the identification of "real" genes, we additionally extracted ORF sequences between 70 and 100 amino acids and then subjected these small

ORFs to additional filtering during the annotation phase of the project. By lowering the amino acid cut-off, an additional 190 novel ORFs not found in the Contig 4 set were identified although many of these ORFs represented only partial gene sequences with frameshifts and premature stop codons. For the purposes of microarray construction, partial gene sequences spotted on an array are sufficient for monitoring expression of a gene. At this time, the current *C. albicans* sequence assembly 19 contains gaps and frameshift mutations making an exhaustive identification of all ORFs a nearly impossible challenge. Altogether, a total of 8017 putative ORFs were identified using the Contig Assembly 5 sequence data.

I have annotated approximately 700 small ORFs (100-150 amino acids) in our database of assembly 4 and assembly 5 ORFs that overlap a larger ORF with an assigned gene name. These small sequences have no BLAST homology and are likely to represent false ORF sequences rather than *bona fide* genes. Approximately 600 ORFs represent duplicates or multiple fragments of a single gene and approximately 400 ORFs are small (less than 200 amino acids) and are likely to be false ORF sequences. Thus, we have identified approximately 6300 putative genes in the *C. albicans* genome.

Preliminary *C. albicans* ORF annotation and *Candida* Gene Database Construction

Genome sequence annotation is important for a thorough analysis of gene-expression data and can be important for determining gene sequences that require special attention in microarray construction (such as gene families and genes with multiple repeat sequences). To annotate the genes we identified in *C. albicans*, the DNA sequence of each putative ORF was translated into amino acid sequence using the non-standard NCBI

translation table 12 which translates the CUG codon as serine in *C. albicans* (28) rather than leucine as it is translated in other organisms. These translated protein sequences were then analyzed by BLASTp homology (BLASTp) against the *S. cerevisiae* sequence available at the *Saccharomyces* Genome Database (SGD) (3) and against the nonredundant protein database sequences downloaded from NCBI (7). The BLASTp results along with the DNA and protein sequence information was assembled into a Johnson *Candida* Gene Database (12) that was designed in Filemaker Pro to facilitate manual annotation of the identified ORFs.

The BLASTp results were evaluated for each ORF and gene names were assigned (B. Braun, M. A. Uhl, D. Inglis, unpublished) based on the following conventions:

1) ORFs matching published *C. albicans* genes were assigned the name of the published gene.
2) ORFs with significant homology to *S. cerevisiae* proteins were assigned the name of the *S. cerevisiae* gene.
3) ORFs with no homology to *S. cerevisiae* proteins yet showed significant homology to a protein in another organism were assigned the name of the gene for that organism.
4) ORFs with no BLASTp homology to any protein were assigned CAORF numbers (CAORF1, CAORF2, etc...).

Putative ORFs identified in the Contig Assembly 5 sequence were annotated in a similar manner however, BLAST identity between the annotated Contig 4 gene database and the

Contig 5 set was used to automatically assign the gene names for identical protein matches to the Contig Assembly 5 derived ORFs. 5,578 putative ORFs were annotated by automated protein and DNA match name assignment. The remaining ORFs were analyzed manually (B. Braun, M. A. Uhl, D. Inglis, UCSF) and assigned gene names as described for the Contig 4 gene set.

Primer design for amplification of predicted *C. albicans* genes

To generate the DNA fragments to spot on the microarrays, PCR primers were designed using a custom Perl algorithm (B. Braun, UCSF) to amplify selected DNA sequences from our annotated ORF set. Four rounds of primer design was used to generate a complete set of primer pairs to PCR amplify products that cover between 80 and 100% of the coding sequence of approximately 6300 genes. The first round of primer design attempted to generate 22 –25 base pair (bp) forward and reverse primer sequences with an optimal annealing temperature of 55°C or greater. Primer pairs were successfully designed to 4032 genes with these criteria. For the second and third round of primer design, the criteria was adjusted to allow for lower annealing temperatures of 50°C and 45°C for amplification of sequences that failed to generate primers with an annealing temperature of at least 55°C. 768 primer pairs were designed to genes with a 50°C or greater annealing temperature and approximately 650 primers were designed at 45°C or greater. A total of 5246 primers pairs were designed and synthesized by these three methods.

To maximize the success of PCR amplification in 96-well plates, ORFs were sorted according to the annealing temperature of the primer pair and then by the size of

the expected PCR products. Each ORF was assigned forward and reverse primer identification numbers and a 96-well plate location for primer synthesis. The first two plate pairs (1FW, 1RV, 2FW, 2RV) were synthesized by the Stanford Genome Technology Center Technology Development Group (4). The remaining 48 plate pairs were synthesized by Research Genetics, (now part of Invitrogen Life Technologies).

Primer Design for Gene Families: A fourth round of primer design was carried out to design specific primers for gene families and for genes with highly repetitive sequences. Two gene families in particular, the ALS gene family (22) and an *HYR1* gene family (10) (Table 4.1) were analyzed to identify small unique regions (M. Miller, UCSF) against which specific primers could be designed. Published primer sequences specific for the ALS gene family (18-22) were also available and were used to amplify ALS sequences for the array. Other gene family members, such as the *MNN2* and *MNN4* families were broken down into small windows of sequence and primers were designed to amplify regions with the least similarity to the rest of the genome (B. Braun, Special Primer Design Database). A list of these custom PCR products is shown in Table 4.1.

Promoter DNA sequences: Twenty-one candidate promoter fragments for use in pilot chromatin immunoprecipitation experiments were designed for the array by Christina Hull. A list of these promoters and the primer names are shown in Table 4.2. The sequence of these primers is available in the Johnson laboratory oligo sequence databases.

Custom Primers for Fusion Proteins and Genetic Markers: Current molecular methods for analyzing gene function in *C. albicans* rely on genetically modified strains, often marked with standard epitope fusion tags. As a means of detecting the presence or expression of several common molecular tags present in our laboratory strains, we generated five DNA fragments, listed in Table 4.3, by PCR against the *HisG* and *rhaB* repeat sequences and against the Myc fusion protein coding sequence, enhanced Green Fluorescent Protein (eGFP) and to the *Streptococcus thermophilus lacZ* gene sequence, optimized for use as a LacZ reporter in *C. albicans* (35). By including these control PCR fragments on the microarray, the relative abundance of the fusion protein messenger RNA (mRNA) transcript can be monitored. The bacterial *HisG* sequence is present in the commonly used Ura-Blaster cassette used for disrupting genes in *C. albicans*. The bacterial *rhaB* gene is present as a repeat flanking *URA3* in the integrating vectors pAU71 and pDI-01 used in the Johnson laboratory for ectopic gene expression (34). These sequences can be used as controls in experiments that compare genomic DNA from laboratory strains containing different numbers of these markers.

Normalization controls: Four *Baccillus subtilus* genes (*DapB*, *LysA*, *PheB*, and *ThrB/C*) were added in duplicate to the array as controls for normalization of the Cy5 and Cy3 signals. Two common methods for normalizing the intensities in the red (Cy5) and green (Cy3) channels obtained by microarray hybridization are the "bulk-scale" and "*in vitro* spike" methods of normalization (32). Bulk-scale normalization assumes that the sum of the signal in the Cy5 channel is equal to the total amount of signal in the Cy3 channel. The "*in vitro* spike" method requires the presence of external genomic DNA

controls such as these four *B. subtilus* genes. With this method, a known quantity of *in vitro* transcribed RNA is added in equal amounts to the samples to be hybridized prior to cDNA synthesis and labeling. The *in vitro* transcribed RNA control spots are used to calculate the normalization factor that sets the Cy5 to Cy3 ratios of these control spots equal to one. The normalization factor is then applied to all the spots on the array.

cDNA Synthesis Controls: As a means of evaluating the success of the reverse transcription reaction (used to generate labeled cDNA for hybridization) for the two samples being compared, PCR primers to amplify a series of non-overlapping *ACT1* fragments (labeled *ACT1*a, *ACT1*b, *ACT1*c, *ACT1*d, *ACT1*e) from the 5 prime to the 3 prime end were provided by M. Lorenz. When both samples are reverse transcribed into cDNA with equal efficiency, the resulting normalized ratios (Cy5/Cy3) for each of these spots will be approximately 1.0. If one of the samples is inefficiently transcribed, the Cy5-labeled sample for example, the normalized ratios for ACT1e could be close to 1.0, however, the ratios for *ACT1*d, *ACT1*c, *ACT1*b, and *ACT1*a would drop below 1.0, indicating a reduced quality of cDNA.

Fink Laboratory Primers: As part of a collaboration between the Johnson Laboratory and the Fink Laboratory at MIT, we acquired an additional 65 primer plate pairs synthesized by GeneMachines that were designed to amplify ~6300 ORFs. The addition of the PCR products obtained with this primer set allows for duplicate representation of most of the ORFs in the *C. albicans* genome to be analyzed and for genes identified by the Fink laboratory and not the Johnson lab to be evaluated. The Fink lab primers were

designed with a 65° C optimal Tm. This primer set amplifies products between 300-600 base pairs with a bias toward the 3 prime end of each gene. The combined set of products amplified from the Johnson and Fink primer sets are estimated to represent approximately 6300 ORFs or more than 98% of the genes in the *C. albicans* genome.

PCR amplification of ORFs for a *C. albicans* microarray

Forward and reverse primers were combined in 96-well PCR plates and amplified by PCR using *C. albicans* strain SC5314 genomic DNA as the template. PCR was performed according to the protocol available at http://www.microarrays.org with the following modifications. PCR plates that produced a high number of multiple-band products were amplified using a "touchdown" PCR conditions with the first round of primer annealing at approximately 5° C above the optimal Tm for that plate and subsequent cycles of annealing decreased to approximately 50 - 45° C, depending upon the original annealing temperature for the plate.

Between 1 and 2 μL of each PCR product was analyzed by electrophoresis on 1% agarose gels designed to accommodate 96-well samples. Images of each gel/PCR plate were saved as tiff files and visually analyzed to determine the relative yield and success of each PCR product. PCR products with prominent bands of the correct estimated size were scored as a "success." Wells with no apparent PCR product, with multiple bands, or with a band of the wrong size were scored as a "fail." A total of 4585 successful PCR products and 661 fails were obtained with the Johnson primers indicating a success rate of 87.4%. A total of 5507 successful products and 910 failed PCRs were obtained with

the Fink primers for a success rate of 85.8%. Altogether, 10,092 *C. albicans* ORF DNA fragments were obtained by PCR and prepared for spotting onto glass-slide microarrays.

Preparation of PCR products for microarray spotting

PCR products were prepared for spotting onto glass-slides using the protocols available at http://www.microarrays.org (see Precipitation.pdf). Briefy, the PCR products were transferred into Costar 96-well U-bottom polypropylene plates (catalog#3790) and precipitated in 0.3M sodium acetate, pH 5.5 with the addition of 1 volume isopropanol. Plates were stored at –20° C overnight to maximize yields. Plates were then centrifuged for two hours at 4500 rpm in a Beckman RC3B centrifuge. The DNA pellets were gently aspirated with a 12-channel Wheaton Aspiration Adaptor (PGC Scientific catalog# 851388). The pellets were washed once with the addition of 100 μL 70% ethanol and centrifuged for one hour at 4500 rpm. The ethanol was aspirated from the wells and the pellets were dried in a speed-vac equipped with 96-well microtiter plate adaptors. The PCR products were then resuspended in ~20 μL of filtered water and transferred to either three or four 384-well plates depending upon the overall yields. Twenty-six empty wells were selected at and filled with either salmon sperm DNA (to assess non-specific hybridization signals on the array) or with SC5314 genomic DNA (to detect general *C. albicans* hybridization signals). The contents of these plates were then dried down at low temperature in a speed-vac and stored at –20° C. Two days prior to printing, each plate was resuspended in ~7 μL of filtered 3X SSC.

Microarray spotting procedure

Approximately two weeks prior to printing, standard glass slides (Gold Seal Microslides) were coated with poly-lysine according to the protocol available at http://www.microarrays.org (see PolylysineSlides.pdf). This coating enables the 3X SSC/ DNA solution to adhere to the glass slides.

The custom-built DeRisi "arrayer" (J. DeRisi, UCSF) was used to spot a total of 11, 325 DNA samples onto 261 glass slides for each print run. Thirty-two precision metal print tips (Majer Precision, Tempe, Arizona) were used to generate an array of spots in 32 blocks with 4 columns and 8 rows on each slide. Each slide is individually labeled with a slide identification number and stored in clean slide storage cases. The glass-slide DNA microarrays are then post-processed and are ready for hybridization of samples to the array.

***C. albicans* Microarray Annotation**

The annotation associated with a microarray is key for the detailed analysis of the genes that are regulated in a set of microarray experiments. Each spot on the microarray is assigned a unique identification number (unique ID) that is used to track the data for each spot in subsequent analyses. In addition to the expression ratio data, a large amount of annotation data is linked to each spot through the unique IDs. The annotation for the Johnson lab microarrays (listed in Table 4.4) has been deposited in the Nomad Microarray Database (5) with the unique IDs and can be extracted with microarray data for detailed gene analyses.

The most basic annotation information typically extracted with a data set is the spot name and spot description. The spot names and descriptions in Nomad include recently updated gene names and ORF DNA overlaps that I identified as well as gene names from the preliminary ORF annotation in the *Candida* Gene Database. PCR reactions that failed are labeled as "fail" in both the spot name and spot description field to simplify filtering these spots out of the microarray data.

The Johnson *Candida* Gene Database sequential number (Lab DB ID) is included for reference to the sequence information and BLAST data contained in the original annotated record. The Fink ORF ID is the unique identification number for the Fink lab ORFs on the array. It is cross-referenced in the Johnson *Candida* Gene Database and is included for identification and record-keeping purposes.

A large amount of the annotation for *C. albicans* comes from the well-studied related yeast, *S. cerevisiae*. Roughly 60% of the *C. albicans* genes are homologous to a protein in *S. cerevisiae* and most of the annotations for these genes were imported directly from SGD or other yeast databases. The *S. cerevisiae* annotation fields for the microarray include the top yeast ID, yeast name, and e-value. The top yeast ID is the systematic name for the best matching *S. cerevisiae* gene. It is used as the unique ID in *S. cerevisiae* PCR microarray experiments and can be used to directly align *C. albicans* and *S. cerevisiae* array data for large-scale cross-species comparisons. The yeast name is the common gene name if one exists. The e-value is from the "expect-value" from the BLASTp results and indicates the significance of the match. The forward and reverse primer sequence data along with the sequence of the predicted amplified fragment is

provided in Nomad for analysis of gene-family hybridizations, sequence overlaps and for determining the specificity of the spotted DNA sequences.

Contig Assembly 6 names

The pair-wise BLAST analysis pages of the Johnson *Candida* Gene Database was used to assign a contig assembly 6 ORF name (orf6 name) to the spots on our microarray. Approximately 150 successful PCR spots on the microarray have no match in the contig assembly 6 ORF set. The orf6 gene set has been annotated by several groups and has been used widely for gene identification in publications of *C. albicans* research. Thus, the orf6 name is an important annotation field associated with our microarrays and serve as a means of universal identification for communicating gene expression results in publications and research presentations. The orf6 matches can also be used to align microarray data from other *Candida* laboratories directly with our own data for comparative analysis.

Contig Assembly 19 names

Contig Assembly 19 was the first to be released as a diploid sequence assembly, and two orf19 alleles are usually present for a single ORF in the haploid assembly 6 genome sequence. Two independent sources of orf19 BLAST alignment with the orf6 matches are provided with our microarray annotation. The first was generated by Burk Braun as a BLAST database of the Contig 19 ORF set against the Contig 6 ORF set. The best matching pair-wise BLASTp result was used to assign the orf6 to orf19 match. The second source of orf6 to orf19 translation was performed by *Candida*DB (13) and was

obtained from the *C. albicans* website developed by A. Nantel and colleagues (http://206.167.190.233/candida/). In many cases, the top orf19 name is the same between the *Candida* DB table and the Johnson orf19 match provided by B. Braun. In some cases however, the orf19 match names are different between the two orf6 BLASTp results most likely indicating the second of the two alleles of in the Contig 19 ORF set.

A new annotation of the Contig 19 ORF set has recently been completed by the *C. albicans* Annotation Work Group that resolves some of the broken genes, connects introns, and filters out the likely false ORFs. This annotation work and the orf19 identification is soon to become the *Candida* community-wide standard for looking up or depositing gene information in the recently funded *Candida* Genome Database (CGD) which will operate on the model of SGD. Thus, the orf19 links are important for future annotations of gene function and updates to the genome sequence. Ideally, microarray data submitted for publication will include the orf19 gene designation in order to unambiguously identify the genes we describe in our studies.

Conclusions

With this annotated *C. albicans* microarray, we now have the ability to simultaneously monitor the genome-wide expression profile and genomic DNA profile of polymorphic fungal pathogen under a wide variety of experimental conditions. These arrays are a powerful tool for identifying genes regulated during various stages of growth and pathogenesis of *C. albicans* during systemic infections in mice and in tissue culture infection experiments. They are also critical for understanding the function of a given

gene; the gene can be deleted and the resulting genome-wide changes in transcription can be monitored. Glass slide DNA microarray technology makes the discovery of entire regulatory pathways possible and simplifies the identification of genes involved in the response to growth under any conditions.

Table 4.1. Custom Primers for Gene Families of *C. albicans*. Genes with custom designed PCR products designed to distinguish between members of multiple gene families are listed by Unique ID and Spot name on our microarray. The Johnson *Candida* Gene Database ID and the Stanford orf6 numbers are also listed except for the ALS gene family whose full-length uninterrupted ORF sequences were obtained from Genbank. The gene products are described in the Spot Description and the source of the custom sequences are listed in the Notes column.

Unique ID	Lab DB ID	orf6 Name	Spot name	Spot Description	Notes
ALS Gene Family					
M11A10	Genbank		ALS1-publ	Specific PCR product for ALS1	L. Hoyer publ. primers
M11A12	Genbank		ALS2/4-publ	Specific PCR product for ALS2 and ALS4 genes	L. Hoyer publ. primers
J14H2	Genbank		ALS3-publ.	Specific PCR product for ALS3	L. Hoyer publ. primers
M11A11	Genbank		ALS3-publ(undig.)	Specific PCR product for ALS3; undigested fragment	L. Hoyer publ. primers
M11B1	Genbank		ALS5-publ	Specific PCR product for ALS5	L. Hoyer publ. primers
M11D4	Genbank	orf6.8574	ALS6-publ.	Specific PCR product for ALS6	L. Hoyer publ. primers
M11D5	Genbank		ALS7-publ.	Specific PCR product for ALS7	L. Hoyer publ. primers
M11B9	Genbank	orf6.2112*	ALS1-MM	Agglutinin-like sequence 1; *orf6.2112 5'eoc fragment	M. Miller custom primers
M11B8	Genbank		ALS2-MM	Specific PCR product for ALS2	M. Miller custom primers
M11B6	Genbank		ALS4-MM	Specific PCR product for ALS4	M. Miller custom primers
M11B5	Genbank		ALS5-MM	Specific PCR product for ALS5	M. Miller custom primers
M11B4	Genbank	orf6.8574	ALS6-MM	Specific PCR product for ALS6	M. Miller custom primers
M11B3	Genbank		ALS7-MM	Specific PCR product for ALS7	M. Miller custom primers
M11B2	Genbank		ALS8-MM	Specific PCR product for ALS8; ALS3 and ALS8 are alleles	M. Miller custom primers
HYR1 Gene Family					
J14F4	4670	orf6.4725	HYR1.2-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11B12	3978	orf6.2179	HYR1.3-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C2	86	orf6.1159	HYR1.5-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C3	6555	orf6.8724	HYR1.7-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C4	4427	orf6.4388	HYR1.8-MM	Similar to HYR1, hyphally regulated gene, FLO11 match	M. Miller custom primers
M11C5	4146	orf6.8279	HYR1.9-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C6	598	orf6.4720	HYR1.10-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C7	935	orf6.3209	HYR1.12-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C8	7265	orf6.8635	HYR1.13a-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C9	7260	orf6.8640	HYR1.13b-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
M11C10	34	orf6.3407	HYR1.14-MM	Similar to HYR1, hyphally regulated gene	M. Miller custom primers
MNN2 Gene Family					
M10G6	6561	orf6.2169	MNN2-1	Mannosyltransferase 2 gene family member	B. Braun custom primers
J43A1	14113	orf6.1866	MNN2-2	Mannosyltransferase 2 gene family member	B. Braun custom primers
J43C2	9979	orf6.4205	MNN2-3	Mannosyltransferase 2 gene family member	B. Braun custom primers
J48B3	20092	orf6.6801	MNN2-4	Mannosyltransferase 2 gene family member	B. Braun custom primers
MNN4 Gene Family					
J43A3	9861	orf6.4390	MNN4-1(MNN4)	Candida Mnn4p mannosyltransferase homolog	B. Braun custom primers
J43C8	19484	orf6.900	MNN4-2	Mannosyltransferase 4 gene family member	B. Braun custom primers
J43C4	11129	orf6.6935	MNN4-3	Mannosyltransferase 4 gene family member	B. Braun custom primers
J43A11	17936	orf6.4127	MNN4-4	Mannosyltransferase 4 gene family member	B. Braun custom primers
M4G7	6167	orf6.4127	MNN4-4	Mannosyltransferase 4 gene family member	B. Braun custom primers
J43B3	18677	orf6.6659	MNN4-5	Mannosyltransferase 4 gene family member	B. Braun custom primers
J43B1	13137	orf6.3982	MNN4-6	Mannosyltransferase 4 gene family member	B. Braun custom primers

Table 4.1. Custom Gene Family Primers

Table 4.2. Custom Promoter DNA Primers. A set of primers designed by C. Hull was used to amplify the promoters of twenty genes. The genes are listed by Unique ID and include the Spot Name, Johnson Candida Gene Database ID, the Stanford orf6 number, the Spot Description, the PCR product size and the names of the primers used.

Unique ID	orf6 name	Lab DB ID	Spot Name	Spot Description	FW primer	RV primer	PCR size
M15A2	orf6.8002	7084	ACT1prom	ACT1 promoter fragment	CHO337	CHO338	369
M15D2	orf6.8002	7084	ACT1prom	ACT1 promoter fragment	CHO337	CHO338	369
M15A1	orf6.9131	4819	ADE2prom	ADE2 promoter fragment	CHO294	CHO295	891
M15D1	orf6.9131	4819	ADE2prom	ADE2 promoter fragment	CHO294	CHO295	891
M15A4	orf6.2920	4679	ALS1-2prom	ALS1-2 promoter fragment	CHO343	CHO344	289
M15D4	orf6.2920	4679	ALS1-2prom	ALS1-2 promoter fragment	CHO343	CHO344	289
M15B3	orf6.2112	9634	ALS1prom	ALS1 promoter fragment	CHO378	CHO379	403
M15E3	orf6.2112	9634	ALS1prom	ALS1 promoter fragment	CHO378	CHO379	403
M15B4	orf6.8423	10186	AXL1prom	AXL1 promoter fragment	CHO380	CHO381	264
M15E4	orf6.8423	10186	AXL1prom	AXL1 promoter fragment	CHO380	CHO381	264
M15A5	orf6.7428	19538	CAG1prom	CAG1 promoter fragment	CHO347	CHO348	319
M15D5	orf6.7428	19538	CAG1prom	CAG1 promoter fragment	CHO347	CHO348	319
M15A12	orf6.695	17802	CPH1prom	CPH1 promoter fragment	CHO369	CHO370	308
M15D12	orf6.695	17802	CPH1prom	CPH1 promoter fragment	CHO369	CHO370	308
M15B5	orf6.3959	15247	DLH1prom	DLH1 promoter fragment	CHO382	CHO383	319
M15E5	orf6.3959	15247	DLH1prom	DLH1 promoter fragment	CHO382	CHO383	319
M15A6	orf6.2854	20156	FUS3 CEK2 prom	FUS3 promoter fragment	CHO351	CHO352	343
M15D6	orf6.2854	20156	FUS3 CEK2 prom	FUS3 promoter fragment	CHO351	CHO352	343
M15A7	orf6.8600	19893	HST6prom	HST6 promoter fragment	CHO353	CHO354	262
M15D7	orf6.8600	19893	HST6prom	HST6 promoter fragment	CHO353	CHO354	262
M15A3	orf6.4399	14502	MTLalpha1prom	MTLalpha1 promoter fragment	CHO339	CHO340	325
M15D3	orf6.4399	14502	MTLalpha1prom	MTLalpha1 promoter fragment	CHO339	CHO340	325
M15A8	orf6.7251	14496	RME1prom	RME1 promoter fragment	CHO355	CHO356	312
M15D8	orf6.7251	14496	RME1prom	RME1 promoter fragment	CHO355	CHO356	312
M15B6	orf6.4841	15619	SGA1prom	SGA1 promoter fragment	CHO384	CHO385	268
M15E6	orf6.4841	15619	SGA1prom	SGA1 promoter fragment	CHO384	CHO385	268
M15B7	orf6.2767	19689	SST2prom	SST2 promoter fragment	CHO386	CHO387	207
M15E7	orf6.2767	19689	SST2prom	SST2 promoter fragment	CHO386	CHO387	207
M15A10	orf6.2924	14450	STA1-1/ALS9prom	agglutinin-like protein promoter	CHO361	CHO362	252
M15D10	orf6.2924	14450	STA1-1/ALS9prom	agglutinin-like protein promoter	CHO361	CHO362	252
M15A11	orf6.6077	12969	STA1-2prom	STA1-2 promoter fragment	CHO363	CHO364	316
M15D11	orf6.6077	12969	STA1-2prom	STA1-2 promoter fragment	CHO363	CHO364	316
M15B8	orf6.2105	18702	STA1-3prom	STA1-3 promoter fragment	CHO388	CHO389	313
M15E8	orf6.2105	18702	STA1-3prom	STA1-3 promoter fragment	CHO388	CHO389	313
M15B9	orf6.7305	19059	STA1-5prom	STA1-5 promoter fragment	CHO390	CHO391	248
M15E9	orf6.7305	19059	STA1-5prom	STA1-5 promoter fragment	CHO390	CHO391	248
M15B1	orf6.4012	8944	STE2prom	STE2 promoter fragment	CHO371	CHO372	333
M15E1	orf6.4012	8944	STE2prom	STE2 promoter fragment	CHO371	CHO372	333
M15B2	orf6.6173	14995	STE3prom	STE3 promoter fragment	CHO373	CHO374	350
M15E2	orf6.6173	14995	STE3prom	STE3 promoter fragment	CHO373	CHO374	350
M15A9	orf6.1353	18185	STE4prom	STE4 promoter fragment	CHO392	CHO393	287
M15D9	orf6.1353	18185	STE4prom	STE4 promoter fragment	CHO392	CHO393	287

Table 4.2. Custom Promoter Primers

Table 4.3. Reporter Genes and Genetic Markers. The names of the reporter genes and genetic markers included in the array are listed along with a description and reference.

Name	Description	Reference
eGFP	enhanced green fluorescent protein	(15)
lacZ	*Streptococcus thermophilus* lacZ reporter coding sequence	(35)
myc-n	approximately 40 bp sequence encoding a myc epitope and linker sequences	(24)
HisG	1.1 kb bacterial HisG repeat flanking *URA3* in Ura Blaster cassettes	(17)
rhaB	bacterial gene flanking *URA3* in pDI-01 and in A. Uhl plasmid pAU71	(34)

Table 4.3. Reporter genes and genetic markers

Table 4.4. Annotation Fields for *C. albicans* DNA Microarrays. Annotation fields with data for each ORF were submitted to the Nomad database for convenient retrieval with microarray results files. The field names are described in the text.

Field Name:
Unique ID
Spot name
Spot description
Johnson Lab DB ID
Top yeast match ID
Top yeast match name
Yeast match e-value
Forward PCR primer name
Reverse PCR primer name
Forward PCR primer sequence
Reverse PCR primer sequence
Expected PCR size
PCR amplicon sequence
top orf6 name
top orf19 name
Candida DB orf19 name
Chromosome

Table 4.4. Annotation Fields

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Appendix A.

Preparation of a Universal Reference mRNA for expression profiling of *Candida albicans*

Acknowledgements

The preparation of this universal reference mRNA for *Candida albicans* genome expression profiling was a joint effort between Mike Lorenz of the laboratory of Gerald Fink (MIT) and me in the Johnson laboratory. I had a great deal of assistance from a wonderful technician, Josie Haduong who optimized our protocol for large-scale poly(A) RNA selection. Matt Miller and Ryan Raisner also contributed mRNAs for some of the growth conditions used in the universal reference pool.

Introduction

Genome-wide expression profiling measures the relative abundance of the each transcript present in the pool of mRNAs from a culture. Hybridization of these transcripts with a complementary spot on a microarray provides a read out or a transcriptional profile for a particular strain and growth condition. DNA microarray data is generally obtained by competitively hybridizing the differentially labeled mRNAs (Cy3 and Cy5) from two different samples (described in detail in Chapter 4). The signals from the Cy3 and Cy5 channels are collected and the ultimate data that is analyzed is the normalized ratios of the Cy5 over Cy3 transcript level for each spot. One problem with this method is that a transcript present in the Cy5 sample but not in the Cy3 sample will result in a fraction (the expression ratio) where the denominator is often close to zero. This situation can generate large variations from experiment to experiment and will often show up as "no data" in subsequent gene cluster analyses.

One solution used to address this problem is the use of a reference pool of mRNA from a wide variety of culture conditions (1, 2). Use of a pool of mRNAs from many growth conditions increases the likelihood that every transcript is represented in the pool and results in more spots on the microarray providing usable data. The universal reference is typically labeled with Cy3 and is competitively hybridized with Cy5 labeled experimental samples. Microarray expression ratios are calculated as the ratio of Cy5/Cy3, thus the universal reference pool is the denominator in the fraction. Since every sample is hybridized to a standard reference, experimental data from many experiments can easily be directly compared.

In conjunction with my efforts to manage the construction of the *C. albicans* DNA microarray, I collaborated with Mike Lorenz, at the time in the Fink laboratory (currently at University of Texas, Houston) to prepare a large quantity of a universal reference pool of mRNA for *C. albicans* expression profiling experiments. We prepared poly(A)-selected mRNA from a wide variety of growth conditions and included several time points or cell densities within each culture condition to maximize the representation of mRNAs present in the *C. albicans* genome. These conditions include early, mid, and late log phase cells growth in rich medium as well as low nutrient conditions such as growth on a poor carbon sources or in low nitrogen media. The culture conditions and amounts of mRNA harvested by the Johnson and Fink laboratories are listed in Table A.1. This universal reference mRNA was produced in June 2001 and has remained stable at –80° for over two and a half years. This universal reference pool is a useful reagent for transcriptional profiling of *C. albicans* and has been used successfully in numerous microarray experiments in both the Johnson and Fink laboratories, including those I performed in Chapter 3 and Appendix C.

Materials and Methods

Strains

Two wild-type strains were used in these experiments; SC5314 was used in the Fink laboratory and CAF2-1 was used in the Johnson laboratory (see chapter 3, Table 3.1 for strain pedigree).

Growth conditions

For most of the experiments, cells were grown overnight in the medium indicated in Table A.1 and diluted to either OD_{600} 0.1 for the log growth experiments or to OD_{600} 1.0 for the hyphal inducing medium (serum, Spider and Lee's pH 6.8) with the following exceptions: Lee's pH 4.5 medium was used for overnight cultures for both the pH 4.5 and pH 6.8 growth conditions, YPD medium was used for overnight growth and subsequent dilution into maltose and Spider medium and YPD was used in the YPD plus 10% serum time courses. Approximately half of the cultures and conditions were grown in the Fink laboratory (see the bottom half of Table A.1). In these cultures, YNB medium was used for overnight culture and dilution into YNB and YNB with acetate and glycerol as alternative carbon sources or into SLAD (synthetic low ammonium dextrose) medium. Media 199 was prepared according to the manufacturer's directions and brought to pH 4.5 and pH 8.0. pH 4.5 was used for overnight growth and for dilution into flasks containing pH 4.5 or pH 8.0 media.

Cell harvesting and RNA preparation

Cells were harvested by centrifugation at 6500 rpm at the time points and cell densities listed in Table A.1. Pellets were "snap" frozen with liquid nitrogen and stored at –80° C. Frozen pellets were thawed and total RNA was isolated as described in Chapter 3, Materials and Methods.

Poly(A) mRNA selection

Poly(A) mRNA was selected using oligo-dT cellulose and Bio-Rad columns essentially as described in the protocol at www.microarrays.org. 4mg of total RNA was used in each poly-A selection. Typical mRNA yields were 1% of the total RNA.

Reference mRNA pools

The mRNA concentrations and OD_{260}/OD_{280} were measured by spectraphotometry. The combined yield of mRNA from all the growth conditions listed in Table A.1 was just over 7 mg. The individual pools of mRNA from each condition were combined in one tube and diluted to a final concentration of 1 mg/mL. For unexplained reasons, the calculated yield from the mixed pool of samples totaled approximately 10 mg. The pooled reference mRNA was then transferred in 100 μL aliquots to screw-cap tubes and stored at –80° C. The aliquots were then divided evenly between the Johnson and Fink laboratories.

Results

Over 7 mg of poly(A)-selected mRNA from more than 25 different culture conditions was pooled to create a universal mRNA reference as a tool for genome-wide expression profiling in *C. albicans*. We used a variety of standard growth conditions and temperatures and took time points at early, middle, and late growth phases (listed in Table A.1). Multiple repeats of many growth conditions were required to obtain sufficient quantities of RNA for poly(A)-selection. Over one hundred poly-A isolation

reactions were used to generate the final pool of universal reference mRNA. This reference mRNA is of high quality and is consistently reverse transcribed and labeled with Cy3 or Cy5 in microarray experiments.

Hybridization of this reference mRNA with itself shows that at least 90% of spots on the microarray hybridize to this pool (unpublished data). The majority of spots that fail to hybridize correspond to unknown genes that may either be pseudogenes or false ORFs that were identified by our ORF-finding program (discussed in chapter 4). It is also possible that some genes may not be expressed at high enough levels under any of the growth conditions used to generate the universal reference pool.

Summary and Conclusions

Through our combined efforts, we generated enough reference mRNA for over 2000 microarray hybridizations. Use of this mRNA provides a common baseline for comparing the data from experiments performed at different times and in different laboratories. This reference pool has been used for *C. albicans* expression profiling in both the Johnson and Fink laboratories and was used in the expression profiling experiments that I presented in Chapter 3 and in Appendix C. This experimental tool has proven to be a useful reagent for both large-and small-scale expression profiling and serves as a reliable control for cDNA synthesis and labeling for microarray experiments in our laboratory.

Table A.1. Culture conditions for mRNAs pooled to generate a universal reference for genome-wide expression profiling of *C. albicans*. The medium, growth temperature, culture density or time point, or stress condition is listed under "growth condition". The amount of poly(A)-selected mRNA for each individual time point for each condition is indicated under "µg added" along with the percentage of each sample represented in the total pool. "Condition total" lists the total amount of mRNA grouped by condition along with the percentages for each major condition in the universal reference mRNA pool. Major conditions are indicated by the alternated shading pattern. The CAF2-1 strain was used in the cultures grown in the Johnson laboratory and strain SC5314 was used for cultures grown in the Fink laboratory. The combined yield was greater than 7 mg of poly(A)-selected mRNA or enough for more than 2000 microarray hybridizations.

	Growth condition	µg added	individual sample %	condition total (µg)	condition %
Johnson mRNAs:	YPD 30° OD=1	250	3.6%	1000	14.3%
	YPD 30° OD=5	250	3.6%		
	YPD 30° OD=10	250	3.6%		
	YPD 30° OD=20	250	3.6%		
	YPD, 37° OD=1	222.6	3.2%	907.8	13.0%
	YPD, 37° OD=2	185.2	2.6%		
	YPD, 37° OD=5	250	3.6%		
	YPD, 37° OD=10	250	3.6%		
	YPD+10% serum, 30° t=2 hr	114.97	1.6%	386.23	5.5%
	YPD+10% serum, 30° t=4 hr	100.74	1.4%		
	YPD+10% serum, 30° t=6 hr	111.18	1.6%		
	YPD+10% serum, 30° t=16.5 hr	59.34	0.8%		
	YPD+10% serum, 37° t=1hr	84.22	1.2%	486.7	6.9%
	YPD+10% serum, 37° t=2hr	104.96	1.5%		
	YPD+10% serum, 37° t=4hr	183.32	2.6%		
	YPD+10% serum, 37° t=6hr	73.3	1.0%		
	YPD+10% serum, 37° t=16hr	40.9	0.6%		
	Lee's pH 4.5, 30° t=2hr	68.92	1.0%	68.92	1.0%
	Lee's pH 6.8, 30° t=2hr	49.58	0.7%	49.58	0.7%
	Spider, 30° t=2hr	60.42	0.9%	154.92	2.2%
	Spider, 37° t=2hr	94.5	1.3%		
	YPD+2% maltose, 30° t=2hr	137.84	2.0%	137.84	2.0%
	YPD, 42° heat shock t=10', 20', 30'	79.46	--	79.46	1.1%
	YPD, 50° heat shock t=10', 20', 30'	628	--	628	9.0%
Fink mRNAs:	YNB-Acetate, OD 1	25	0.4%	178	2.5%
	YNB-Acetate, OD 2	108	1.5%		
	YNB-Acetate, OD 3.5	45	0.6%		
	YNB-glycerol, OD 0.8	178	2.5%	373	5.3%
	YNB-glycerol, OD 3	169	2.4%		
	YNB-glycerol OD ~4	26	0.4%		
	YPD +1.5M NaCl, OD1	62	0.9%	213	3.0%
	YPD +1.5M NaCl, OD1.4	85	1.2%		
	YPD +1.5M NaCl, OD4.4	66	0.9%		
	SLAD, OD 1	239	3.4%	292	4.2%
	SLAD, OD 1.5	25	0.4%		
	SLAD, OD 2.4	28	0.4%		
	YNB, OD 1	500	7.1%	1049	15.0%
	YNB, OD 5	500	7.1%		
	YNB, OD 9	49	0.7%		
	Media 199, pH4.5 (1 hr, 5 hr, 24 hr)	1000	--	1000	14.3%
	Media 199, pH8.0 (1 hr, 5 hr, 24 hr)				
	poly(A)selected mRNA TOTAL	7004.5	100.0%	7004.5	100.0%

Table A.1. Percentage of each growth condition present in the universal reference mRNA pool.

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Appendix B.

Staining the nucleolus of *Candida albicans*

Introduction

The microarray analysis presented in Chapter 3 revealed that a large number of genes involved in ribosome biogenesis and nucleolar functions showed decreased expression. This suggests that *ash1* mutants may have differences in nucleolus. To test whether differences in nucleolar function could be observed by cell staining, I used an antibody directed against the Nop1 protein of *S. cerevisiae*. The Nop1 proteins of *S. cerevisiae* and *C. albicans* are highly conserved (BLASTp score 455. expect value e-129) and were predicted to cross-react with *C. albicans* Nop1. *C. albicans* was grown in the yeast form of growth using M199 pH 4.5 medium at 30° C. To induce hyphal development, yeast form cells in the stationary phase were diluted into M199 pH 7.0 medium at 37° C.

Materials and Methods

Strains and growth conditions

Lee's pH 4.5 medium at 30° C was used for growth of yeast cells. Overnight cultures grown in M199 pH 4.5 medium at 30° C were diluted into M199 pH 7.0 at 37° C to induce filamentous growth. The yeast form cells were harvested at OD_{600} 1.0 and the hyphal cells were harvested after 2 hrs of growth in serum medium. Between 5 and 10 mLs of cells were transferred to 15 mL Falcon tubes and fixed for 1 hour in the growth medium with 0.7 mLs of 37% formaldehyde for every 5 mL of cells. The cells were pelleted and washed three times before resuspension in sterile PBS.

Indirect immunofluorescence

Cells were prepared for antibody stain as described in Chapter 2 Materials and Methods. The mouse monoclonal anti-Nop1 antibody (MCA-28F2, EnCor Biotechnology, Alachua, FL) was diluted in PBS with 1% BSA to a concentration of 1:400 for the hyphal cells and 1:800 for the yeast form cells. A goat anti-mouse secondary antibody (Oregon Green-488 #O6380, Molecular Probes, Eugene OR) was diluted 1:100 in PBS with 1% BSA.

Microscopy

The images of hyphal cells were taken in the Cyster laboratory with a Leica DMLB microscope. The yeast cell images were obtained using the Guthrie lab microscope. All images were taken with 100X objective lenses. The images were reduced by 60% in Adobe Photoshop for presentation.

Results

The nucleolus is the location of rDNA, rRNA synthesis and processing, and ribosomal subunit assembly. Changes in nucleolar function and structure have been linked to rDNA silencing and aging in *S. cerevisiae* (1-3). Microarray analysis of *C. albicans ash1* mutants showed that expression of a large number of nucleolar genes, that function in rRNA processing and ribosome biogenesis, were reduced relative to wild-type expression levels (chapter 3). We, therefore, tested whether changes in the nucleolus could observed by comparing the staining pattern of wild-type and *ash1* mutant cells using a highly conserved nucleolar protein, Nop1.

The anti-body Nop1 antibody, produced against the *S. cerevisiae* protein, cross-reacts with *C. albicans* cells and produces a crescent-shaped staining pattern in yeast form cells (Figure B.1). This pattern is similar to what is observed in *S. cerevisiae*. The nucleolus is usually observed at the periphery of one end of the DAPI-staining nucleus. The nucleolar staining pattern of *C. albicans* wild-type and *ash1* mutant cells in the hyphal form are shown in Figures B.2 – B.4 at various stages of development. Overall, the staining pattern of *ash1* mutants and the wild-type add-back strain, YDI-199 (see Materials and Methods), appear similar.

Figure B.2 shows wild-type cells during the first two rounds of hyphal cell division. In Figure B.2 A, cell division has just been completed in the germ tube and the mother cell nucleus is migrating back towards the mother cell body. Figure B.2 B shows a similar mother and hyphal daughter cell pair with the migration of the mother cell nucleus is slightly farther along. A small nucleous is visible in both mother and daughter cells. In Figure B.2 C, the mother cell nucleus has completed its migration back into the

mother cell body. In both cells, a bright nucleolar stain is observed. The *ash1* mutant cells shown in Figure B.3 B are comparable in stage of hyphal development although the daughter-cell nucleolus of the *ash1* mutant appears smaller than wild-type in this particular example. Other examples, such as the wild-type nucleoli shown in Figure B.2 B, also appear small. The size of the nucleoli in hyphal cells grown in M199 are typically smaller than when YPD with serum is used as the hyphal-inducing medium.

Discussion

These results show that the pattern of nucleolar stain visualized with the anti-Nop1 antibody are similar between a wild-type reintegrant strain and an *ash1* mutant strain of *C. albicans*. We did not observe any gross morphological differences between these strains, therefore, we conclude that down-regulation of nucleolar genes in *ash1* mutants effects some aspect of nucleolar function other than morphology or inheritance. The wild-type strain used in these experiments was derived from the *ash1* mutant strain. It has one functional copy of *ASH1* tagged with myc6 epitope. It is formally possible that this strain is only partially complemented for Ash1 function and thus, the nucleolar staining pattern appears similar to *ash1* mutants. However, I briefly examined the Nop1 staining pattern of another wild-type strain, CAF2-1 (data not shown) and they appeared similar to the YDI-199 cells used in this experiment.

Figure B.1. Staining pattern of wild-type (YDI-199) yeast form cells stained with anti-Nop1 antibodies. Nop1 stain is on the left, DAPI stained nuclei are shown on the right. Arrowhead indicates a cell in a position that shows the crescent-shaped nucleolar staining pattern similar to *S. cerevisiae*.

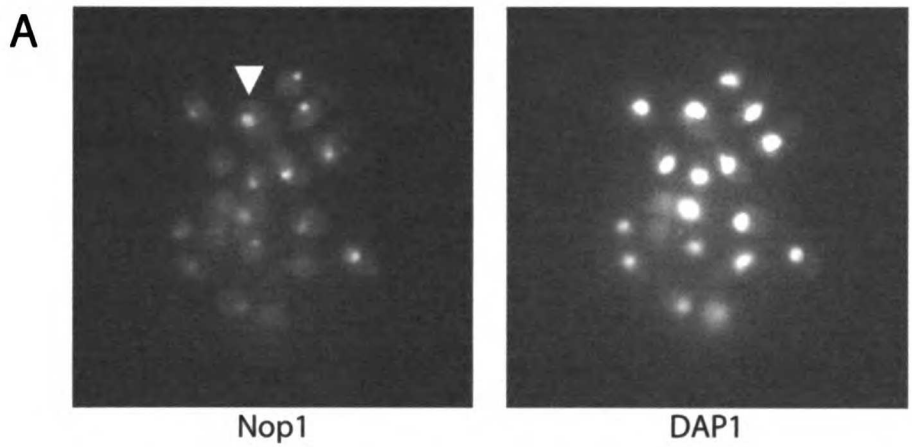

Figure B.1 Nop1 staining pattern of wild-type strain, YDI-199 .

Figure B.2. Nucleolar staining pattern of wild-type strains during the first two rounds of hyphal cell division. Cells were grown in M199 pH 7.0 medium for two hours at 37° C. (A) A mother and daughter cell that just completed cell division in the germ tube. The mother cell nucleus is migrating back towards the mother cell body. (B) Migration of the mother cell nucleus is slightly farther along than in A. A small nucleous is visible in both mother and daughter cells. (C) A mother cell with a mother cell nucleus that has completed its migration back into the mother cell body. Both mother and daughter cell show a bright nucleolar stain. Septa, the sites of cell division, are indicated with an arrowhead.

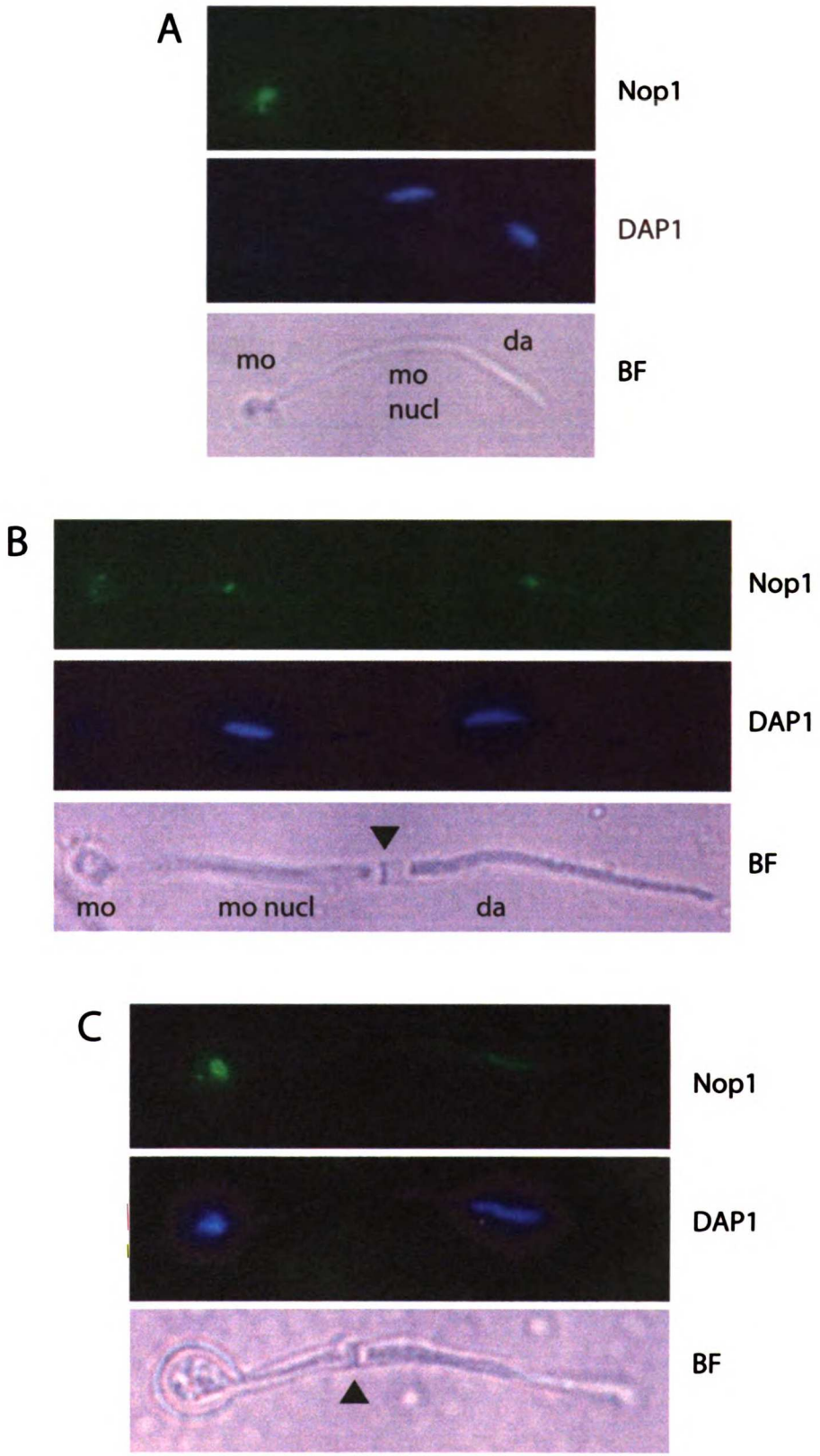

Figure B.2 Nucleolar staining pattern of wild-type strain, YDI-199 in the two-cell stage of hyphal development.

Figure B.3. Nucleolar staining pattern of *ash1* mutants during the two-cell stage of hyphal development. (A) An elongated mother cell nucleus prior to cell division within the growing germ tube. (B) The mother cell nucleus has re-entered the mother cell body and appears similar to the wild-type pattern observed in Figure B.2 C. Arrowhead indicates a small dot that is detached from the main nucleolar structure at the leading edge of the tip-migrating daughter cell nucleus.

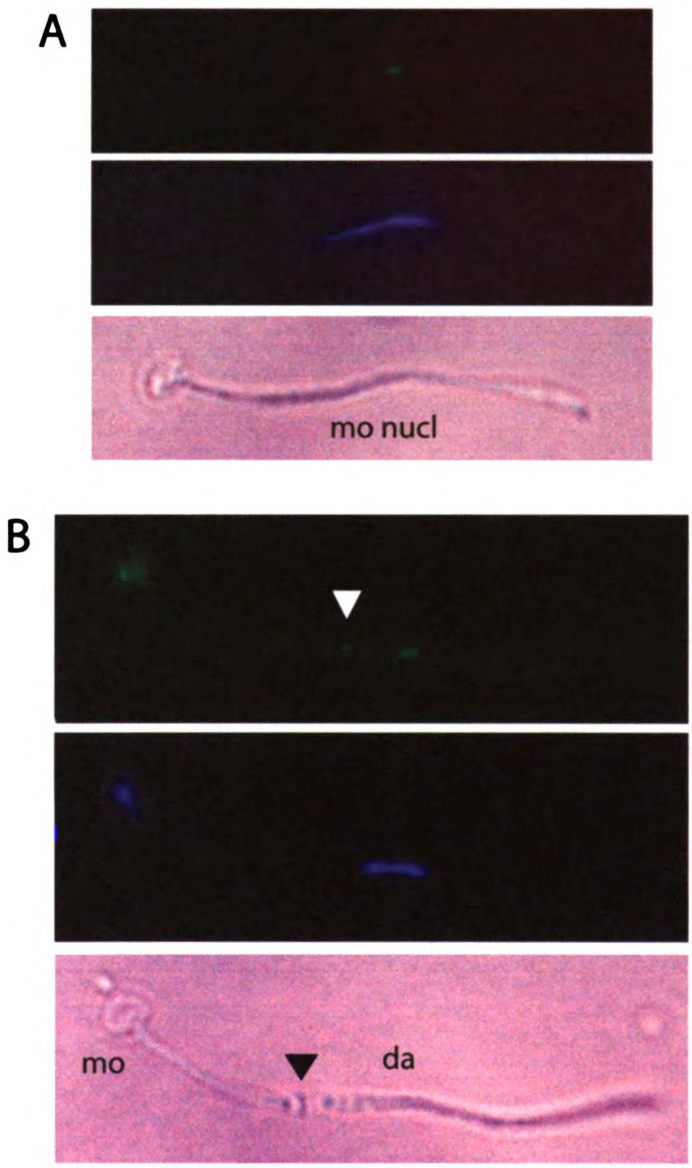

Figure B.3. Nucleolar staining pattern of ash1 mutants during the two-cell stage of hyphal development.

Figure B.4. Nucleolar stain of *ash1* mutant strain in the three-cell stage of hyphal development. An additional daughter cell is also budding from the original mother cell. (A) The Nop1 stain is absent from the highly vacuolated sub-apical cell but is present in the hyphal tip cell, the original yeast form mother cell, and the second daughter cell budding from the original mother cell.

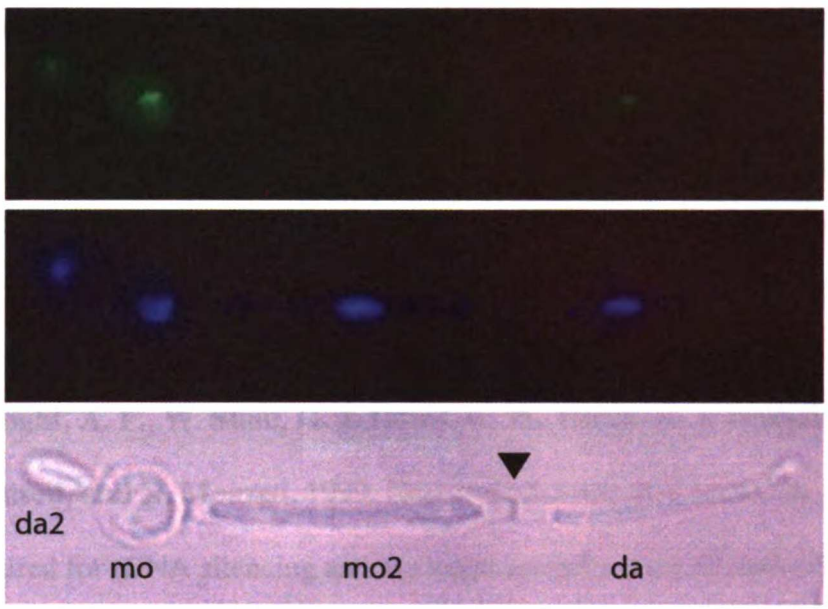

Figure B.4. ash1 mutant strain at the three-cell stage of hyphal development.

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Appendix C.

Candida albicans response regulator gene *SSK1* regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress

Acknowledgements

The construction of a DNA microarray for *C. albicans* brought with it the opportunity to share this valuable research tool with others in the research community. The paper presented in this appendix is the result of a productive collaboration with Neeraj Chuajan and Richard Calderone at Georgetown University. I performed the microarray hybridizations with technical assistance from Bethann Hromatka in the Johnson laboratory and I performed the analysis of the microarray data. The results of these microarray experiments nicely complemented the rest of the experiments in the paper which were the work of Neeraj Chuajan and colleagues. Permission to reprint this publication from Eukaryotic Cell was generously granted by ASM Journals.

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Candida albicans Response Regulator Gene *SSK1* Regulates a Subset of Genes Whose Functions Are Associated with Cell Wall Biosynthesis and Adaptation to Oxidative Stress

Neeraj Chauhan,[1] Diane Inglis,[2] Elvira Roman,[3] Jesus Pla,[3] Dongmei Li,[1] Jose A. Calera,[4] and Richard Calderone[1]*

Department of Microbiology and Immunology, Georgetown University School of Medicine, Washington, District of Columbia 20057[1]; Department of Microbiology and Immunology, University of California at San Francisco, San Francisco, California[2]; and Departamento de Microbiología II, Universidad Complutense de Madrid, Madrid,[3] and Departamento de Microbiología, Universidad de Salamanca, Salamanca,[4] Spain

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Ssk1p of *Candida albicans* is a putative response regulator protein of the Hog1 two-component signal transduction system. In *Saccharomyces cerevisiae*, the phosphorylation state of Ssk1p determines whether genes that promote the adaptation of cells to osmotic stress are activated. We have previously shown that *C. albicans SSK1* does not complement the *ssk1* mutant of *S. cerevisiae* and that the *ssk1* mutant of *C. albicans* is not sensitive to sorbitol. In this study, we show that the *C. albicans ssk1* mutant is sensitive to several oxidants, including hydrogen peroxide, *t*-butyl hydroperoxide, menadione, and potassium superoxide when each is incorporated in yeast extract-peptone-dextrose (YPD) agar medium. We used DNA microarrays to identify genes whose regulation is affected by the *ssk1* mutation. RNA from mutant cells (strain CSSK21) grown in YPD medium for 3 h at 30°C was reverse transcribed and then compared with similarly prepared RNA from wild-type cells (CAF2). We observed seven genes from mutant cells that were consistently up regulated (three-fold or greater compared to CAF2). In *S. cerevisiae*, three (*AHP1*, *HSP12*, and *PYC2*) of the seven genes that were up regulated provide cells with an adaptation function in response to oxidative stress; another gene (*GPH1*) is regulated under stress conditions by Hog1p. Three other genes that are up regulated encode a cell surface protein (*FLO1*), a mannosyl transferase (*MNN4-4*), and a putative two-component histidine kinase (*CHK1*) that regulates cell wall biosynthesis in *C. albicans*. Of the down-regulated genes, *ALS1* is a known cell adhesin in *C. albicans*. Verification of the microarray data was obtained by reverse transcription-PCR for *HSP12*, *AHP1*, *CHK1*, *PYC2*, *GPH1*, *ALS1*, *MNN4-4*, and *FLO1*. To further determine the function of Ssk1p in the Hog1p signal transduction pathway in *C. albicans*, we used Western blot analysis to measure phosphorylation of Hog1p in the *ssk1* mutant of *C. albicans* when grown under either osmotic or oxidative stress. We observed that Hog1p was phosphorylated in the *ssk1* mutant of *C. albicans* when grown in a hyperosmotic medium but was not phosphorylated in the *ssk1* mutant when the latter was grown in the presence of hydrogen peroxide. These data indicate that *C. albicans* utilizes the Ssk1p response regulator protein to adapt cells to oxidative stress, while its role in the adaptation to osmotic stress is less certain. Further, *SSK1* appears to have a regulatory function in some aspects of cell wall biosynthesis. Thus, the functions of *C. albicans SSK1* differ from those of *S. cerevisiae SSK1*.

Candidiasis is among the most common nosocomial diseases in the United States, occurring in ~8 to 10% of high-risk patients (7, 8, 50). The disease has a high attributable mortality, most often because detection of the organism occurs late in the course of the disease or because therapy fails. Treatment with amphotericin B invariably results in toxicity to the patient, often necessitating withdrawal of the drug. The popularity of certain of the azole antifungals has been tempered by the development of resistance, especially among the nonalbicans species of *Candida*, such as *Candida glabrata*, *Candida krusei*, and *Candida dubliniensis* (18).

The virulence of *Candida albicans* appears to be multifactorial and includes the requirement for yeast-hyphal transition (morphogenesis), which is probably needed for invasion (9, 32), although direct persorption of yeast cells by mucosal cells has also been observed to precede blood-borne invasion (17). Virulence is also dependent upon the expression of cell surface adhesins that promote host cell recognition, as well as extracellular enzymes, such as phospholipases and proteases (9, 32).

While much is known about the virulence factors of *C. albicans*, its adaptation to stress conditions in the host, for example, oxidative stress following phagocytosis, is less understood. On the other hand, considerable effort has been devoted to characterizing the role of neutrophils (polymorphonuclear leukocytes [PMNs]) and macrophages, whose oxidative activities in protection against candidiasis are well known (43, 45–49, 52, 53). The importance of these cells in protection is reflected in the increased susceptibility of patients to candidiasis due to either defects in neutrophil function or reduction in their total number (neutropenia) (8). In vitro killing of *C. albicans* is increased if neutrophils are activated or if the organism is opsonized (46, 48). Wysong et al. (53) have demonstrated that

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Georgetown University School of Medicine, 312 SE Med-Dent Building, 3900 Reservoir Rd., NW, Washington, DC 20057. Phone: (202) 687-1137. Fax: (202) 687-1800. E-mail: calderor@georgetown.edu.

a respiratory burst by neutrophils is Ca^{2+} dependent only if hyphae are opsonized. Neutrophil killing is less efficient in vitro if cell wall mannan is included in assays, but the active component of mannan that inhibits phagocytic killing is unknown (52). Reactive oxygen species (ROS), which appear to be candicidal alone or in combination, include hydrogen peroxide, superoxides, hydroxy radicals, and peroxynitrites (47). Nonoxidative components include defensins, small peptides with a broad spectrum of antimicrobial activity, and other proteins associated with specific granules of neutrophils (48). Survival of the organism following phagocytosis by PMNs can occur, but the mechanism(s) that promotes its escape from oxidative killing is unknown.

One of the most important ways in which prokaryotes and lower eukaryotes, such as yeasts and other fungi, adapt to stress conditions is through two-component signal transduction (2, 5, 9, 10, 26, 28, 32). For example, the HOG (hyperosmotic glycerol) MAP kinase two-component signal pathway regulates growth of *Saccharomyces cerevisiae* when cells are confronted with high-osmotic conditions (23, 37). The HOG MAP kinase pathway consists of a sensor, a transmembrane histidine kinase (Sln1p), a phosphohistidine intermediate protein (Ypd1p), and a cytoplasmic response regulator protein (Ssk1p). The phosphorylation state of Ssk1p determines whether the HOG MAP kinase pathway is activated, and glycerol synthesis is induced to adapt cells to osmotic stress. In *S. cerevisiae*, *ssk1* mutants are deficient in growth on high-osmotic media, but the *C. albicans* *SSK1* gene does not complement that mutant, indicating that perhaps the *SSK1* of *C. albicans* is functionally divergent (14). In *C. albicans*, two-component homologues of the HOG pathway (*SLN1*, *YPD1*, *SSK1*, and *HOG1*) have been identified, and strains with *SLN1*, *SSK1*, and *HOG1* deleted are defective in morphogenesis and are attenuated or avirulent (2, 3, 9, 14–16, 31, 54). Other histidine kinases found in *C. albicans* and *Aspergillus fumigatus* have been characterized, and their functions in morphogenesis, virulence, phenotypic switching, and cell wall biosynthesis have been established (1, 9–13, 31, 38, 40, 44, 54). However, the *SLN1* and *SSK1* mutants of *C. albicans* are only partially sensitive to hyperosmotic conditions (15, 31). Therefore, in this study we determined the sensitivity of the *ssk1* mutant to a variety of stress conditions and inhibitors, including antifungal drugs; ROS, such as H_2O_2; and heat shock. We show that *C. albicans* *SSK1* contributes to the adaptation of cells to oxidant stress. Also, microarray data support a role for *SSK1* in the expression of cell wall structural or enzymatic proteins.

MATERIALS AND METHODS

Strains, media, and culture conditions. The following *C. albicans* strains were used in this study: CAF2 (*Δura3::imm434/URA3*) (21), CSSK21 (*Δura3::imm434/Δura3::imm434 Δssk1::hisG/Δssk1::hisG-URA3-hisG*) (15), and CSSK23 (*Δura3::imm434/Δura3::imm434 Δssk1::hisG SSK1::URA3-hisG*) (15). Strains with genes deleted were constructed by the "urablaster" procedure (21) and have been described previously (15). All strains were maintained as frozen stocks and then cultured on YPD (1% yeast extract, 2% glucose, and 2% peptone) agar prior to use.

H_2O_2 and drug sensitivity assay. The sensitivities of *C. albicans* strains CAF2, CSSK21, and CSSK23 (a gene-reconstituted strain) to oxidants were assayed by spotting dilutions of 5×10^1 to 5×10^5 cells (each in a total volume of 5 μl) from an overnight culture of yeast cells grown in YPD broth at 30°C onto YPD agar plates containing H_2O_2, menadione, *t*-butyl hydroperoxide, and KO_2. The growth of each strain was examined after 48 h. The sensitivities of CSSK21, CSSK23, and CAF2 to antifungal drugs (miconazole, amphotericin B, nikkomycin Z, and caspofungin), calcofluor white, and caffeine were also evaluated.

TABLE 1. Primer sequences for RT-PCR experiments

Primer	Sequence (5′-3′)
HSP12 5′	AGAGTCAATTTCTCTAGTAGAGGT
HSP12 3′	AACTCCACTCACGTATTCAGCA
FLO1 5′	AGTGCATTATTTGCTGCTACC
FLO1 3′	AGCATGACAAGCATCATTATGA
MNN4-4 5′	AGTTGAAAATCCAAGTGAAGGATACGG
MNN4-4 3′	CAAAATCATGGAATCTTGTCAACGATCTC
CHK1 5′	TCCCCAAATGCCTGAAATGGAC
CHK1 3′	GCTACGATCACGGGTAACCAAC
AHP1 5′	ATGTCATTTAAAGAAGGTTCC
AHP1 3′	CTTGGCTAAAACAGCATCAAT
PYC2 5′	GAAGGTGATGATATTGAAGAT
PYC2 3′	GACCAACATAGAATCATAATG
ALS1 5′	CAGGATACCCAACTTGGAAT
ALS1 3′	CCAGTATTCAGTAGTAGTGA
GPH1 5′	GGATTATCTTACCCCATCAACT
GPH1 3′	GTCATTTGGATACAACACTGA
STL1 5′	AGAACCACAACTGCTGGATTA
STL1 3′	AGCTTCAGCAACAATAGCATC
ACTIN 5′	GACGGTGAAGAAGTTGCTGC
ACTIN 3′	CAAACCTAAATCAGCTGGTC

Heat shock experiments. Heat shock experiments were performed as described by others (33, 34). Exponentially grown cells were diluted to 10^4 to 10^7 per ml in phosphate-buffered saline–Tween buffer (0.05 M Tris, pH 7.5). At each dilution CAF2, CSSK21, and CSSK23 cells were incubated at either 30 or 42°C for 30 min; subsequently, dilutions were plated on YPD agar and cultured at 37°C. The number of CFU was then determined for each strain after 48 h of incubation.

Microarray analysis. The *C. albicans* strains CAF2 and CSSK21 were inoculated into YPD medium and grown to a final concentration of 10^7 cells/ml, transferred to fresh YPD medium, grown at 30°C for 3 h to a constant optical density, and harvested by centrifugation. Total RNA was isolated according to the standard protocols (39, 41). Microarray analysis was repeated three times, each with different RNA extracts of cells; 1 mg of total RNA from each sample was used for poly(A) selection with an Oligotex kit from Qiagen (Chatsworth, Calif.).

Protocols and material source information for the microarray experiments were obtained from http://www.microarrays.org. DNA microarrays were fabricated at the University of California at San Francisco by spotting *C. albicans* (strain 5314) open reading frames (ORFs) that were derived from PCR products onto glass slides. Approximately 6,000 ORFs were screened. Four micrograms of mRNA from each strain was used for cDNA synthesis in the presence of aminoallyl dUTP. cDNA was then labeled with Cy5. For each experiment, 4 μg of reference mRNA (REF) derived from pooled CAF2 cells grown under a variety if growth conditions (D. Inglis and M. Lorenz, unpublished data) was labeled with Cy3. The Cy3- and Cy5-labeled samples were hybridized to the array in 50% formamide, 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.4 μg of poly(A) DNA/μl, and 0.1% sodium dodecyl sulfate (SDS) at 44°C. The microarrays were scanned with a GenePix 4000 microarray scanner (Axon Instruments Inc., Foster City, Calif.). Genes were assigned to the resulting microarray image with GenePix Pro version 3.0 software. Data were uploaded to the database program NOMAD (available at http://derislab.ucsf.edu/), which also normalized the data over the entire array. To analyze the mutant (CSSK21)/wild-type (CAF2) ratio, the values for *cssk1*/REF were divided by the values for CAF2/REF. The normalized ratios were filtered, and a self-organizing map for genes was created using the Cluster software program (19). The hierarchical cluster was viewed with TreeView (19). We used a ≥3-fold increase in the gene expression of strain CSSK21 to identify up-regulated genes compared to CAF2. Up-regulated genes were ≥4-fold in two experiments, 4-fold in three experiments, 3-fold in four experiments, and 3-fold in three experiments.

RT-PCR. Total RNA was extracted as described above from strains CAF2, CSSK21, and CSSK23 grown in YPD broth at 30°C for 3 h. Primers for all PCR amplifications are indicated in Table 1. Actin expression served as an internal control. RT-PCR was performed by using the Qiagen one-step PCR kit; 1 μg of total RNA was used for each PCR. All PCR products were resolved on 1% agarose gels, which were then scanned using an Alpha Imager 2000 (Alpha

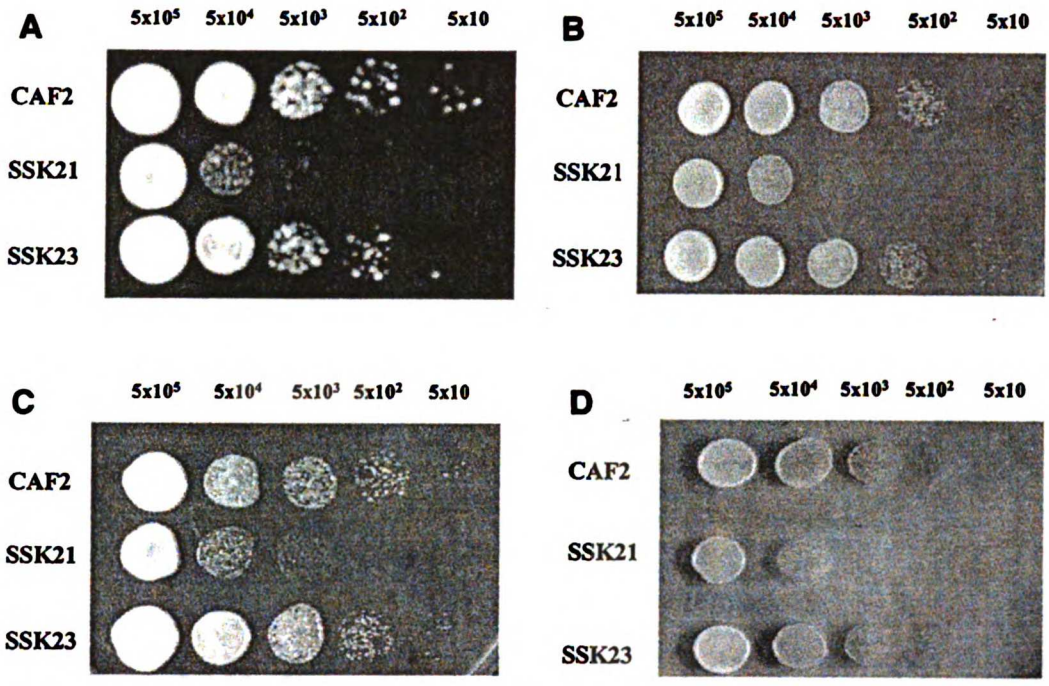

FIG. 1. Growth of CAF2 (wt), SSK21 (*ssk1/ssk1*), and SSK23 (*ssk1/SSK1*) at 30°C for 48 h on YPD agar containing (A) 0.1 mM menadione, (B) 1.0 mM KO_2, (C) 1 mM *t*-butyl hydroperoxide, and (D) 5 mM H_2O_2. Five microliters of cell dilutions (5×10^5 to 5×10^1 cells) was spotted on YPD agar containing each compound.

Innotech Corp.), imported as TIFF files, and evaluated for differential expression.

Phosphorylation of Hog1p assay. Yeast strains (CAF2 [*SSK1* Ura$^+$], CSSK21 [*ssk1* Ura$^+$ mutant], and CSSK23 [Ura3$^+$; SSK1 reconstituted]) were grown in YPD medium supplemented with 20 mg of uridine/ml at 37°C. Overnight cultures were suspended in prewarmed YPD medium to an optical density at 600 nm of 0.05, and experiments were performed when the cultures reached an optical density at 600 nm of ~1.0. Under these conditions, the culture was challenged with 1.5 M NaCl or 10 mM hydrogen peroxide (final concentrations). Samples were taken after the addition of each stimulus at different times (0 to 60 min), and cell extracts were obtained using glass beads in a fast-prep cell breaker as previously indicated (2, 30). Similar amounts of proteins, as determined by absorbance at 280 nm and SDS-polyacrylamide gel electrophoresis–Coomasie Blue staining, were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nylon membranes (Boehringer Mannheim, Mannheim, Germany) following standard procedures (2). The blots were first probed with an ScHog1p polyclonal antibody (anti-ScHog1; Santa Cruz Biotechnology). Subsequently, the blots were stripped and reacted with a phospho-p38 MAP kinase (Thr180/Tyr182) 28B10 monoclonal antibody (anti-TGY^P; Cell Signaling Technology, Inc.). Blots with anti-ScHog1p or anti-TGY^P were developed according to the conditions recommended by the manufacturer using the Hybond ECL kit (Amersham Pharmacia Biotech).

RESULTS

Sensitivity of the *ssk1* mutant of *C. albicans* to inhibitors and heat shock. The reference strain CAF2 (*SSK1 URA3*), the mutant strain CSSK21 (*ssk1 URA3*), and the reconstituted strain CSSK23 (*SSK1 URA3*) were incubated at 30°C for 48 h on YPD agar containing 0 to 8 mM H_2O_2 and 0 to 1.0 mM menadione (which generates superoxide), *t*-butyl hydroperoxide, or KO_2. We found that, compared to CAF2 and CSSK23, strain CSSK21 was sensitive to H_2O_2 at concentrations as low as 5 mM, as well as 0.1 mM menadione, *t*-butyl hydroperoxide, or KO_2 (Fig. 1). The inhibition of CSSK21 was also observed by incubating cells with the same concentrations of oxidants described above for 1 h and then culturing the cells on YPD agar (data not shown). No differences between the sensitivities of strain CSSK21 and those of strains CSSK23 and CAF2 were observed with amphotericin B, miconazole, nikkomycin Z, calcoflour white, caspofungin, or caffeine (data not shown). Heat shock experiments were conducted with CAF2, CSSK21, and CSSK23. We observed that only the viability of the *ssk1* mutant strain (CSSK21) was reduced when cells were incubated at 42°C for 30 min and then cultured at 37°C. The reduction in growth was >2 log units in CSSK21 (data not shown).

Gene array studies. In order to determine the effect of the *SSK1* deletion on gene expression, we performed DNA microarray analysis experiments using RNA preparations of the *ssk1* mutant and wild-type cells (CAF2). Cells of each strain were grown at 30°C for 3 h in YPD broth, and RNA was isolated, reverse transcribed, and hybridized to SC5314 DNA ORFs. Analysis was done with a total of four RNA preparations from four cell preparations. The genes with the greatest amount of up regulation appear red, and those most down regulated appear green. A total of seven genes were up regulated at least three- to fourfold for all experiments with the *ssk1* mutant (Fig. 2 and Table 2). Annotation of the up-regu-

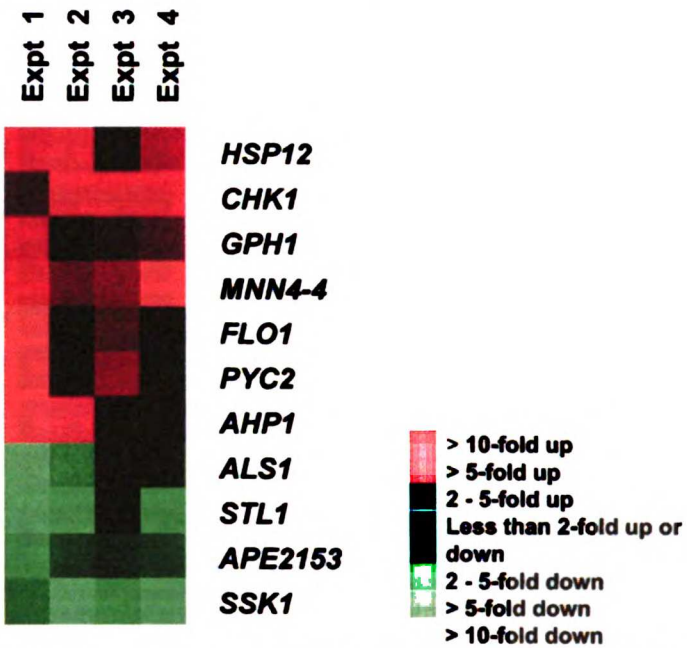

FIG. 2. Microarray analysis of genes up (red) and down (green) regulated in strain SSK21 compared to wild-type (CAF2) cells. A total of four microarray experiments (Expt) were done.

lated genes was performed using the *Saccharomyces* genome database (www.yeastgenome.org) or, when possible, the *C. albicans* genolist.pasteur.fr/CandidaDB website. We determined that three of the seven genes that were up regulated provide stress adaptation functions in *S. cerevisiae*. Those genes included *AHP1* (4.89-fold), *HSP12* (3.8-fold), and *PYC2* (3.6-fold). A fourth up-regulated gene (*GPH1*) encodes a glycogen phosphorylase, which is regulated via the Hog1p pathway during osmotic shock (3.2-fold) (51). The three other up-regulated genes, *CHK1* (4.37-fold), *FLO1* (4.4-fold), and *MNN4-4* (4.44-fold), either encode cell wall biosynthetic enzymes (*MNN4-4*), regulate cell wall biosynthesis (*CHK1*) (27), or encode a cell surface protein (*FLO1*). As for the genes encoding a cell wall function, we observed that *ALS1* is down regulated (0.35-fold) (Fig. 2 and Table 2). The genes, whose expression was altered in strain CSSK21 were subjected to RT-PCR from similarly grown cells and compared to CAF2 and CSSK23 (Fig. 3). Actin was used as an internal loading control. Compared to CAF2 and CSSK23, we observed that expression levels of *HSP12*, *AHP1*, *MNN-4*, and *FLO1* were greater in strain CSSK21, while *ALS1* was down regulated. Levels of *PYC2*, *CHK1*, and *GPH1* were slightly but consistently higher than those of CAF2 and CSSK23. By Northern analysis, the up regulation of *GPH1* and *PYC2* in the mutant was more readily noted (data not shown). As expected, *SSK1* was weakly detected in mutant cells, while differences among strains were not apparent by RT-PCR for *STL1* and *APE2153* (not shown). RT-PCR was also done with SSK23, the gene-reconstituted strain (Fig. 3). Expression in SSK23 was either similar to that in CAF2 cells or, in the case of *MNN4*, up regulated, as with strain SSK21.

Phosphorylation of Hog1p. In *S. cerevisiae*, phosphorylation of Hog1p by Ssk1p is critical in the adaptation of cells to

TABLE 2. Summary of microarray data showing the up-regulated and down-regulated genes of the *ssk1* mutant.

Gene name	Stanford name	Average median ratio[a]	Description[b]
HSP12	orf6.1668	3.81 (8)	Heat shock protein; oxidative stress (12kDa)
CHK1	orf6.7978	4.37 (19)	Histidine kinase
GPH1	orf6.7947	3.22 (12)	Glycogen phosphorylase regulated by Hog1p
MNN4-4	orf6.4127	4.44 (11)	Mannosyl transferase; multiple-gene family member
FLO1	orf6.3288	4.40 (4)	Flocculin; cell surface protein
PYC2	orf6.2989	3.61 (4)	Pyruvate carboxylase; NADPH regeneration
AHP1	orf6.6590	4.89 (4)	Alkyl hydroperoxide reductase
ALS1	orf6.2112[c]	0.35 (8)	Agglutinin-like cell surface protein
STL1	orf6.1416	0.50 (12)	Hexose transporter of the major facilitator family
SSK1	orf6.7978	0.16 (4)	Response regulator, two-component phosphorelay

[a] The number of spots used for calculating the average median ratio is in parentheses.
[b] Putative gene function(s) in *S. cerevisiae* or *C. albicans*.
[c] Partial ORF sequence.

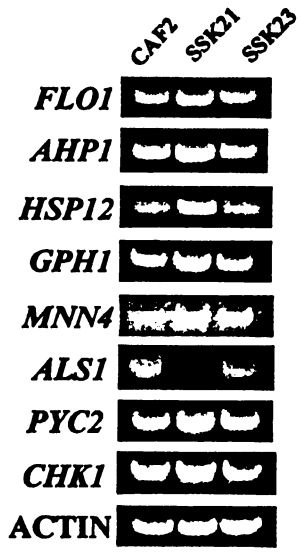

FIG. 3. RT-PCR of selected genes in CAF2, SSK23, and SSK21. For each experiment, β-actin was used as an internal control. RT-PCR gels were scanned, and the images were stored as TIFF files. A representative RT-PCR analysis is shown. Results were similar for three analyses of each gene.

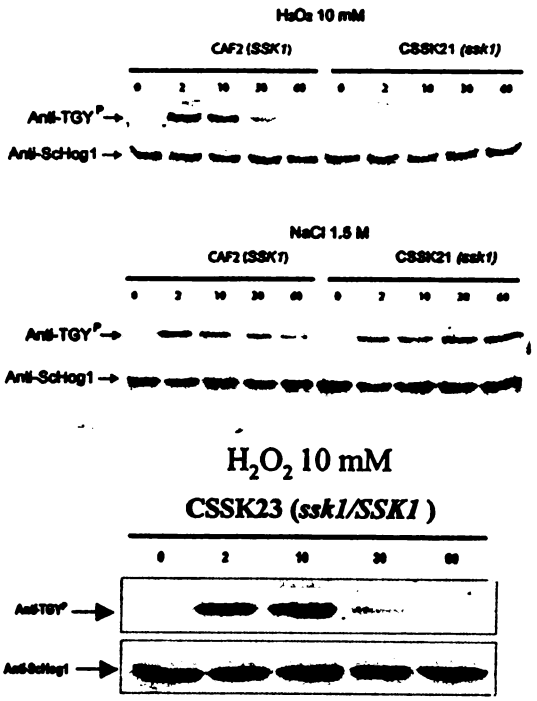

FIG. 4. Western blot analysis. (A) Strains CAF2 (*SSK1*) and CSSK21 (*ssk1*) were grown in either 10 mM H_2O_2 (upper blots) or 1.5 M sodium chloride (lower blots). At the indicated times (in minutes, above the lanes), samples were obtained and Western blots were performed using either an anti-*S. cerevisiae* Hog1p antibody (lower reactive band) or an anti-TGY antibody (upper band) that recognizes the phosphorylated p38 protein of mammalian cells (Hog1p is a homologue of this protein). (B) Phosphorylation of Hog1p in SSK23 cells that were oxidant stressed as described for panel A. Restoration of the phosphorylation is observed in the SSK1 gene-reconstituted strain.

osmotic stress. Thus, we were interested in the relationship of Ssk1p to Hog1p in *C. albicans* under conditions of osmotic or oxidative stress. Previously, it was shown that strain CSSK21 is insensitive to 1.0 M sorbitol and 2 mM H_2O_2 (15). To determine the relationship of Ssk1p and Hog1p, we used Western blot analysis with an antibody that recognizes phosphorylated Hog1p (anti-TGY^P) (Fig. 4) (2). Cells of strains CAF2 and CSSK21 were grown in the presence or absence of 10 mM H_2O_2 or 1.5 M NaCl for 0 to 60 min. Extracts of cells at each time point were prepared, electrophoresed, transferred to nitrocellulose filters, and reacted with anti-ScHog1p, an antibody that recognizes both the *S. cerevisiae* and *C. albicans* Hog1p proteins regardless of the phosphorylation state. A reactive band of 38 kDa was observed in both types of stress treatments and in both CAF2 and CSSK21 at all time points (Fig. 4A). The specificity of the antibody was shown in previous studies, since a *hog1* mutant of *C. albicans* lacked this reactive protein (2). Subsequently, the Western blot was stripped (with 2% SDS at 70°C, then with 62.5 mM Tris-HCl [pH 6.8; 20°C], and finally with 100 mM β-mercaptoethanol) and reacted with anti-TGY^P, which recognizes the phosphorylated Hog1p (Fig. 4A). In CAF2, Hog1p was phosphorylated within 2 min in cells treated with either NaCl or H_2O_2. By 10 min, the signal decreased in wild-type cells treated with NaCl, but the signal remained strongly positive in cells treated with H_2O_2 at 10 min; subsequently, the phosphorylation signal decreased in both treatments. No phosphorylation of Hog1p was observed in the SSK21 mutant treated with H_2O_2 (Fig. 4A, top), but phosphorylation of Hog1p did occur in the *ssk1* mutant exposed to NaCl. Thus, *SSK1* is required for phosphorylation of Hog1p in cells exposed to H_2O_2 but is not required for cells grown in high concentrations of NaCl. Interestingly, the temporal phosphorylation of Hog1p in the CSSK21 mutant grown in 1.5 M NaCl remains strongly positive after 60 min, but not in cells of CAF2. The phosphorylation of CSSK23 was also studied under the same conditions (Fig. 4B). The introduction of *SSK1* in this strain restored Hog1p phosphorylation when the cells were oxidatively stressed. We did not examine the phosphorylation of Hog1p in cells stressed with NaCl, since that signal was already apparent in the *ssk1* mutant (Fig. 4A).

DISCUSSION

The adaptation of bacteria, archaea, lower eukaryotes, and higher plants, but not human cells, to stress conditions is regulated through two-component signal transduction (5, 26, 37, 42). The Hog1 pathway of *S. cerevisiae* is the most studied of the two-component systems in fungi (23, 37). In *C. albicans*, homologues of the HOG pathway are found (Sln1p, Ypd1p, and Ssk1p), but the Sln1p and Ssk1p proteins seem to have minor roles in adapting cells to osmostress, as *SLN1* and *SSK1* mutants are only partially sensitive (15, 31). Likewise, *C. albi-*

cans SSK1 does not complement the *ssk1* mutant of *S. cerevisiae* (14). Recent data suggest that the Hog1p MAP kinase is essential for adaptation of *C. albicans* to oxidative stress due to ROS, such as oxidants (menadione, hydrogen peroxide, and KO_2), and UV light (2). It follows that the two-component proteins that regulate the phosphorylation of Hog1p may also be involved in the adaptation of cells to oxidant stress. Since adaptation to high-osmotic conditions appears to be a minor function (if any) of the *C. albicans* Ssk1p, our preliminary experiments focused upon the sensitivity of strain CSSK21 (*ssk1/ssk1*) to other stress conditions. We tested a variety of antifungal compounds and oxidants in order to assess the sensitivity of the mutant strain. Strain CSSK21 was not sensitive to nikkomycin Z, miconazole, amphotericin B, caffeine, caspofungin, or calcofluor white. However, the strain was sensitive to hydrogen peroxide at a concentration of 5 mM and to other oxidants, which explains why in previous studies carried out using ≤2 mM H_2O_2, no effect was observed (15).

The sensitivity of CSSK21 to peroxide stress was supported by microarray data. Three of the seven genes that were up regulated threefold or greater in strain CSSK21 are among those whose functions in *S. cerevisiae* are associated partially or totally with adaptation to stress conditions, including oxidative stress. *AHP1* of *S. cerevisiae* encodes one of several oxidoreductases whose function is to maintain cells in a reduced state (redox potential) during oxidative metabolism (22, 36). The oxidoreductases are small, heat-stable proteins that contain two conserved cysteine residues in their active sites and are of two types, the thioredoxins and glutathione-glutaredoxins (22, 36). While they are highly conserved with overlapping functions, differences exist between the two types of oxidoreductases. For example, the thioredoxins utilize NADPH as an H donor, while the glutaredoxins use reduced glutathione (22). Importantly, the expression of thioredoxin-related genes, such as *AHP1*, is regulated by the transcription factors YAP-1p and Skn7p (22). The latter transcription factor is a response regulator two-component signal protein in *S. cerevisiae*. Thus, a consequence of cell growth and oxidative metabolism in *S. cerevisiae* is the generation of ROS that are highly toxic to cells unless proteins such as Ahp1p are produced. While the oxidoreductases of *C. albicans* have not been characterized, it would appear that *C. albicans* processes oxidative stress in a manner that is somewhat different from the *S. cerevisiae* process (20). The organism also produces an adaptive response that protects it from lethal effects of a subsequent challenge (24). In addition to *AHP1*, *HSP12*, and *PYC2*, up regulation of *GPH1* (glycogen synthesis) was observed; Gph1p is regulated by Hog1p in osmotically stressed cells (51). Recent data also point to an increase in trehalose production during oxidative stress (4), although our array analysis did not indicate changes in the expression of genes involved in trehalose biosynthesis. Finally, *CSSK1* appears to regulate the expression of certain cell wall proteins (Mnn4p, Als1p1, and Flo1p [25, 35]) and a two-component histidine kinase, *CHK1*, that regulates cell wall synthesis (27). Up regulation of *MNN4* in *S. cerevisiae* during stress responses has been demonstrated (35). The array data for up-regulated genes obtained with the *ssk1* mutant were verified by RT-PCR. Importantly, expression of the adhesin *ALS1* (25) is down regulated. This observation is correlated with a previous study that showed a reduced adherence to human esophageal cells by the *ssk1* mutant of *C. albicans* (6, 29).

The relationship of Ssk1p and Hog1p was established through Western blot assays. We used anti-ScHog1p antibody to identify in Western blotting the *C. albicans* Hog1p. Following exposure of the wild type (CAF2) and the *ssk1* mutant to hydrogen peroxide, phosphorylation of Hog1p, as determined using the anti-TGY antibody, occurred only in CAF2 cells (2). Thus, Ssk1p is used for phosphorylation of Hog1p, at least in peroxide-stressed cells. On the other hand, Hog1p is phosphorylated in both wild-type and *ssk1* mutant cells in the presence of 1.5 M NaCl. Thus, it would appear that in *C. albicans*, Ssk1p may or may not interact with Hog1p during osmotic stress; however, other proteins carry out this activity in the absence of Ssk1p.

In summary, Ssk1p in *C. albicans* represents at least one of the signal proteins that adapt cells to peroxide stress. This activity appears to be at least partially mediated through the HOG MAP kinase pathway. Importantly, we show that Ssk1p is not essential for adaptation to osmotic stress, unlike *S. cerevisiae* Ssk1p, which is essential. We have recently determined that the *ssk1* mutant is also more readily killed by human PMNs (unpublished data), suggesting that its avirulence in a murine model of hematogenously disseminated candidiasis may in part be associated with this phenotype (15). Down regulation of *ALS1* in the *cssk1* mutant also suggests a reason for its reduced adherence to human esophageal cells (6, 29). Since two-component signal proteins are found only in fungi, prokaryotic organisms, and plants, they may be suitable as targets for the development of new antifungals (5, 26). Among the fungal pathogens of humans, other histidine kinases have been identified in *A. fumigatus* (*FOS1*) (30) and *C. albicans* (*COS1/NIK1* and *CHK1*), and mutants of each are attenuated or avirulent (54). Importantly, while strains with single genes deleted are still viable, deletions of both *sln1* and a second histidine kinase gene (*cos1/nik1*) are lethal (54). Thus, an anti-two-component drug should be relatively broad spectrum in its activity and fungicidal.

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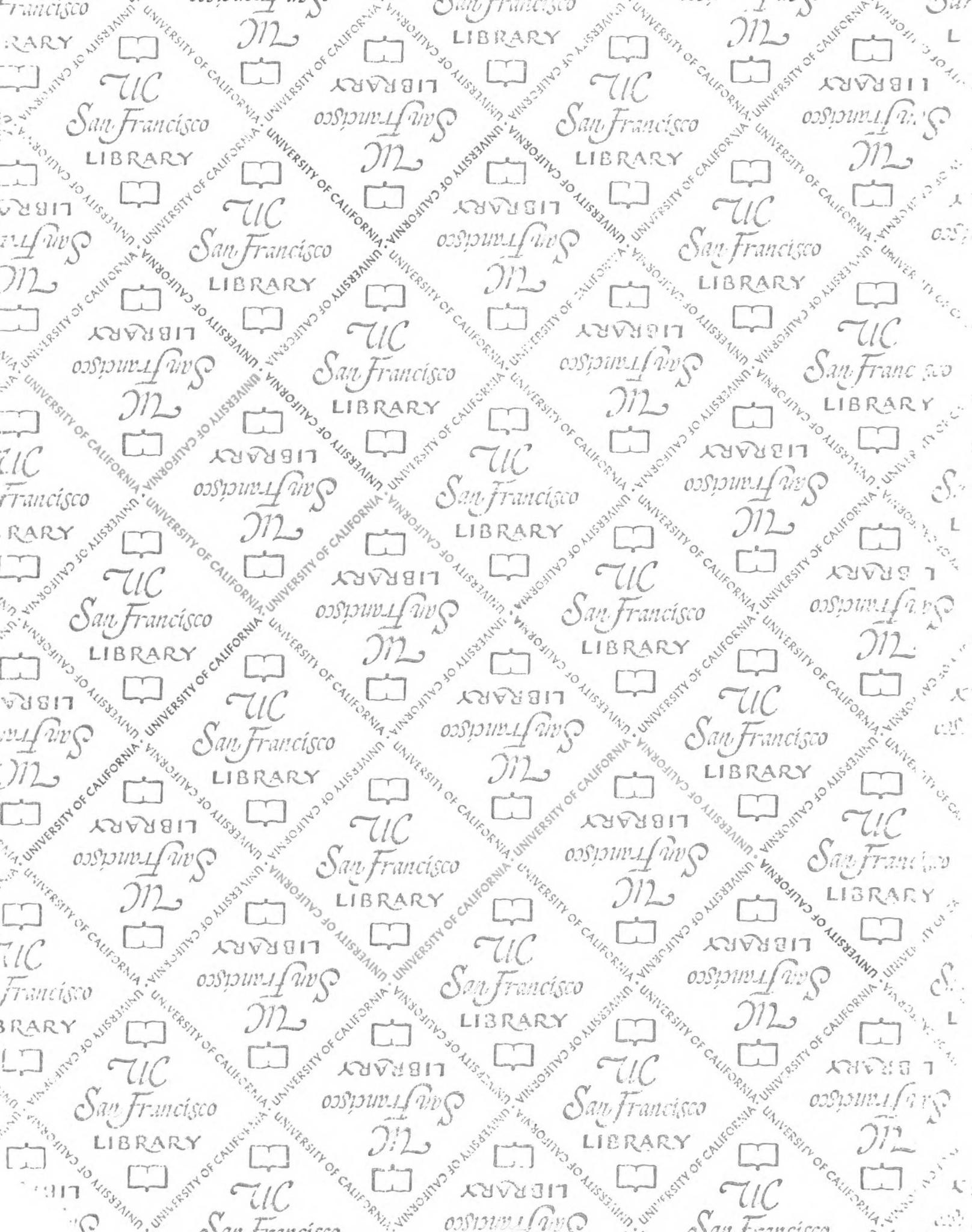
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