CHAPTER III

LITERATURE REVIEW

Candida and Candidiasis

Candida spp. are frequently present as members of the normal flora of the mouth, throat, large intestine, vagina, and skin and are often contaminants in exudates or other specimens taken from these areas. Essentially all areas of the gastrointestinal (GI) tracts of humans can harbor *Candida* spp. The overall carriage rate in healthy individuals has been estimated to reach 80%. The most commonly isolated species (50 to 70% of yeast isolates) from the GI tracts of humans is *C. albicans* (32). In patients whose immune defenses have been compromised by disease or by the secondary effects of drugs used to treat diseases, normal flora organisms may invade deeper tissues, producing severe, life-threatening infections. Candidiasis is a primary or secondary infection involving a member of the genus *Candida*. Involvement may be localized to the mouth, throat, skin, scalp, vagina, fingers, nails, bronchi, lungs, or the gastrointestinal tract, or become systemic as in septicemia, endocarditis, and meningitis (33). Candidiasis has been and continues to be a major disease problem of immunocompromised hosts (34). Several species of *Candida* have been implicated in candidiasis. Historically, *C. albicans* accounted for 70 to 80% of the isolates recovered from infected patients. *C. glabrata* and *C. tropicalis* each accounted for approximately 5 to 8% of isolates, while other non-*albicans Candida* species occur only rarely. However, more recent epidemiological data reveal a mycological shift from *C. albicans* to the non-*albicans Candida* species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (32).

Taxonomy of *Candida*

In older taxonomic treatises, species of the genus *Candida* that lacked a sexual stage (teleomorph) were designated Deuteromycetes, the class of fungi that does not

reproduce sexually. On the other hand, the teleomorph stages of several pathogenic (Table 1) and nonpathogenic *Candida* species have been described, but for familiarity's sake, those organisms (especially the pathogens) retain their designation as *Candida* species. *C. krusei*, for example, is not routinely refered to as *Issatchenkia orientalis*, in spite of the fact that teleomorph stage is known for this species. Therefore, the anamorph (asexual stage name) is specified in distinguishing species of *Candida*, even if the sexual stage (teleomorph) is identified (Table 1). The older classification of many species of *Candida* as Dueteromycetes was based on the fact that they lacked a sexual stage. However, for those species of *Candida* for which a teleomorph has been described (Table 1), the sexual stages are ascomycetous. Further, comparative sequence data from a number of studies support the idea that both the sexual and nonsexual *Candida* species are related phylogenetically to the Ascomycete rather than to the Basidiomycete class of fungi (35).

Table 1. A partial list of pathogenic species of *Candida* (35)

Reported by 1988	Current list	Sexual (teleomorph form)
C. albicans	*C. albicans*	Not described
C. tropicalis	*C. tropicalis*	Not described
C. glabrata	*C. glabrata*	Not described
C. parapsilosis	*C. parapsilosis*	Not described
C. krusei	*C. krusei*	*Issatchenkia orientalis*
C. guilliermondii	*C. guilliermondii*	*Pichia guilliermondii*
C. kefyr	*C. kefyr*	*Kluyveromyces marxianus*
C. viswanathii	*C. viswanathii*	Not described
	C. lusitaniae	*Clavissopora lusitaniae*
	C. dubliniensis	Not described
	C. famata	*Debaryomyces hansenii*
	C. inconspicua	Not described
	C. utilis	*Pichia jadinii*

Growth Forms of *Candida*

The pathogenic *Candida* spp. are listed in Table 1 along with the teleomorph (sexual) stage binomial designation, if known. The genus *Candida* is composed of an extreamly heterogenous group of organisms that grow as yeasts (Fig. 1A, blastoconidia; see Table 2). Most members of the genus also produce a filamentous type of growth (pseudohyphae, pseudomycelium, Fig. 1C; Table 2), but *C. albicans* and *C. dubliniensis* also form true hyphae (true mycelium) (Fig. 1B) in addition to pseudohyphae (pseudomycelium). Therefore, both species are considered to be polymorphic. The distinction that is made between hyphae and pseudohyphae is related to the way in which they formed. Pseudohyphae form from yeast cells or form hyphae by budding, but the new cell remains attached to the parent cell and elongates, resulting in filaments with constrictions at the cell-cell junctions of the filaments. In comparison, true hyphae form from yeast cells or as branches of existing hyphae. Outgrowths of the yeast cell (germ tubes) grow by apical extension and cross walls (septa) are formed behind the growing tip of the hyphae. Budding occurs laterally just behind the septa (Fig. 1B), the latter of which are perpendicular to the main axis of the hyphae. The appearance of hyphae differs somewhat when grown in serum, where hyphal branching occurs and budding is delayed. The pseudohypha appears to be an intermediate growth form of yeast and hyphal morphologies. Because *C. albicans* and *C. dubliniensis* produce hyphae from yeast cells, these species are also referred to as being germ tube positive (Fig. 2A; Table 2) (35).

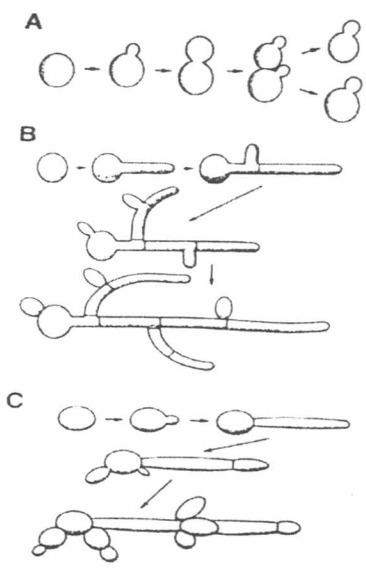
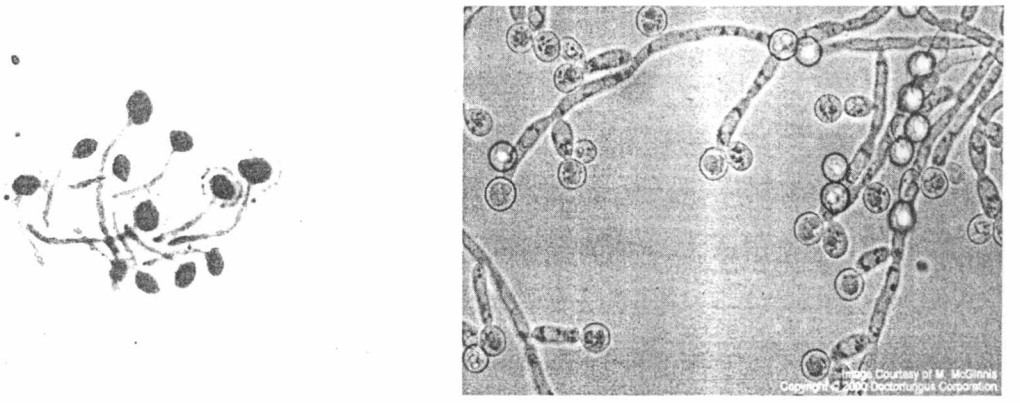


Figure 1. The growth form of *Candida* species. All species grow as yeasts (A), and most produce some type of pseudohyphae (C). *C. dubliniensis* and *C. albicans* also form true hyphae (B). Budding in *Candida* species occurs at numerous sites on the cell surface. The newly formed bud increases in size, then becomes separated following the final stages of mitosis. Hyphal formation is initiated by an outgrowth (germ tube) followed by apical extension. Septa (cross walls) form, and branching of the hyphae or budding occurs just behind the septa.

The germ tube test is commonly employed in the diagnostic mycology laboratory to identify isolates rapidly from patients as either of these two species. Germination can be induced in complex media (peptone broth), chemically media, and serum. The environmental factors that favor germination include a temperature greater than 35°C and a pH of 6.5 to 7.0 or slightly alkaline. *C. albicans* and *C. dubliniensis* also produce large (8 to 12 μm), refractile, thick-walled cells referred to as chlamydospores (Table 2 and Fig. 2B) that are most often observed in vitro on nutrient poor media, such as cornmeal-Tween agar and other equally nutritionally poor media (35).

Table 2. Morphological features of selected pathogenic species of *Candida* (35)

Species	Chlamydospore	Germ tube	Pseudohyphae	Size of yeasts (μm)
C. albicans	+	+	+	4-6 x 6-10
C. tropicalis	±	-	+	4-8 x 5-11
C. glabrata	-	-	-	1-4
C. parapsilosis	-	-	+	2.5-4 x 2.5-9
C. krusei	-	-	+/-	3-5 x 6-20
C. dubliniensis	+	+	+	3-7 x 3-14
C. guilliermondii	-	-	Primitive	2-5 x 3-7
C. lusitaniae	-	-	Variable	2-6 x 3-10
C. kefyr	-	-	+	2.5-5 x 5-10
C. inconspicua	-	-	Primitive	1.5-3 x 5-10
C. famata	-	-	Primitive	3.7-5 x 2.7-4.7



A B

Figure 2. The germ tubes formation in serum for 3 hours (A) and chlamydoconidia from cultures grown on cornmeal-Tween agar (B).

Oral Candidiasis

Candida albicans is normal part of the gastrointestinal flora, and as an opportunistic pathogen, *Candida* species may establish an infection when immunologic and mechanical host defenses are immunocompromised. Infections of the skin and the mucous membranes due to *Candida* spp. may occur either in immunocompromised or in nonimmunocompromised patients. This is in contrast to systemic candidiasis (e.g., candidemia), which is only seen in severely immunocompromised patients. It may be assumed that infections of the skin and the mucous membranes due to *Candida* spp. are growing in incidence because of the increasing numbers of immunocompromised patients. Essentially, the beginning of the AIDS epidemic has focused almost all studies and research on oral *Candida* infections in human immunodeficiency virus (HIV)-infected individuals. Therefore, two groups of patients may be characterized as at risk of developing oropharyngeal candidiasis (OPC), the HIV-infected and non-HIV-infected patients (35).

1. Epidemiology

Oral carriage of *Candida* spp. in "healthy" persons and in various patients groups has been described in numerous reports. The majority of reports are descriptive in nature and considered *C. albicans* as the most prevalent yeast. However, considering the applicability of modern molecular typing techniques, it remains open as to how frequently misidentification of clinical isolates occurs. At least for the new species, the misidentification of *Candida dubliniensis* as *C. albicans* has been estimated to be as high as 17% (36). Data about the "true" incidence of oral *Candida* colonization or infection in different patient groups other than HIV-infected individuals are rare. From the more than 150 *Candida* spp. that are known, only a few are considered to be pathogenic for humans. *C. albicans* is the most commonly isolated species (>70%) from samples taken from mucous membranes such as oral cavity and digestive and vaginal tracts. Less than (<7%), other *Candida* spp., including *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. kefyr*, *C. guilliermondii*, rarely *C. sake*, and relatively new species *C. dubliniensis*, are isolated from nonsterile superficial sites (37). Non-*C. albicans* species are more likely to occur in patients who receives or have received antifungal therapy with azoles. The vast majority of data on OPC published in the last 10 years emphasizes the HIV-infected patients who frequently receives longterm or intermittent therapy with fluconazole and other agents (2). Almost all cases of OPC are caused by *C. albicans*, but rarely it has been claimed (in HIV-positive patients) that *C. glabrata, C. parapsilosis*, *C. tropicalis*, *C. krusei*, or *C. dubliniensis* by itself may cause OPC or even esophagitis (38, 39).

In a small study by Dronda et al., the clinical significance of mixed oropharyngeal *Candida* infections (*C. albicans* cultivated together with non-*C. albicans* strains such as *C. krusei* or *C. glabrata*) was examined in 12 HIV-infected patients. It was concluded that non-*C. albicans* strains of *Candida*, less sensitive to azole drugs than their *C. albicans* counterparts , are not clinically relevant in episodes of mixed oropharyngeal candidiasis in HIV-1-infected patients (8).

2. Pathogenesis

Adhesion of yeasts to the mucosa of the oral cavity and dentures is an important initial step in the pathogenesis of oral yeast infections. Furthermore, a direct

relationship between the adhesion capacity of various *Candida* species and their ability to cause infection has been observed (40, 41). Adherence mechanisms known to be used by *C. albicans* are hydrophobicity, protein-protein binding via integrin-like surface proteins, and lectin-like proteins (42). Local defense mechanisms play a central role in preventing yeast colonization of the oral cavity. These mechanism include the physical local barrier of the epithelia, antimicrobial peptides, secretory immunoglobulin A, and salivary factors such as saliva flow rate and salivary molecules (e.g., lysozyme, histatins, and lactoferrin) (43). Observations in the late 1960s have shown that persistent *Candida* infection is common in patients with cell-mediated immunodeficiencies (44). Consequently, it was suggested in subsequent studies in chronic mucocutaneous candidiasis (CMC) that impaired cell-mediated immune response leads to this chronic infection (45). In the pre AIDS era the scant published data proposed a relationships between immunological parameters and response to therapy in oral candidiasis other than CMC (46). Experimental evidence supporting a role for T lymphocytes in resistance to mucosal candidiasis has come from studies with germ-free athymic mice. Further experimental studies supported the clinical observation in HIV infected individuals that $CD4^+$ lymphocytes play a critical role in resistance to mucosal candidiasis (47). The most important predisposing factor for OPC other than T-cell immunodeficiency is the wearing of removable dentures (48). Other conditions that may contribute to OPC are radiation therapy for head and neck cancer, reduced salivary flow rates, diabetes mellitus, and gastrointestinal disorders (49, 50).

3. Oral Candidiasis in HIV-Infected Individuals

Oral candidiasis is seen in nearly 10 to 30% of asymptomatic HIV-1-infected patients in the United States and Europe, in 40 to 60% of patients with symptomatic HIV infection (formerly described as AIDS-related complex), and in 50 to 90% of AIDS patients. Oral candidiasis is not only the most common fungal infection in HIV-infected individuals but is by far the most common opportunistic infection observed in this patients group, affecting nearly 90% of subjects at some stage during the course of HIV disease progression. The reports from the early 1980s have identified OPC as a clinical predictor of progression to AIDS (51, 52). Other host factors besides advanced HIV disease alone have been claimed to contribute to OPC : low CD4 lymphocyte count, xerostomic, age > 35 years, intravenous drug use, and certain

racial populations, such as black intravenous drug users compared to white subjects (53, 54). More recently, Gootfredsson et al. concluded that the amount of asymptomatic oropharyngeal yeast carriage in persons with HIV infection is more significantly correlated with HIV viremia than with CD4 cell count (55). It was discussed by several authors that HIV viral load suppression induced by highly contribute to the clinically observed reductions of oral and esophageal candidiasis and other opportunistic infections (56). It may be argued that highly active antiretroviral therapy can induce recovery of CD4 T-cell reactivity against *C. albicans* similar to other opportunistic pathogen, and therefore reconstitute immune defense mechanisms (57). *C. albicans* is the most commonly isolated species from samples taken from the oral cavity. However, from molecular epidemiological studies, we now understand that several strains of *C. albicans* can colonize the mucous membranes. Colonization with up to three genotypically different strains of *C. albicans* may occur either in parallel or sequentially, but in most cases one "dominant" strain (better described as genotype) persists and is associated with recurrent infections. For a patient, intrastrain variation may occur (58). These persistent strains may be transferred via intimate contact between sexual partners (59). However, even when the genotype of *C. albicans* persists, the phenotypical expression of the strains may change (60).

4. Clinical presentation of oral candidiasis in HIV-infected patients

Symptoms of OPC usually include an alteration of taste ("furry" taste), inflammation in the mouth with oral pain and burning of mount, and xerostomia. Occasionally patients complain that their breath smells like a brewery. In most cases, patients are able to maintain their oral nutritional intake, despite discomfort, and do not loss weight. Laboratory tests may confirm the diagnosis of candidiasis by microscopy with a 10 to 20% potassium hydroxide preparation of lesions for detection of pseudohyphae and yeast forms or by fungal culture on specialized media (e.g., CHROMagar, Albicans ID, and others) allows the detection of yeasts in mixed cultures by differences in colony color (61).

Various presentation of OPC have been described, from which the pseudomembranous candidiasis represents the most prevalent form. According to the classification of the EEC-Clearing-house on oral problems related to HIV infection, four subtypes were defined in HIV-infected patients : erythematous, hyperplastic, pseudomembranous candidiasis and angular cheilitis (62).

4.1 Pseudomembranous Candidiasis (Thrush)

Pseudomembranous candidiasis is characterized by extensive white pseudomembranes consisting of desquamated epitherial cells, fibrin, and fungal hyphae. These white patches occur on the surface of the labial and buccal mucosa, hard and soft palate, tongue, periodontal tissues, and oropharynx. The membrane can usually be scraped off with a swab to expose an underlying erythematous mucosa. Diagnosis can be confirmed microbiologically either by staining a smear from the affected area or by culturing a swab from an oral (62). In addition to patients with HIV-infection, the occurrence of pseudomembranous candidiasis has been described in neonates, the elderly, the patients with malignancies and/or undergoing chemotherapy, and those with nutritional deficiency, deficiencies of cell-mediated immunity, or phagocyte dysfunction and after broad-spectrum antibodies or steroids (49).

4.2 Erythematous OPC

Erythematous OPC has been reported primarily in patients with HIV infection, and has been suggested that this form is more difficult to recognize and hence is underdiagnosed (63). The definition for erythematous OPC is red area without removable white spots or plaques. It often located on palate, dorsum of the tongue and buccal mucosa (62). In a study by Dodd et al. of 169 HIV-positive patients with OPC, 92 had pseudomembranous candidiasis, 37 had erythematous candidiasis, and 40 patients had both types of lesion (63). Data from Samaranayake et al. suggested that almost equal numbers of cases present with either the erythematous or pseudomembranous form, but Dodd et al. found a threefold-higher incidence of the pseudomembranous form compared to the erythematous presentation, and the examination of 456 consecutive HIV-infected patients in a study by Palmer et al. revealed erythematous candidiasis in a 24%, pseudomembranous candidiasis in 14%, and angular cheilitis in 6% (63). These data may reflect the fact that the erythematous form is more difficult to diagnose and may be missed on oral examination. However, a clear definition of erythematous candidiasis (other than a red area without removable white spots or plaques) as seen in HIV-infected individuals is lacking (62). In non-HIV-infected individuals the presentation of erythematous candidiasis has been observed in patients with denture stomatitis, but definite epidemiologic data are rare (64).

4.3 Hyperplastic Candidiasis (Candida Leukoplakia)

Hyperplastic candidiasis characteristically occurs on the buccal mucosa or lateral border of the tongue as firm of adherent white patches or pin-head-sized nodules on an erythematous area that can not attributed to any other diagnosable lesion. The lesion usually occur on the buccal mucosa or lateral borders of the tongue *Candida* spp. are not always isolated from lesions of oral leukoplakia and it has been suggested that finding of *Candida* spp. in these premalignant lesions is a complicating factor rather than a causative one (62).

4.4 Angular Cheilitis

Angular cheilitis characteristic is a fiery red commissures with a fissuring or cracking appearance. The majority of cases is associated with *C. albicans*. Angular cheilitis should raise suspicion when present in young, non-denture wearing patients (62).

5. Treatment

A number of options, either local or systemic, are available for the treatment of OPC. Table 3 lists the 6 antifungal drug preparations approved for marketing in the United States the Food and Drug Administration to manage mucosal fungal disease.

Table 3. Antifungal drugs for treatment of oropharyngeal candidiasis (65)

Generic name	Proprietary name	Formulation	Typical treatment course
Amphotericin B	Fungizone	100 mg/ml oral suspension	Rx: 100 mg QID for 14 d
Clotimazole	Mycelex	10-mg troche	Rx: 10 mg 5xd for 10 d
Flucoazole	Diflucan	100-mg tablet	Rx: 200 mg stat and 100 mg QD for 14 d
		10 mg/ml oral solution	Rx: 100 mg QD for 14 d
		40 mg/ml oral solution	Rx: 100 mg QD for 14 d
Itraconazole	Sporanox	100-mg capsule	Rx: 200 mg QD for 14 d
		10 mg/ml oral solution	Rx: 200 mg QD for 14 d
Ketoconazole	Nizoral	200-mg tablet	Rx: 400 mg stat and 200 mg QD for 14 d
Nystatin	Mycostatin	100,000 u/ml oral suspension	Rx: 500,000 U QID for 10 d
		200,000 u/oral pastille	Rx: 200,000 U 5xd for 10 d
		500,000 u/oral tablet	Rx: 500,000 U QID for 10 d
		100,000 u/vaginal tablet	Rx: 100,000 U QID for 14 d

Prior to 1980, the compound most commonly used for treatment of oral thrush was gentian crystal violet, but this approach became unpopular due to local necrosis and staining of oral mucosa (66). Oral polyenes and azoles have finally replaced this compound. Several antifungal agents including nystatin (a polyene antifungal), clotimazole (a topical imidazole), and amphotericin B oral suspension can be used topically. Systemic antifungals, including ketoconazole, fluconazole, and itraconazole, have the advantage of once-daily dosing and simultaneous treatment of fungal infections at multiple body sites. However, these antifungals have more side effects, and selection requires consideration of important drug interactions. Several studies have been conducted comparing local (topical) and systemic therapy. Clinical response rates were similar, but signs and symptoms were more rapidly resolved with systemic treatment. Most patients will respond to topical therapy with clotimazole troches (one 10 mg troche 5 times daily), or nystatin (as a suspension of 100,000 U/ml 4 times daily) for 7 to 14 days, but longer treatment for up to 28 days may be needed in some cases due to the lower clinical response rate compared to that with azoles. In Europe amphotericin B suspension (100 mg 4 to 6 times per day) is used frequently instead of clotimazole because clotimazole is marketed for vaginal candidiasis only (67). Oral fluconazole (100 mg per day for up to 14 days) has become the drug of choice in many patients, because the clinical response is usually apparent within 1 to

2 days and side effects due to therapy are minimal (68). The early studies have shown that 50 mg of fluconazole daily is effective for most cases of OPC, but more recently a 100 mg dosage was advocated and used in most studies to prolong the disease-free interval in severely immunocompromised patients (69). Itraconazole as a solution (100 mg 2 times per day up to 14 days) is regarded as efficacious as fluconazole. Absorption of ketoconazole and itraconazole capsules depends on gastric acidity and may be, therefore, regarded only as second choice treatment in patients with hypochorhydria. Single-dose treatment has been shown to be effective in HIV-infected individuals, but prolonged therapy for 14 days and longer has shown somewhat higher mycological responses. However, clinical evidence of relapse and recurrence is a common finding irrespective of treatment, as seen in the majority of studies. Recurrences of oral or esophageal candidiasis can be prevented in HIV-infected individuals with low CD4 cell counts (<200/μl) when a maintenance suppressive treatment is given either dialy (50 to 200 mg/day) or once weeky (150 to 200 mg), and continuous therapy is associated with a lower annual relapse rate than intermittent therapy.

6. Resistant Oral Candidiasis

Oral candidiasis refractory to systemic therapy with antifungal agents resulting in treatment failure as well as development of resistance in *C. albicans* to antifungal agent in vitro, has been associated with the repeated or continuous use of azole antifungal agents in general, but fluconazole in particular (70). Common factors that led to the presence of resistant *Candida* strain in patients may be due to replacement by a more resistant species (e.g., *C. krusei*, *C. glabrata*) or replacement with a more resistant strain of *C. albicans*. Azole drug resistance did not become a significant problem until this group of compounds began to be used in patients with HIV infection. A correlation of in vitro susceptibility testing of *C. albicans* with in vivo response to antifungal therapy has been observed for oral candidal infections in HIV-infected patients. Fichtenbaum and Powderly have summarized data from recent studies and defined fluconazole-refactory mucosal candidiasis as a progressive disease with lack of improvement after treatment with fluconazole 200 mg orally or intravenously daily for at least 7 days. Recent reports suggest that approximately 30% of patients with advanced AIDS will develop azole-resistant *Candida* infection.

Clinical patterns that were associated with fluconazole refractory mucosal disease include very advanced HIV disease with a $CD4^+$ cell counts of <50/μl, a history of recurrent candidiasis, extensive previous fluconazole exposure, history of opportunistic infections (e.g., tuberculosis or toxoplasmosis), and treatment with antituberculosis drugs (35).

Generally the same strain of *C. albicans* is repeatedly isolated and is associated with progressiely increasing MICs over repeated courses of azole therapy, the dose of which has been gradually increased to achieve a therapeutic response. A fluconazole dose of 100 mg/day begins to be less effective in AIDS patients with OPC when the MIC for the infecting *Candida* isolate reaches 8 μg/ml and is generally ineffective at an MIC of 16 μg/ml. Higher doses of 400 to 800 mg/day may effectively treat the infection until the MIC is 64 μg/ml or higher. Loss of a fluconazole-resistant *C. albicans* isolate which emerged during treatment of OPC with intermittent fluconazole (50 to 200 mg three times weekly or more) was reported in a patient following resolution of clinical signs of infection after itraconazole treatment. Non-*C. albicans* isolates resistant to fluconazole, including *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, and *C. krusei*, have been recovered from AIDS patients with OPC and have been associated with therapeutic failure in some cases. Typically, these species have been isolated from patients who have recived prior courses of azole therapy, and increased MICS usually have been associated with clinical failures (35).

The Genome of *Candida albicans* and Other *Candida* Species

1. Genome Size of *Candida* Species

C. albicans is the diploid and has about 32 Mbp of nuclear DNA per cell; hence, the haploid genome size is 16 Mbp. That the organism is diploid was first suggested by Olaiya and Sogin, based on the fact that its sensitivity to UV light is similar to that of diploid *Saccharomyces cerevisiae*. However, since radiation sensitivity is a function of repair capacity as well as gene number, the ploidy could not be definitively determined in this way. Whelan and Magee demonstrated that natural isolates were heterozygous for several genes and suggested that the most likely explanation for this was that the organism is diploid. Riggsby et al. used DNA-DNA hybridization to show that the amount of unique DNA per cell (15 to 20 pg) is

about half the cellular DNA content (37 pg), providing physical confirmation of the genetic results (71). The estimate of repeated nuclear DNA was about 3% (71). Whelan later showed genetically that *Candida parapsilosis* and *Candida krusei* are also diploid (72). *Candida (Torulopsis) glabrata*, on the other hand, is haploid. Since this organism is more closely related to *S. cerevisiae* than to *C. albicans*, it is perhaps not so surprising that it differs from other *Candida* strains (73). Exhaustive analysis of the information content of species other than *C. albicans* has not been carried out.

2. Genomic Variability

Early efforts at separation of the *C. albicans* chromosomes by use of pulse-field electrophoresis reveals that the electrophoretic karyotype of this organism is highly variable from isolate to isolate (74). The variability in the genome reflects a variety of genomic alterations. These include translocation, deletion, amplification, and loss of particular chromosomes (75), aneuploidy and ploidy change (76) and chromosome length polymorphism (CLP) (77). Many of the karyotypic changes are translocations, which take place around a repeated element of intermediate frequency. This element, called the major repeat sequence (MRS), will be described in detail below.

The standard karyotype of *C. albicans* is defined as that in which chromosomes containing largely homologous genetic information migrate together on a pulse-field gel. In this karyotype, the number of chromosomal bands is 8; hence, the diploid chromosome number of *C. albicans* is 16. The chromosomes are numbered from 1 (largest) to 7 (smallest). The largest chromosome is not numbered; it is denoted chromosome R because it contains the ribosomal DNA (rDNA) cistrons. The rDNA cistrons are found in tandem arrays on the two homologs of this chromosome, but the number of copies of the repeat usually differs between the two homologs. Furthermore, the number on given homolog can change upon cultivation due to sister-chromatid exchange, which changes the number of tandem repeats on the chromosome. Chromosome R was not given a number, since the positions of its homologs on a pulse-field gel are variable because of the variation in distribution of the rDNA cistrons between them.

Length polymorphism in *C. albicans* chromosomes is common. Analysis of chromosome 7 in several strains with different-sized homologs suggests that much, if

not all, of the length polymorphism is due to differences in the size of the MRS on different homologs (77). Other CLPs may be due to variation in the size of telomeres or to small translocations, which add or subtract only a few tens of kilobases in size to the chromosome affected.

2.1 Role of The MRS in Genome Variability

It now seems clear that a great deal of the karyotypic variability found in *C. albicans* is associated with the intermediate repeat element called MRS. This element has been extensively studied by Tanaka and coworkers. It consists of three elements: HOK, about 5,200 bp; RPS, varying in size from 2 to 2.9 kb; and RB2, about 4 kb (29). The numbers are approximate because it is likely that copies on different chromosomes differ in size. The MRS is found on all chromosomes except chromosome 3; an analogous but largely nonhomologous sequence (78) is found in *C. dubliniensis*. There is sequence variation in the separate copies of the MRS; for example, the different HOK sequences cloned from strain FC18 vary in homology from 82 to 97%. The arrangement of the MRS is shown in Fig. 3.

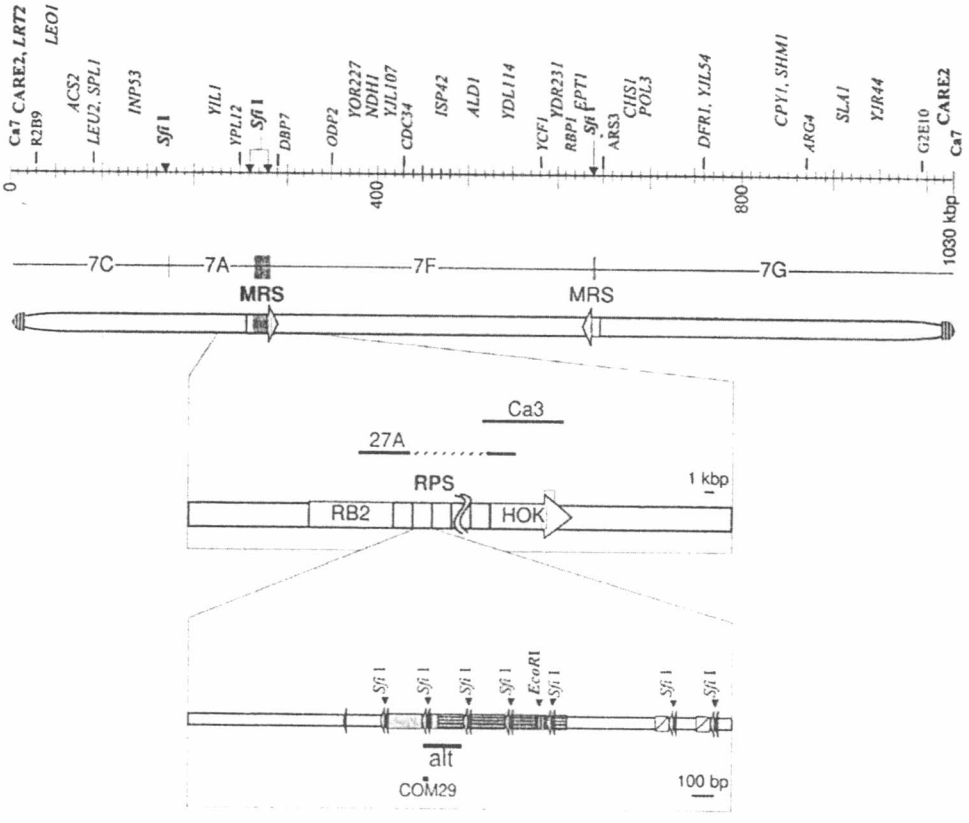


Figure 3. A map of chromosome 7 showing the orientation and fine structure of the MRS (35)

The RPS is usually tandemly repeated in the MRS, with the number of repeats of the whole unit varying from a few to as many as 50 (28). Within the RPS there are smaller tandem repeats called alts; these are about 172 bp in size. The alts are composed of 143 bp, which can vary in sequence between the different kinds of alt, followed by a sequence that is very highly conserved, COM29. This sequence contains a site for the 8-bp specific restriction enzyme, *Sfi*I. There are at least four different alts. The size of the RPS depends on the number of alt repeating units within it. The repeating alt region is flanked by DNA with high homology among the different RPS units. Homology of the RPS region is much higher than that of HOK, with the major difference coming from the number and kind of internal alt repeats (Fig. 4) (28). Because of the *Sfi*I sites in the RPS, most of the *Sfi*I fragments generated from a digestion of the genome are generated by digestion in these repeats; however, there are several *Sfi*I sites that are in unique DNA. Fig. 4. shows the structure of the members of RPS family interms of arrays of alts.

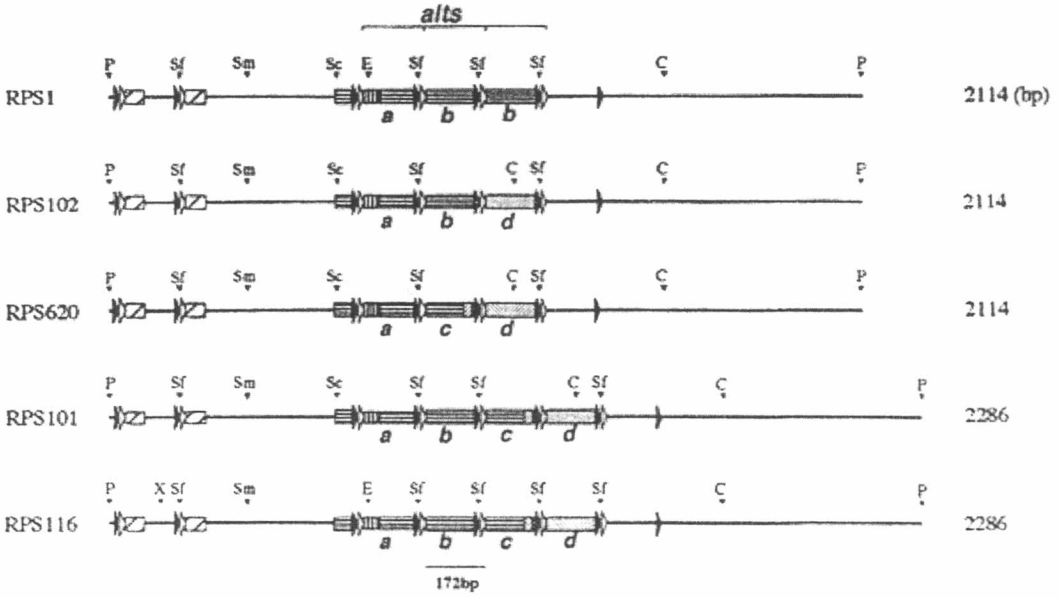


Figure 4. Schematic representation of the structure of RPS units. COM29, the 29 bp sequence, is indicated by a filled arrowhead (COM16) and an open arrowhead. alts are indicated by COM29 and a striped and /or stippled square, and different alts are distinguished by letters of the alphabet. The same pattern in squares means homology in terms of sequence. The downward-pointing closed arrowheads indicate restriction sites as follows: *Cla*I (C), *Eco*RI (E), *Pst*I (P), *Sca*I (Sc), *Sma*I (Sm), *Sfi*I (Sf), and *Xba*I (29).

The variability of composition of the MRS is the basis of a great deal of the genomic variability of the *C. albicans*. Chibana et al. have shown that chromosome length polymorphism (CLP) is usually the result of variation in the size of the MRS on the specific chromosomes. In an intensive investigation of chromosome 7 they showed that size differences in the MRS accounted for almost all of the differences in length of the homologs both within and between strains (29). It seems likely, although it is of course not known at this time that this will be true throughout the genome.

Ca3 (79), 27A (80), and RPS (30) have all been used as tools for the epidemiological analysis of *C. albicans*. Each of the three probes, Ca3, 27A, and RPS, exhibits restriction fragment length polymorphisms (RFLPs) when used as probe on genomic Southern blots of various strains. Ca3 and 27A are included in the MRS, and a part of each overlaps with RPS (Fig. 3). The number of RPSs is alterable during culture in vitro and also in vivo, so fragments that include the RPS cluster display RFLP within a strain lineage (81).

The MRS also seems very important in the frequent chromosomal translocation that are found among *C. albicans* isolates. In WO-1, the strain whose translocations have been most completely characterized, all seem to have occurred at MRS sites. Translocations at one of the repeated MRS sequences do not affect the organization of genes in the unique DNA between the repeats (77). Since RPS has several sites for the 8-bp-specific restriction enzyme *Sfi*I, the *Sfi*I fragment patterns of most *C. albicans* strains are similar. Chibana et al. demonstrated that translocations involving chromosome 7 in several clinical strains also occurred at the MRS and suggested that in *C. albicans*, synteny should be defined not as the association of genes on a chromosome but as association on an *Sfi*I fragment, since these fragments seem to be the conserved units of the *C. albicans* genome (77).

2.2 Telomeres and Subtelomeric Repeats

A sequence corresponding to the *C. albicans* telomeric repeat was isolated in 1991 by Sadhu et al. (79). They cloned it by chance in a study of repeated genomic fragments. The *C. albicans* telomere is more complicated than that of most fungi: it consists of a 23-bp repeat (82). However, this repeat shares a conserved sequence motif with many budding yeasts (83). The size of the telomere in strain WO-1 is dependent on temperatures; the number of repeats is greater at higher

temperatures. Determination of the complete map of chromosome 7 (84) revealed that one of the intermediate repeated sequences previously isolated in two laboratories, CARE2 (Rel-1) (85), constitutes a subtelomeric repeat in *C. albicans*. The absence of this repeated sequence was shown to be a differential diagnostic characteristic between *C. albicans* and *C. dubliniensis* (86). The subtelomeric region contains sequences homologous to the reverse transcriptase of LINE-type retrotransposon.

2.3 Chromosome Loss or Grain

A relatively common occurrence in *C. albicans* is chromosome loss. Aneuploid strains of *C. albicans* have been reported since the early 1980s (87). If a strain has lost one homolog of a chromosome, it grows slowly (74). Janbon et al. found that selection for growth on L-sorbose resulted in loss of one homolog of chromosome 5. Growth on glucose selects for strains that have two copies of the remaining homolog. The same group later showed that the loss of chromosome 4 is associated with resistance to fluconazole in one *Candida* strain (88). It is possible that there are other conditions that select strains that have undergone chromosome nondisjunction.

3. *C. albicans* Chromosomes of strain WO-1 (35)

Chromosome R

Chromosome R is the largest of the chromosomes, comprising more than 4,000 kbp. It carries the rDNA cistrons. The size of the homologs varies in different strains because the partitions of these cistrons is usually unequal. Chromosome R contains at least one copy of the MRS, but the exact number has not yet been determined. There are four *Sfi*I sites on one homolog and three on the other in strain 1006. No translocations involving chromosome R have been reported in *C. albicans*.

Chromosome 1 (3,400 kb)

Chromosome 1 has four to five *Sfi*I sites on each homolog. There is at least one MRS site located.

Chromosome 2 (2,600 kb)

Chromosome 2 has an MRS sequence located close to one end.

Chromosome 3 (1,800 kb)

Chromosome 3 is only one of the eight *C. albicans* chromosomes that does not contain at least one copy of the MRS. It dose have one of the repeated flanking sequences RB2 (89), but it lacks HOK and RPS. No translocations involving this chromosome have been found.

Chromosome 4 (1,700 kb)

Chromosome 4, like chromosome 7, has two copies of the MRS sequence. The two are arranged as inverted repeat. In addition, thare is one other *Sfi*I sute on one homolog. Chromosome 4-chromosome 7 translocations have been identified in several strain (90).

Chromosome 5 (1,200 kb)

Chromosome 5 contains one copy of the MRS. This copy can contain as many as 50 repeats of the RPS perhaps because of large MRS, chromosome 5 seems to be very frequently involved in translocations.

Chronosome 6 (1,100 kb)

Chromosime 6 contains one copy of the MRS. It is involved in a translocation with chromosome 5 in WO-1.

Chromosome 7 (1,000 kb)

Chromosome 7 is the most completely characterized of the *C. albicans* chromosomes, since a complete sequence-tagged site map has been published. Because it was the first chromosome for which the complete physical map was analyzed in several strains. This analysis has shown that both translocations and variations in the size of the MRS can lead to this polymorphism (77).

5. Multiple Karyotype Variations

Although most laboratory strains of *C. albicans* show relatively stable karyotypes in vitro (except for chromosome R) (91), several studies have demonstrated that karyotype variation can be associated with phenotypic variation can be associated with phenotypic variation. The first to do so were Suzuki and coworkers, who demonstrated that the reversible transition in a clinical isolate from a smooth to semirough colony morphology was accompanied by correlated changed in the electrophoretic karyotype (92). The karyotypes of the variants contained extra chromosomal bands but retained the five bands of the parent strain.

In one case translocation was shown to occur at a frequency high enough so that it can be selected for by growth conditions. This first clear demonstration of a specific chromosome rearrangement related to a specific phenotypic change was the observation by Wickes et al. that when *Candida stelatoidea*, a subspecies of *C. albicans* that is normally unable to grow on sucrose, is plated on medium containing the sugar as sole carbon and energy source, spontaneous Suc^+ strains arise. These sucrose-metabolizing variants all contained four particular chromosome rearrangements (93). Rustchenko et al. found that karyotypic rearrangements were associated with colony morphology phenotypes in strain 3153A. However, there was no specific correlation of variant karyotype with colony phenotype (75). These authors suggested that karyotype variation is a means of achieving genetic variability in *C. albicans*. Many of the variations involved chromosome R, these variations seem to occur without affecting the phenotype in many strains (75).

Molecular-based methods of strain typing of *Candida* species

The methods originally adopted to characterize and type specific *Candida* strains were based on the analysis of phenotypic traits, including colony morphology and color on indicator media, biochemical and serological tests, carbohydrate assimilation tests, and resistotyping. Although all of these tests are relatively easily performed, their specificity tends to be low since many unrelated strains of *Candida,* and sometimes even different *Candida* species, often share phenotypic characteristics. In addition, phenomena such as phenotypic switching can affect the reproducibility of results obtained with such tests. For these reasons, strain discrimination methods based on the analysis of genotypic differences have been developed. In particular, these methods rely most often on comparisons of the genomic content and organization or on the nucleotide sequence of specific loci of individual strains. To be useful in epidemiological studies each method must, above all, be very specific and allow the accurate identification of each strain reproducibly. Ideally, the same method should also be applicable to multiple species. In cases of large-scale studies, the method used should have the capacity for use with large sample numbers; it should also be rapid and inexpensive and ideally should not be labor-intensive. A wide variety of methods have been developed and adapted to detect genetic polymorphisms in yeast species. These methods vary in their resolution, and each has specific

disadvantages and advantages that should be taken into account when choosing a method to investigate a particular clinical problem. Indeed, it is often recommended that more than one method be used to achieve optimal results. In tandem with the development and refinement of molecular epidemiological techniques, improved computer-assisted data analysis packages, such as DENDRON and GelCompar, have been developed and applied (94). These systems have the capacity to automatically correct gel electrophoresis anomalies, calculate fingerprint pattern relatedness, and draw dendrograms. Most important, these systems permit fingerprint patterns generated in different experiments to be validly compared by reference to the fingerprint patterns of control reference strains used in each experiment. This allows the accurate and quantitative comparison of strains from large data sets and the development of comprehensive databases with which new strains can be easily compared (35).

1. Electrophoretic Karyotyping Analysis

Chromosome size variation is assayed via an electrophoretic technique, electrophoretic karyotype analysis, which uses electric fields of alternating orientation to move intact chromosome through the agarose gel matrix. The approach developed by Schwartz and cantor (95) and further modified by Carle and Olson (96) and Chu et al. (97), which involves the use of pulsed-field electrophoresis (PFGE) to separate very large pieces of DNA. The original apparatus devised for this technique used two sets of electrodes set at an angle of 90° with respect to each other, with the current being switched from one set to the other at regular intervals. Carle and Olson called this sort of technique orthogonal field alternation gel electrophoresis (OFAGE) (96) and called a second pulsed-field device field inversion gel electrophoresis (FIGE) (98). The latter technique uses a normal gel box but reverses the field at regular intervals. The OFAGE approach tends to give gels in which the DNA fails to run in a straight line due to field inhomogeneities. To overcome this problem, Chu et al. (97) devised contour-clamped homogeneous electric field gel electrophoresis (CHEF). This requires a special hexagonal electrodes set. A commercial adaptation of OFAGE which also avoids the effects of field in homogeneity is available from Beckmann (TAFE, transverse alternating field electrophoresis (99). To separate a particular collection of DNA molecules, a specific set of conditions must be used. The variables

include the length of the pulse, the voltage applied, the buffer used, the percentage of agarose in the gel, and the temperature (100).

With the invention of pulse-field gel electrophoresis chromosome-sized fragments of the yeast genome were readily separated in a gel using an alternating electric field, which allows the separation of DNA fragments that are too large to be electrophoresed (95). The general technology is straight forward. Cells are mixed with enzymes to remove the cell wall and then embedded in an agarose plugs. Protease and detergent are added, and the cells are incubated to remove membranes and digest protein. The agarose gel protects the large DNA molecules from shearing forces. The agarose plug is placed in a well at the top of an agarose slab gel, and electrophoresis is carried out according to the specifications of the particular separating system. Yeast chromosome-sized DNA fragments are separated according to size and can be identified by ethidium bromide staining. Specific chromosomes can be identified using Southern blot hybridization with chromosome-specific DNA probes (94).

PFGE is one of the techniques used most widely in *Candida* epidemiology. This method can be used to discriminate between individual strains due to the fact that the sized of individual chromosome in different *Candida* strains can vary widely, resulting in different patterns of chromosome-sized bands known as karyotypes. Althought effective at strain typing in many *Candida* species (21, 101), karyotypes can vary as a result of phenotypic switching, a feature commonly found in *C. albicans* isolates in particular. In addition, the equipment required is extensive, sample preparation is labor-intensive, and gel can take 72 h or more to run. Consequently, PFGE is not suitable for the analysis of large numbers of samples. In addition to the separation of chromosomes from *Candida* isolates, PFGE can also be used in RFLP in which restriction enzymes that cleave genomic DNA infrequently (e.g., *Sfi* I and *Not* I) generate large restriction fragments that are not resolvable by conventional agarose gel electrophoresis. The pattern of fragments thus obtained can be used to discriminate between strains (31, 102).

2. Restriction Fragment Length Polymorphism (RFLP) Without Hybridization

RFLP was the first method used to compare the genotypes of individual strains of *Candida* species and was used extensively in the earliest studies investigating the

molecular epidemiology of *Candida* infections (35). Separation depends upon the percentage of agarose in the gel, the electrophoresis time, the voltage, and the particular endonuclease(s) employed. All of the experimental conditions must be determined empirically. The pattern is based on different fragment lengths determined by the restriction sites identified by the particular endonuclease(s) employed. Variation among strains can occur as a result of changes in restriction site sequences, secondary modification of restriction sites, deletion of recognition sites, or deletions and insertions in the sequences between recognition sites (94).

RFLP is a simple method for genotypes of *Candida* species that relies on the purification and subsequent digestion of total genomic DNA with specific restriction enzymes (e.g., *Eco*RI or *Hin*FI). Following digestion, the resulting DNA fragments are stained with ethidium bromide, and the resulting banding patterns compared visually. In most cases the restriction patterns obtained are very complex, often comprising thousands of bands, and are therefore very difficult to compare objectively, whether visually or using computer-assisted methods (35). In some cases patterns of bands of above-average intensity, due to the presence of repeated sequences such as rDNA, can facilitate strain identification in species (12, 102-104). However, despite the fact that RFLP is a simple and relatively inexpensive technique, its lack of discriminatory ability has largely led to it being replaced by other methods.

3. Restriction Fragment Length Polymorphism (RFLP) and Species-Specific DNA Fingerprinting Probes

A general RFLP pattern of eukaryotic cell DNA visualized by ethidium bromide staining is poorly resolved primarily because all restriction fragments are staining. Fragments within the pattern are in fact highly resolved, and a method for selectively visualizing a limited number of fragments will provide a more highly resolved fingerprint pattern for analysis. To visualize particular fragments in the pattern, one can probe a southern blot of the RFLP gel with a radiolabeled or biotinylated DNA sequence that recognize one or more fragments as a result of sequence homology (94).

The efficacy of DNA profiling of *Candida* strains has been greatly enhanced by the identification of species-specific repeated sequences that are distributed throughout the genome. To simplify the banding patterns obtained by restriction

endonuclease digestion, usually with *Eco*RI, following electrophoresis (i.e., as in RFLP analysis), these can be transferred to nitrocellulose or nylon membranes using Southern blotting and subsequently hybridized with labeled fingerprinting probes corresponding to species-specific repetitive elements (35).

1. rDNA and mDNA Probes

DNA probes that hybridized to repeat sequences dispersed throughout the genome were sought, since it was belived that either the repeat sequences or bordering sequences would vary among strains and that the resulting data from one Southern blot hybridization pattern would be complex enough to reflect genetic distance (24). In the case of *C. albicans*, among the first probes used were probes that corresponded to RNA genes (24) and mitochondrial DNA sequences (105). However, these probes yielded too few bands (e.g., three to five) for meaningful analysis. rDNA and mitochondrial DNA, therefore, have not been generally used in broad epidemiological studies of the infectious fungi, and neither method has been validated for the different levels of genetic resolution (94).

2. Repetitive and Complex DNA Probes

To date, the most successful and popular hybridization probes for fungi have been cloned fragments containing repetitive genomic sequence (94). In the case of *C. albicans*, Ca3 (79), 27A (80), and RPS (30) probes (Fig. 5) have been used as tools for epidemiological analysis of *C. albicans* infections. When genomic Southern blots of various strains are probed with any of these sequences, restriction fragment-length polymorphisms are found (106). These probes yield strain-specific fingerprint patterns composed of 10 to 20 bands of varying intensity (89). Ca3, 27A, and RPS are included in the Major Repeat Sequence (MRS) that described above (in genome of *Candida*), and all share some common sequences (84, 89) (Fig. 3 and Fig. 5). A major cause of the variation revealed by these probes is the diversity in the size of RPS unit, ranging from 1.7 to 3.5 kbp, due to the number of short periodic internal repeats that found in different strains (29, 107). A second reason is heterogeneity in sites for the restriction enzyme *Eco*RI. Sites for this enzyme, which is used in many epidemiological studies, are not highly conserved in the RPS family (29), so that *Eco*RI cuts the whole RPS cluster out occasionally. The number of RPSs varies

among MRSs, chromosomes, and strains. The combination of all these factors leads to different profiles of hybridization with Ca3, 27A, and RPS. Sister chromatid exchange and interchromosomal gene conversion presumably operate to change the number of RPS repeats and the specific sequence of some of the RPSs at MRSs (81). These changes result in the diversity in the Southern-hybridization profiles of strains as they diverge from a common ancestor (77). Another *C. albicans* probe that yields highly variable fingerprints is the CARE-2 probe (85); however, the extreme variability of the bands generated (e.g., the lack of monomorphic bands) decreases the efficacy of this probe for the analysis of distantly relates (94).

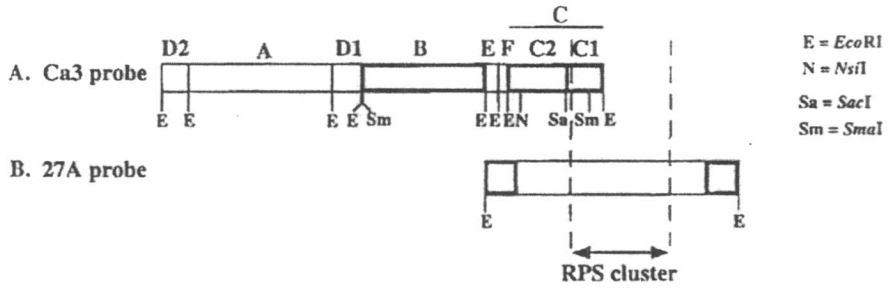


Figure 5. Physical map of the DNA probe Ca3, 27A and an RPS cluster of *C. albicans.* Known DNA sequences are represented by boxes with thick walls. Relative positions are assigned by DNA homology. RPS regions contain different numbers of full-length RPS units and are demarcated by dashed borders (94).

Species specific probes have since been developed for use in the epidemiological analysis of other clinically important *Candida* species, including *C. glabrata* (108), *C. tropicalis* (78), *C. krusei* (109), and *C. dubliniensis* (78). The simple and reproducible DNA fingerprints obtained with these probes greatly facilitate the comparison of strains. The DNA patterns obtained with species-specific fingerprinting probes are amenable to computer-assisted analysis that can provide much more reliable and efficient quantitative comparison of large numbers of moderately related strains. Although this method is highly accurate and reproducible, Southern hybridization experiments are labor-intensive and time-consuming; therefore, this method is not particularly suitable for the rapid analysis of very large numbers of strains. Despite this, DNA fingerprinting using *Candida* species-specific

semirepetitive probes is now the "gold standard" strain typing method for *Candida* species (35).

4. Randomly Amplified Polymorphic DNA (RAPD) Analysis

PCR technology, in addition to its application in the rapid identification of *Candida* species, has also been applied to the rapid differentiation of individual *Candida* strains belonging to particular species. However, RAPD differs from conventional PCR in that it is based on the use of a single oligonucleotide (usually 10 to 15 bp) of arbitrary sequence using low stringency amplification reaction conditions. Due to the low annealing temperatures used, primers can bind to many sites throughout the genome, thus allowing the amplification of products that vary in size. If the optimum amplification conditions and primer sequence have been determined empirically, these fragments, when separated by agarose gel electrophoresis and subsequently stained with ethidium bromide, can yield strain-specific fingerprint patterns comprising three to six bands. Differential banding patterns between strains occur as a result of nucleotide variation as the primer annealing sites (110). The facts that so little sample DNA is required for RAPD and that PCR is rapid and easy to perform have led to the widespread use of this technique in the analysis of Candida populations (106, 111, 112). However, extreme care must be taken to prevent variation in experimental conditions, as even subtle changes in temperature or magnesium concentration can lead to the appearance or disappearance of bands, thus affecting the reproducibility of the technique (113).

5. Multilocus Enzyme Electrophoresis (MLEE)

Nucleic acid are not the only targets used to detect variation between strains. Nucleotide polymorphisms can result in amino acid substitutions in proteins that can lead to a change in the charge of the protein that can affect the proteins' mobility. Thus, when cell extracts are separated on starch gels and stained using specific enzyme strains, strain-specific straining profiles can be obtained. As with DNA fingerprinting methods, these protein-derived data can be analyzed by computer-assisted methods. MLEE is a powerful technique that has been widely applied to the analysis of *Candida* species and has proved particularly useful in population studies

(106, 114). However, despite its usefulness, MLEE is a time-consuming technique that required specialized equipment.

In vitro Antifungal Susceptibility Testing of Yeasts

In vitro antifungal susceptibility tests are similar in design to tests with antibacterial agents and are performed for the same reasons. Ideally, in vitro susceptibility tests (i) provide a reliable measure of the relative activities of two or more antifungal agents, (ii) correlate with in vivo activity and predict the likely outcome of therapy, (iii) provide a means with which to monitor the development of resistance among a normally susceptible population of organisms, and (iv) predict the therapeutic potentials of newly discovered investigational agents.

With the use of both established and investigational agents has come the recognition of resistance to one or more antifungal agents in selected isolates. As a result, clinical laboratories are now being asked to assume a greater role in the selection and monitoring of antifungal chemotherapy. The methods that have been applied to antifungal susceptibility testing include broth dilution (macro-and micro dilution), agar dilution, and disk diffusion. Currently, two commercial antifungal susceptibility tests are being evaluated. The colorimetric microdilution test developed by Alamar bioscience, Inc. (Sacraments, Calif.) and the agar diffusion antifungal strip E test by AB Biodisk (Solna, Sweden).

1. NCCLS Broth-based Methodology for Yeasts

After passing through the stages of being a (P)roposed document (M27-P) in 1992 (115) and a (T)entative document (M27-T) (116) the NCCLS M27 methodology for yeasts became an (A)pproved level docoment (M27-A) in 1997 (117) Publication of M27A was the culmination of approximately 15 years of collaborative work. Although imperfect, this method has the great advantage of high interlaboratory reproducibility. Combined with the secondary knowledge generated during its development concerning factors that both add to and detract from reproducibility, M27 has become a valuable tool for all investigators in this field. The method specifies inoculum size and preparation, test medium, incubation time and temperature, and end-point reading for flucytosine, amphotericin B, fluconazole, ketoconazole, and itraconazoe (Table 4). The reference method was initially defined

solely as a macrodilution methodology, but this was extended with a microdilution method that was included beginning with the T-level revision of the document. The microdilution method has become the method of choice because of its less cumbersome nature. Microdilution plates may easily be prepared and frozen well in advance of use.

The M27 method continues to be augmented. The M27-A document provides Quality Control (QC) limits at 48 h for amphotericin B, flucytosine, fluconazole, ketoconazole, and itraconazole. QC data were recently expanded to include 24-h QC limits as well for these previously mentioned agents (118). In addition, QC limits at 24 and 48 h for voriconazole (UK-109,496), caspofungin (MK-0991), ravuconazole (BMS 207147), posaconazole (SCH 56592), and anidulafungin (LY303366, V-echinocandin) have been provided (118).

Table 4. Summary of the M27-A method developed by the NCCLS (119)

Characteristic	**Implementation in the M27-A methodology**
Suitability	Yeasts
Inoculum	0.5×10^3 CFU/ml
Inoculum standardization	Spectrophotometric, with reference to 0.5 McFarland $BaSO_4$ turbidity standard
Test medium	RPMI 1640, buffered to pH 7.0 with 0.165 M MOPS
Format	Macrodilution or microdilution
Temperature	35 ^{0}C
Duration of incubation	48 h (*Candida* spp.) 72 h (*C. neoformans*)
End point	Optically clear well for amphotericin B, ~80% reduction in growth (macrodilution testing with azoles), prominent decrease in turbidity (microdilution testing with flucytosine and the azoles antifungals)
QC isolates and drug	Two isolates of *Candida* against amphotericin B, flucytosine, fluconazole, voriconazole, ketoconazole, itraconazole, caspofungin (MK-00991), revuconazole (BMS 207147), posaconazole (SCH 56592), and anidulafungin (LY303366, V-echinocandin)

2. Commercial Broth-based MIC Systems.

Recently studied broth-based commercial systems include Candifast (International Microbio/Stago Group, Milan,Italy), Integral Systems Yeasts (Liofilchem Diagnostic, L'Aquila, Itary), and Fungitest (Bio-Rad SDP [formerly Sanofi Diagnostics Pasture], Paris, France). These kits provide reagents for testing a limited number of drug concentrations selected as critical breakpoint values. Direct comparisons have found these methods to possess limited correlation with the M27-A reference method. ATB Fungus (API-bioMerieux, Marcy l'Etoile, France), Mycostandard (Institut Pasteur, Paris, France), and Mycototal (Behring Diagnostic, Rueil-Malmaison, France) are similarly designed systems for which only limited data are available on correlation with the M27-A methodology (119).

A commercial system that includes a colorimetric response based on the Alamar Blue redox marker is marketed as Sensititre Yeast One Colorimetric Antifungal Panel (Trek Diagnostic Systems, Inc., Westlake, Ohio). This kit tests a full range of drug concentrations, and rates of concordance of ≥85% with the reference method are generally obtained, although one group noted some difficulties with testing of *C. neoformans.* The ASTY colorimetric panel (Kyokyuto Pharmaceutical Industrial, Ltd., Tokyo, Japan) demonstrated a similarly good correlation with the M27 reference method. Some of these kits are currently being evaluated by the U.S. Food and Drug Administration; however, to date none have received approval for marketing in the United States (119).

3. Etest

Etest (AB Biodisk, Solna, Sweden) is a proprietary, commercially available method for antimicrobial susceptibility testing. MICs are determined from the point of intersection of a growth inhibition zone with a calibrated strip impregnated with a gradient of antimicrobial concentration and placed on an agar plate lawned with the microbial isolate under test. This methodology has been adapted to a number of antifungal agents. Both nonuniform growth of the fungal lawn and the frequent presence of a feathered or trailing growth edge can make end-point determination difficult. However, with experience and standardized techniques, the correlation between this method and the reference method has been acceptable for most *Candida* spp. and the azole antifungal agents. One study, however, did observe less than 50%

concordance between the methods for *C. tropicalis* and *C. glabrata* with fluconazole , and another found a limited correlation for ketoconazole and *C. lusitaniae*. These results suggest that users of the Etest methodology should carefully validate their local procedures against the reference methodology (119).

The choice of growth medium appears critical with this technique, with RPMI-based agars generally appearing most useful (e.g., this agar gave >96% correlation with the reference microdilution method in one study). The NCCLS method has limited ability to identify isolates of *Candida* and *Cryptococcus* resistant to amphotericin B. By substituting antibiotic medium 3 broth buffered to pH 5 to 7 for RPMI 1640 medium buffered to pH 7, Rex et al. were able to discriminate between amphotericin B-resistant and–susceptible isolates of *Candida*. Further studies to identify suitable quality control isolates and to establish interlaboratory reproducibility are in progress. However, the Etest may provide a simpler alternative. A comparision of the Etest and the NCCLS broth macrodilution method with 91 clinical *Candida* isolates found that agreement within ±2 dilutions between the two methods was 95% for fluconazole and 96 to 97% for amphotericin B. Moreover, the Etest was able to identify amphotericin B-resistant isolates on glucose-supplemented RPMI 1640 agar as well as on undefined antibiotic medium 3. QC Etest limits for the two NCCLS M27 QC isolates (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) against amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole have been proposed (119).

Antifungal Drug in Current Use

Given that ergosterol biosynthesis is specific to fungi and is necessary for their growth, this feature has been largely exploited by scientists responsible for the design and isolation of antifungal agents. As a result, most of the antifungal agents used in medicine target the ergosterol biosynthetic pathway and include polyenes, azole, allylamines, or morpholine derivatives (Fig. 6). Among currently used antifungal agents, 5-fluorocytosine (5-FC) is one of the few that does not directly interfere with the ergosterol biosynthesis pathway (35).

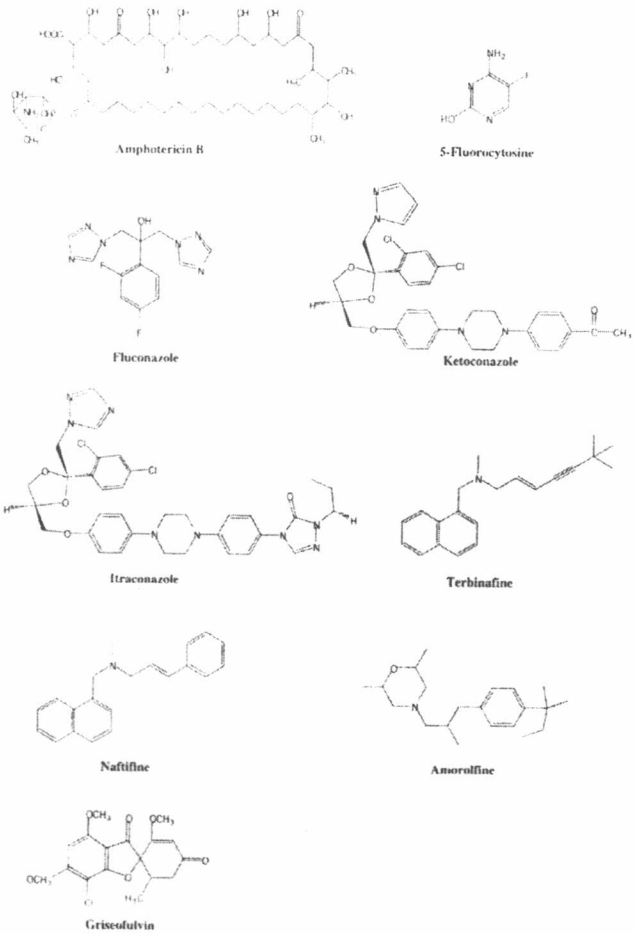


Figure 6. Chemical structures of antifungal agents in current use (35)

1. Antifungal Agents Affecting Fungal Sterols

The three major groups of antifungal agents in clinical use azoles, polyenes, and allylamine/thiocarbamates, all owe their antifungal activities to inhibition of synthesis of or direct interaction with ergosterol. Ergosterol is the predominant component of the fungal cell membrane (120).

1.1 Azole-based Antimycotic Agents

Azole antifungal agents discovered in the late 1960s are synthetic compounds belonging to the largest group of antifungal agents. Azole antifungal agents used in medicine are categorized into N-1 substituted imidazoles (ketoconazole, miconazole, clotimazole) and triazoles (fluconazole, itraconazole)

(Fig. 6). The new generation of azole antifungal agents under development (posaconazole, revuconazole, voriconazole) belongs also to triazoles (35).

Mechanism of action. Ergosterol served as a bioregulator of membrane fluidity and asymmetry and consequently of membrane requires in fungal cells. Integrity of the cell membrane requires that inserted sterols lack C-4 methyl groups (35). Azoles have a cytochome P450 as a common cellular target in yeast or fungi. This cytochrome P450 is named CYP51A1, according to an internationally recognized nomenclature, and is involved in the 14α-demethylation of lanosterol. CYP51A1 is also now referred to as Erg11p, the product of the *ERG11* gene. The unhindered nitrogen of the imidazole or triazole ring of the azole antifungal agents binds to the heme iron of the cytochrome P450 as a sixth ligand, thus inhibiting the enzymatic reaction. The affinity of imidazole and triazole derivatives is not only dependent on this interaction, but is also determined by the N-1 substituent, which is actually responsible for the high affinity of azole antifungal agents to their target. Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14α-methylated sterols (lanosterol, 4, 14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of a plasma membrane with altered structure and function (120).

1.2 Polyenes

From the 1950s until the discovery of the azoles, polyene antifungal agents such as amphotericin B (AmB) (Fig. 6) represented the standard of therapy for systemic fungal infections (120). AmB is produced by *Streptomyces nodosus* (121). AmB can form soluble salts in both basic and acidic environments, is not orally nor intramuscularly absorbed, and is virtually insoluble in water. AmB has been solubilized in a micellar preparation with deoxycolate for its clinical use for more than 3 decades (122). Although AmB is the most effective antifungal drug available, it possesses several drawbacks limiting its clinical utility. Systemic and renal problems are often encountered with AmB (123). To reduce its unwanted side effects, AmB has been formulated in liposomes (Ambisome) (124), lipid complexs (Abelcet) (125), and colloidal suspension (Amphotec) (119,288) to allow the use of higher doses of AmB against fungal pathogens and at the same time reduce its toxic effect to mammalian cells. AmB incorporated into liposomes may participate in a selective

transfer mechanism from the "donor" liposome to the ergosterol-containing "target" in the fungal cell membrane or with the help of the fungal and/or host phospholipases (126). AmB has a strong fungicidal effect on most important yeast and fungal pathogens. A liposomal preparation of nysatin, another polyene antifungal agent (Nyotran), is currently undergoing preclinical and clinical evaluation (127).

Mechanism of action. The primary mode of action of polyenes including AmB or nystatin is to bind ergosterol in the membrane bilayer of susceptible fungi. This interaction is thought to result in the production of aqueous pores consisting of polyene molecules linked to the membrane sterols. This configuration gives rise to a pore like structure in which the polyene hydroxyl residues face inward, leading to altered permeability, leakage of vital cytoplasmic components (mono- or divalent cations), and death of the organism (128).

1.3 Allylamines

Allylamines, such as terbinafine and nafitine, have been developed as a new class of ergosterol biosynthetic inhibitors that are functionally as well as chemically distinct from the other major classes of ergosterol-inhibiting antifungal agents. Terbinafine (Fig. 6) is highly effective against dermatophytes in vivo and in vitro. A recent study of terbinafine by the Natonal Committee for Clinical Laboratory Standards M27 method showed that its genometric mean MIC against 179 clinical isolates of *C. albicans* was 1.2 μg/ml. Furthermore, preliminary evidence from our group and from Ryder and coworkers indicates that terbinafine has good activity against at least some azole-resistant *C. albicans* strains (35).

Mechanism of action. Allylamines act by inhibiting early steps of ergosterol biosynthesis. This inhibition coincides with accumulation of the sterol precursor squalene and the absence of any other sterol intermediate, suggesting that allylamine inhibition of sterol synthesis occurs at the point of squalene epoxidation, a reaction catalyzed by aqualene epoxidase. Fungal cell death is related primarily to the accumulation of squalene rather than to increase membrane permeability, leading to disruption of cellular organization (120).

2. Compounds Active Against Fungal Cell Walls

The fungal cell wall contains conpounds, such as mannan, chitin, and α- and β -glucans, that are unique to the fungal kingdom. Since these compounds are not found elsewhere in nature, they have been identified as possible targets that provide selective toxicity advantages. The cell wall of this yeast is a multilayered structure composed of chitin, β-glucans and mannoprotein, with the last two constituents making up to 80% of the wall mass. The outer layers are composed of mannan, mannoprotein, and β-(1,6)-glucan, while the inner layers are predominantly β-(1,3)-glucan and chitin with some mannoprotein (120).

Inhibitors of Glucan Synthesis

Of the three groups of compounds (aculeacins, echinocandins, and papulacandins) that are specific inhibitors of fungal 3β-glucan synthase, only echinocandin are being activity pursued in clinical trials to evaluate their safety, tolerability, and efficacy against candidiasis. Echinocandins, which are lipopeptides, have fungicidal activity both in vitro and in vivo against *Candida* and *Aspergillus* species (120).

Mechanism of action. β-Glucan inhibitors act as specific noncompetitive inhibitors of β-(1,30-glucan synthetase, a large (210-kDa) integral membrane heterodimeric protein. Treatment of fungal with these compounds inhibits the synthesis of the structural glucan component without affecting nucleic acid or mannan synthesis. Inhibitors of glucan synthesis also have secondary effects on other components of intact cells including a reduction in the ergosterol and lanosterol content and an increase in the chitin content of the cell wall. Inhibition of β-(1,3)-glucan synthetase results in cytological and ultrastructural changes in fungi characterized by growth as pseudohyphae, thickened cell wall, and buds failing to separate from mother cells. Cells also become motically sensitive, with lysis being restricted largely to the growing tips of budding cells (120).

3. Compounds Inhibiting Nucleic Acids

5-Fluorocytosine (5FC)

5FC (Fig. 6) is a fluorinated pyrimidine with inhibitory activity against many yeasts, including *Candida* and *Cryptococcus neoformans*. It is fungicidal in susceptible yeasts and fungi. The initial promise of this agent has been diminished by the high prevalence of primary resistance in many fungal species. Two surveys of *C. albicans* conducted by Stiller et al. and Defever et al. provided estimates of resistance frequencies. The majority of the candidal isolates studied were susceptible (60 and 50%), but significant percentages were partially resistant (36 and 37%) or highly resistant (4 and 6%). Today, 5FC is used in combination with other antifungals, such as amphotericin B and fluconazole, but only rarely as a single agent (35).

Mechanism of action. 5-FC is taken up by fungal cells by a cytosine permease and is deaminated by a cytosine deaminase to 5-fluorouracil (5-FU), a potent antimetabolite. 5-FU can be converted to a nucleoside triphosphate and, when incorporated into RNA, causes miscoding. In addition, 5-FU can be converted to a deoxynucleoside, which inhibits thymidylate synthase and, thereby, DNA synthesis. 5-FC shows little toxicity in mammalian cells, since cytosine deaminase is absent or poorly active in these cells. 5-FU is, however, a potent anticancer agent but is impermeable to fungal cells (129, 130). The conversion of 5-FC to 5-FU is possible by intestinal bacteria, and, therefore, 5-FC can show toxicity in oral formulation. (131).