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A *Candida albicans* Petite Mutant Strain with Uncoupled Oxidative Phosphorylation Overexpresses *MDR1* and Has Diminished Susceptibility to Fluconazole and Voriconazole[▽]

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We showed that a *Candida albicans* petite mutant in which oxidative phosphorylation is uncoupled was eightfold more resistant to fluconazole and voriconazole than SC5314 but equally susceptible to ketoconazole, itraconazole, and amphotericin B. Strain P5 significantly overexpressed *MDR1*, which likely accounts for the decreased drug susceptibility.

The azole antifungal agents are widely used to treat diverse *Candida albicans* infections. Resistance to these drugs can contribute to treatment failures (28, 29). The molecular bases of resistance are best characterized for fluconazole (12, 23, 42). Mechanisms include drug efflux due to overexpression of ATP-binding cassette (ABC) transporters encoded by *CDR1* and *CDR2* (10, 30, 32) and major facilitator transporters encoded by *MDR1* and *FLU1* (5, 15, 30); increased expression of *ERG11*, which encodes the target enzyme 14-α-demethylase; mutations in *ERG11* (21, 38); and mutations in *ERG3* that inactivate $\Delta^{5,6}$ desaturase and cause accumulation of growth-supporting 14α-methylfecosterol (19, 20, 27). More recently, aneuploidy, particularly of chromosome 5, has been implicated and considered likely due to increased copy numbers of *ERG11* and other genes (36).

Petite mutant strains of *Candida glabrata* and *Saccharomyces cerevisiae* are resistant to fluconazole and other azoles through mechanisms that are not fully defined (1–4, 34, 42). Although a *C. albicans* petite mutant was tolerant to amphotericin B (14), susceptibility of such strains to azoles is unknown. In a previous study, we serially passed *C. albicans* strain SC5314 through the spleens of mice and recovered a petite mutant (called P5) in which oxidative phosphorylation was uncoupled (7). The primary objectives of this study were to determine the susceptibility of SC5314 and strain P5 to azoles and, in the event of differences, to study previously characterized mechanisms of resistance.

Using a standard broth macrodilution method (24), we found the MICs of fluconazole and voriconazole to be eightfold higher against strain P5 than SC5314 but still within susceptible ranges (Table 1). The MICs of ketoconazole and itraconazole did not differ between the strains, nor did MICs of amphotericin B (Table 1). Moreover, amphotericin B time-kill curves (8) were similar at drug concentrations 0.25× and 1× MIC (Fig. 1).

TABLE 1. In vitro susceptibilities of strains SC5314 and P5 to antifungal agents and other drugs

Drug	MIC (μg/ml)[a]			
	24 h		48 h	
	SC5314	P5	SC5314	P5
Fluconazole	**0.125**	**1.0**	**0.125**	**1.0**
Ketoconazole	0.064	0.064	0.064	0.064
Itraconazole	0.016	0.016	0.016	0.016
Voriconazole	**0.004**	**0.032**	**0.004**	**0.032**
Amphotericin B	0.06	0.06	0.125	0.125
Brefeldin A	**4**	**16**	**8**	**32**
Cerulenin	**2**	**4**	**2**	**8**
Rhodamine 6G	8	8	8	8
Cycloheximide	400	400	400	800
Nitroquinoline-*N*-oxide	0.05	0.05	0.05	0.05
Benomyl	20	20	20	20
Crystal violet	0.2	0.2	0.4	0.4
Sulfometuron	10	10	10	10

[a] Values in bold indicate MICs that differ between strains SC5314 and P5.

We used a broth microdilution method to measure the MICs of several drugs that are structurally unrelated to fluconazole but subjected to efflux by ABC and/or Mdr1p transporters

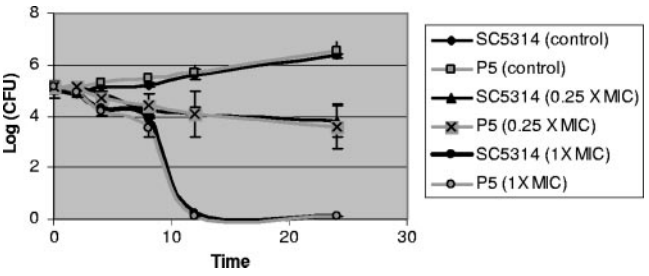

FIG. 1. Amphotericin B time-kill curves against strains SC5314 and P5. Data presented are means ± standard deviations from three experiments.

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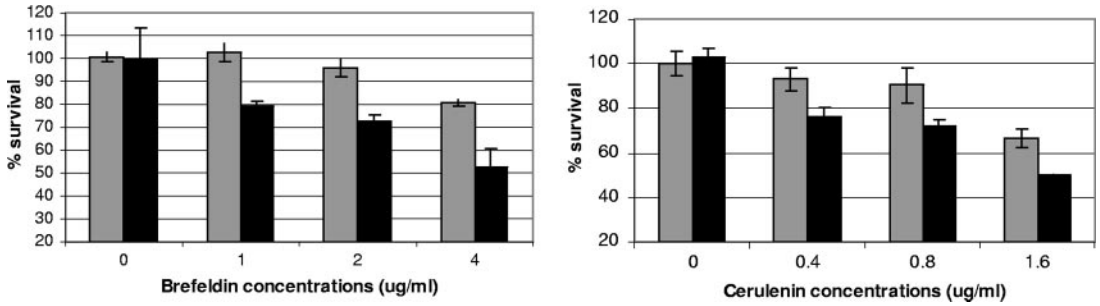

FIG. 2. Susceptibility of strains SC5314 (black bars) and P5 (gray bars) to brefeldin A and cerulenin at concentrations below the MIC. Data presented are means ± standard deviations from three experiments.

(Table 1). MICs were identical against both strains for all drugs except brefeldin A and cerulenin. MICs of these agents were consistently 4- and ≥2-fold higher, respectively, against strain P5. As a further measurement of susceptibility, we diluted overnight cultures of the strains (yeast extract-peptone-dextrose at 30°C) to an optical density at 599 nm (OD_{599}) of 0.1 in fresh yeast extract-peptone-dextrose and coincubated them at sub-MIC concentrations of brefeldin A and cerulenin (Fig. 2). As shown, survival of strain P5 was greater at all concentrations.

Using semiquantitative reverse transcription-PCR (RT-PCR) (Table 2), we demonstrated that *MDR1* expression was up-regulated in strain P5 and *CDR1* was up-regulated in SC5314 (data not shown). Levels of *CDR2* expression were very low in both strains. The expression of *ERG11*, *ERG3*, and *FLU1* did not differ between the strains. To corroborate and better quantify our findings for *MDR1*, *CDR1*, and *CDR2*, we performed real-time RT-PCR (Table 2). We demonstrated that expression of *MDR1* by strain P5 was 8.3- to 12.5-fold greater than by SC5314, whereas expression of *CDR1* was 3.1- to 5.7-fold lower (Table 3). The expression of *CDR2* by strain P5 was slightly higher than by SC5314 (Table 3).

The sequence of *ERG11* in strain P5 differed at two residues from that of our SC5314 strain and the published sequence (http://www.candidagenome.org/): F14S and A409T. *ERG3* sequences did not differ from the published sequence. Using whole-genome single-nucleotide polymorphism (SNP) microarrays to analyze the strains' genotypes (11), we found that the SNP profiles were identical. These results effectively excluded aneuploidy (A. Forche, personal communication).

To our knowledge, this is the first study of azole susceptibility in a *C. albicans* petite mutant strain. Similar to strain P5, previously described *C. glabrata* petite mutants have shown diminished susceptibility to fluconazole. Our results differed in several ways from earlier reports, however. Most notably, the *C. glabrata* petite mutants were fully resistant to both fluconazole and itraconazole, which was attributed to up-regulation of *CDR1* with a lesser contribution from *CDR2* (2, 3, 34). In strain P5, the diminished susceptibilities to fluconazole and

TABLE 2. Primers and probes used for RT-PCR and real-time PCR

Gene	Expt	Probe or primers	Sequence(s)[a]	Reference
EFB1	RT-PCR	Primers	5′-ATTGAACGAATTCTTGGCTGAC 5′-CATCTTCTTCAACAGCAGCTTG	35
ERG3	RT-PCR	Primers	5′-GGAAGAACCCATCAACTGGATGG 5′-GTGCCACTACTGCCATTCCA	22
ERG11	RT-PCR	Primers	5′-ATTGGTATTCTTATGGGTGGTCAACATAC 5′-CCCAATACATCTATGTCTACCACCACC	16
CDR1	RT-PCR	Primers	5′-TTTCTGGTGCCATGACTCCTGCTAC 5′-CAATATAAATGGCCAAAAAGAATACG	25
CDR2	RT-PCR	Primers	5′-GGGTATTGGCTGGTCCTAATGTGATTC 5′-CTAGCCAACCAGTAAAAGAAAATAGTAA	25
MDR1	RT-PCR	Primers	5′-AGAGCCATCACCGGTAACGACAG 5′-CCAACCAAAAATGAAAAGACCTGAAG	25
FLUI1	RT-PCR	Primers	5′-CACTGCCTTGGCTGGTAAC 5′-ACATCGTGCAAAAGGAAGAAC	25
CDR1	Real-time PCR	Probe	6-FAM-TTAACCCATATGTCAGAAGTGCCCGGG-TAMRA	6
		Primers	TTTAGCCAGAACTTTCACTCATGATT TATTTATTTCTTCATGTTCATATGGATTGA	6
CDR2	Real-time PCR	Probe	6-FAM-TCCCGGGTTTTGGATTTTCATGTACAGA-TAMRA	6
		Primers	GGTATTGGCTGGTCCTAATGTGA GCTTGAATCAAATAAGTGAATGGATTAC	6
MDR1	Real-time PCR	Probe	6-FAM-TCGCAAGGCTAAAAGATTGAGAGCCATCA-TAMRA	6
		Primers	TTACCTGAAACTTTTGGCAAAACA ACTTGTGATTCTGTCGTTACCG	6

[a] 6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

TABLE 3. Differences in expression of *CDR1*, *CDR2*, and *MDR1* by strain P5 compared to SC5314, as measured by real-time RT-PCR

Expt no.	Fold difference in expression		
	CDR1	*CDR2*	*MDR1*
1	↓ 5.74 (3.41–9.65)	↑ 1.59 (0.96–2.63)	↑ 12.5 (7.14–20.0)
2	↓ 3.10 (1.74–5.50)	↑ 1.54 (1.01–2.38)	↑ 8.33 (5.55–12.50)

voriconazole were most likely mediated by increased drug efflux due to overexpression of *MDR1* and, to a lesser extent, *CDR2*.

Several pieces of evidence are consistent with a role for *MDR1* overexpression. First, *MDR1* was found by real-time RT-PCR to be significantly up-regulated in strain P5. Previous studies have shown that activation of *MDR1* is associated with diminished fluconazole susceptibility but not necessarily full resistance (40). Second, strain P5 exhibited decreased susceptibility to brefeldin A and cerulenin, drugs that are also effluxed from cells by Mdr1p and by Mdr1p and Cdr2p, respectively (18, 41). Strain P5 and SC5314 were equally susceptible to ketoconazole, itraconazole, benomyl, sulfoneturon, and crystal violet, drugs that are not substrates for Mdr1p (17, 26, 30, 31, 37, 41), cycloheximide, which is pumped by both Mdr1p and ABC transporters, and rhodamine 6G, which is pumped by ABC transporters only. Finally, we excluded other molecular causes of resistance, including overexpression of *ERG11*, mutations in *ERG3*, or aneuploidy. The *ERG11* mutations in strain P5 have not been previously associated with diminished azole susceptibility (33). Nevertheless, we cannot definitively conclude that they did not contribute to our results, particularly since the levels of drug resistance were low. Indeed, we must acknowledge that *C. albicans* is likely to possess as-yet-uncharacterized resistance mechanisms (39) which might also have influenced our findings.

Whereas petite mutant yeasts have generally demonstrated diminished azole susceptibility, amphotericin B results have been less consistent. In addition to our findings, amphotericin B-tolerant *C. albicans* and hypersusceptible *C. glabrata* strains have been previously reported (2, 3, 9, 13, 14). The diverse types of mitochondrial damage that cause petite mutant phenotypes are known to alter sterol synthesis in different ways (13, 14). Results to date suggest that the alterations to synthetic pathways have more consistent effects on susceptibility to azoles than amphotericin B. Along these lines, strain P5 is potentially a unique tool with which to study relationships between mitochondrial function, oxidative phosphorylation, sterol synthesis, and mechanisms of azole resistance.

Experiments were performed in C. Clancy and K. Nguyen's labs at the North Florida/South Georgia Veterans Health System.

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REFERENCES

1. **Bouchara, J.-P., R. Zouhair, S. L. Boudouil, G. F. R. Renier, D. Chabasse, J.-N. Hallet, and A. Defonatine.** 2000. In-vivo selection of an azole-resistant petite mutant of *Candida glabrata*. J. Med. Microbiol. **49:**977–984.
2. **Brun, S., C. Aubry, O. Lima, R. Filmon, T. Berges, D. Chabasse, and J. P. Bouchara.** 2003. Relationships between respiration and susceptability to azole antifungals in *Candida glabrata*. Antimicrob. Agents Chemother. **47:** 847–853.
3. **Brun, S., T. Berges, P. Poupard, C. Vauzelle-Moreau, D. Chabasse, and J.-P. Bouchara.** 2004. Mechanisms of azole resistance in petite mutants of *Candida glabrata*. Antimicrob. Agents Chemother. **48:**1788–1796.
4. **Brun, S., F. Dalle, P. Saulnier, G. Renier, A. Bonnin, D. Chabasse, and J. P. Bouchara.** 2005. Biological consequences of petite mutations in *Candida glabrata*. J. Antimicrob. Chemother. **56:**307–314.
5. **Calabrese, D., J. Bille, and D. Sanglard.** 2000. A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. Microbiology **146:**2743–2754.
6. **Chau, A. S., C. A. Mendrick, F. J. Sabatelli, D. Loebenberg, and P. M. McNicholas.** 2004. Application of real-time quantitative PCR to molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. Antimicrob. Agents Chemother. **48:**2124–2131.
7. **Cheng, S., C. J. Clancy, Z. Zhang, B. Hao, W. Wang, K. A. Iczkowski, M. A. Pfaller, and M. H. Nguyen.** 2007. Uncoupling of oxidative phosphorylation enables *Candida albicans* to resist killing by phagocytes and persist in tissue. Cell. Microbiol. **9:**492–501.
8. **Clancy, C. J., H. Huang, S. Cheng, H. Derendorf, and M. H. Nguyen.** 2006. Characterizing the effects of caspofungin on *Candida albicans*, *Candida parapsilosis*, and *Candida glabrata* isolates by simultaneous time-kill and postantifungal-effect experiments. Antimicrob. Agents Chemother. **50:**2569–2572.
9. **Defontaine, A., J. P. Bouchara, P. Declerk, C. Planchenault, D. Chabasse, and J. N. Hallet.** 1999. In-vitro resistance to azoles associated with mitochondrial DNA deficiency in *Candida glabrata*. J. Med. Microbiol. **48:**663–670.
10. **Dominique, I. F. M., and M. B. Jacques.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology **134:**405–416.
11. **Forche, A., G. May, and P. T. Magee.** 2005. Demonstration of loss of heterozygosity by single-nucleotide polymorphism microarray analysis and alterations in strain morphology in *Candida albicans* strains during infection. Eukaryot. Cell **4:**156–165.
12. **Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morshhauser.** 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. **42:**3065–3072.
13. **Geraghty, P., and K. Kavanagh.** 2003. Erythromycin, an inhibitor of mitoribosomal protein biosynthesis, alters the amphotericin B susceptibility of *Candida albicans*. J. Pharm. Pharmocol. **55:**179–184.
14. **Geraghty, P., and K. Kavanagh.** 2003. Disruption of mitochondrial function in *Candida albicans* leads to reduced cellular ergosterol levels and elevated growth in the presence of amphotericin B. Arch. Microbiol. **179:**295–300.
15. **Gupta, V., K., K. Avmeet, P. Shankarling, A. Neeti, S. Panwar, and R. Prasad.** 1998. Identification of polymorphic mutant alleles of *CaMDR1*, a major facilitator of *Candida albicans* which confers multidrug resistance, and its in vitro transcriptional activation. Curr. Genet. **34:**192–199.
16. **Henry, K. W., J. T. Nickels, and T. D. Edlind.** 2000. Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. Antimicrob. Agents Chemother. **44:**2693–2700.
17. **Hiller, D., D. Sanglard, and J. Morschhauser.** 2006. Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in *Candida alibicans*. Antimicrob. Agents Chemother. **50:**1365–1371.
18. **Holmes, A. R., S. Tsao, S. W. Ong, E. Lampling, K. Niimi, M. C. Monk, M. Niimi, A. Kaneko, B. R. Holland, J. Schmid, and R. D. Cannon.** 2006. Heterozygosity and functional allelic variation in the *Candida albicans* efflux pump genes CDR1 and CDR2. Mol. Microbiol. **62:**170–186.
19. **Kelly, S. L., D. C. Lamb, D. E. Kelly, J. Loeffler, and H. Einsele.** 1996. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. Lancet **348:**1523–1524.
20. **Kelly, S. L., D. C. Lamb, D. E. Kelly, N. J. Manning, J. Loeffler, and H. Hebart.** 1997. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol Δ5,6-desaturation. FEBS Lett. **400:**80–82.
21. **Marichal, P., L. Koymans, S. Willemsens, D. Bellens, P. Verhasselt, W. Luyten, M. Borgers, F. C. S. Ramaekers, F. C. Odds, and H. V. Bossche.** 1999. Contribution of mutations in the cytochrome P450 14 α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology **145:**2701–2713.
22. **Miyazaki, T., Y. Miyazaki, K. Izumikawa, H. Kakeya, S. Miyakoshi, J. E. Bennett, and S. Kohno.** 2006. Fluconazole treatment is effective against a *Candida albicans erg3/erg3* mutant in vivo despite in vitro resistance. Antimicrob. Agents Chemother. **50:**580–586.
23. **Morschhauser, J.** 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. Biochim. Biophys. Acta **1587:**240–248.
24. **National Committee for Clinical Laboratory Standards.** 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. NCCLS document M27-A. National Committee for Clinical Laboratory Standards, Wayne, PA.

25. **Niewerth, M., D. Kunze, M. Seibold, M. Schaller, H. C. Korting, and B. Hube.** 2003. Ciclopirox olamine treatment affects the expression pattern of *Candida albicans* genes encoding virulence factors, iron metabolism proteins, and drug resistance factors. Antimicrob. Agents Chemother. **47:**1805–1817.
26. **Niimi, K., K. Maki, F. Ikeda, A. R. Holmes, E. Lamping, M. Niimi, B. C. Monk, and R. D. Cannon.** 2006. Overexpression of *Candida alibicans CDR1*, *CDR2*, or *MDR1* does not produce significant changes in echinocandin susceptibilty. Antimicrob. Agents Chemother. **50:**1148–1155.
27. **Nolte, F. S., T. Parkinson, D. J. Falconer, S. Dix, J. Williams, C. Gilmore, R. Geller, and J. R. Wingard.** 1997. Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. Antimicrob. Agents Chemother. **41:**196–199.
28. **Pfaller, M. A., D. J. Diekema, and D. J. Sheehan.** 2006. Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. Clin. Microbiol. Rev. **19:**435–447.
29. **Rex, J. H., M. A. Pfaller, J. N. Galgiani, M. S. Bartlett, A. Espinel-Ingroff, M. A. Ghannoum, M. Lancaster, F. C. Odds, M. G. Rinaldi, T. J. Walsh, A. L. Barry, et al.** 1997. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and Candida infections. Clin. Infect. Dis. **24:**235–247.
30. **Sanglard, D., K. Kuchler, F. Ischer, J.-L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39:**2378–2386.
31. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1996. Susceptibilites of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob. Agents Chemother. **40:**2300–2305.
32. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology **143:** 405–416.
33. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1997. Amino acid substitutions in the cytochrome P-450 lanosterol 14 α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to antifungal agents. Antimicrob. Agents Chemother. **42:**241–253.
34. **Sanglard, D., F. Ischer, and J. Bille.** 2001. Role of ATP-binding cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. Antimicrob. Agents Chemother. **45:**1174–1183.
35. **Schaller, M., W. Schafer, H. C Korting, and B. Hube.** 1998. Differential expression of secreted aspartyl proteinases in a model of human oral candidiasis and in patient samples from the oral cavity. Mol. Microbiol. **29:**605–615.
36. **Selmecki, A., A. Forcher, and J. Berman.** 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. Science **313:**367–369.
37. **White, T. C.** 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41:**1482–1487.
38. **White, T. C., K. A. Marr, and R. A. Bowden.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. **11:**382–402.
39. **White, T. C., S. Holleman, F. Dy, L. F. Mirels, and D. A. Stevens.** 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob. Agents Chemother. **46:**1704–1713.
40. **Wirsching, S., S. Michel, and J. Morschhauser.** 2000. Targeted gene disruption in *Candida albicans* wild-type strains: the role of the *MDR1* gene in fluconazole resistance of clinical *Candida albicans* isolates. Mol. Microbiol. **36:**856–865.
41. **Wirsching, S., S. Michel, G. Kohler, and J. Morschhauser.** 2000. Activation of the multiple drug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a trans-regulatory factor. J. Bacteriol. **182:**400–404.
42. **Yang, Y.-L., and J.-L. Lo.** 2001. Mechanisms of antifungal agent resistance. J. Microbiol. Immunol. Infect. **34:**79–86.