Outwitting Multidrug Resistance to Antifungals

Brian C. Monk, *et al.*

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The presence of human commensal (and human-pathogenic) bacteria in the environment can be considered yet another form of contamination. Because any antibiotic resistance gene needs to coexist in the same environment as the human pathogen to which it may transfer, the increase in human population and the widespread lack of efficient wastewater treatment bring with them a risk of transfer of antibiotic resistance. Finally, it seems reasonable to speculate that a human-driven increase in the concentrations of antibiotics in natural ecosystems may not only influence antibiotic resistance, but also affect the broader microbial population dynamics in different natural environments.

Natural (nonclinical) habitats represent the main source of antibiotics and where antibiotic resistance has primarily evolved. The functional role these elements play in such environments is likely to be distinct from their "weapon/shield" function in clinical settings. In spite of the ecological relevance that antibiotics and resistance determinants have in nonclinical environments, there remains much to learn about the effect that human-driven changes of natural ecosystems may have on the evolution and dissemination of resistance in nature. Yet, the relevance this is likely to have for the future of human health is clear.

References and Notes

1. A. Alonso, P. Sanchez, J. L. Martínez, *Environ. Microbiol.* **3**, 1 (2001).
2. J. L. Martínez, F. Baquero, D. I. Andersson, *Nat. Rev. Microbiol.* **5**, 958 (2007).
3. S. A. Waksman, H. B. Woodruff, *J. Bacteriol.* **40**, 581 (1940).
4. E. B. Goh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 17025 (2002).
5. A. Fajardo, J. L. Martínez, *Curr. Opin. Microbiol.* **11**, 161 (2008).
6. J. F. Linares, I. Gustafsson, F. Baquero, J. L. Martínez, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 19484 (2006).
7. M. Gerber *et al.*, *J. Med. Microbiol.* **57**, 776 (2008).
8. V. M. D'Costa, K. M. McGrann, D. W. Hughes, G. D. Wright, *Science* **311**, 374 (2006).
9. G. Dantas, M. O. A. Sommer, R. D. Oluwasegun, G. M. Church, *Science* **320**, 100 (2008).
10. A. Fajardo *et al.*, *PLoS One* **3**, e1619 (2008).
11. J. Lubelski, W. N. Konings, A. J. Driessen, *Microbiol. Mol. Biol. Rev.* **71**, 463 (2007).
12. L. J. Piddock, *Nat. Rev. Microbiol.* **4**, 629 (2006).
13. J. F. Linares *et al.*, *J. Bacteriol.* **187**, 1384 (2005).
14. T. Kohler, C. van Delden, L. K. Curty, M. M. Hamzehpour, J. C. Pechere, *J. Bacteriol.* **183**, 5213 (2001).
15. D. R. Macinga, P. N. Rather, *Front. Biosci.* **4**, D132 (1999).
16. J. L. Martínez, F. Baquero, *Antimicrob. Agents Chemother.* **44**, 1771 (2000).
17. J. Davies, *Science* **264**, 375 (1994).
18. K. Franklin, A. J. Clarke, *Antimicrob. Agents Chemother.* **45**, 2238 (2001).
19. D. Mazel, *Nat. Rev. Microbiol.* **4**, 608 (2006).
20. S. Demaneche *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 3957 (2008).
21. F. C. Cabello, *Environ. Microbiol.* **8**, 1137 (2006).
22. D. L. Smith, J. Dushoff, J. G. Morris, *PLoS Med.* **2**, e232 (2005).
23. L. Poirel, J. M. Rodriguez-Martinez, H. Mammeri, A. Liard, P. Nordmann, *Antimicrob. Agents Chemother.* **49**, 3523 (2005).
24. L. Martinez-Martinez, A. Pascual, G. A. Jacoby, *Lancet* **351**, 797 (1998).
25. V. Cattoir, L. Poirel, C. Aubert, C. J. Soussy, P. Nordmann, *Emerg. Infect. Dis.* **14**, 231 (2008).
26. A. Hernandez, R. P. Mellado, J. L. Martínez, *Appl. Environ. Microbiol.* **64**, 4317 (1998).
27. J. W. Beaber, B. Hochhut, M. K. Waldor, *Nature* **427**, 72 (2004).

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PERSPECTIVE

Outwitting Multidrug Resistance to Antifungals

Brian C. Monk and Andre Goffeau*

The economic cost of fungal infection and its mortality associated with multidrug resistance remain unacceptably high. Recent understanding of the transcriptional regulation of plasma membrane efflux pumps of modest specificity provides new avenues for the development of broad-spectrum fungicides. Together with improved diagnosis and indirect intervention via inhibition of the energy supply for drug efflux, we envisage multifunctional azole analogs that inhibit not only ergosterol biosynthesis and drug efflux-pump activity but also activation of the transcriptional machinery that induces drug efflux-pump expression.

Eight hundred million years of evolution have generated ~1.5 million fungal species that occupy many distinct ecological niches, yet only ~300 fungi cause disease in humans (*1*). The identification of antifungals that act specifically against these pathogens is a particular challenge because of fungal diversity, individualized pathways for infection, and fungal use of multiple mechanisms that circumvent exogenous toxins. These highly regulated mechanisms include innate resistance to specific antifungal drugs, formation of biofilms, selection of spontaneous mutations that increase expression or decrease susceptibility of the drug target (*2*), stress-related tolerance that enhances short-term survival (*3*, *4*), modification of chromosomal ploidy (*5*), and overexpression of multidrug efflux pumps (*6*). Fortunately, compared with infections caused by drug-resistant bacteria, those caused by resistant fungal pathogens and their spread to other patients occur relatively infrequently. However, the economic cost of fungal infection and its associated mortality, especially in debilitated and high-investment patients, remain unacceptably high.

Department of Oral Sciences, Faculty of Dentistry, University of Otago, Post Office Box 647, Dunedin, New Zealand; and Institut des Sciences de la Vie, Université Catholique de Louvain, Louvain-la-Neuve, 1348, Belgium.

*To whom correspondence should be addressed. E-mail: andre.goffeau@uclouvain.be

A Clinical Perspective

The most prominent fungal pathogens affecting humans include *Aspergillus fumigatus*, *Candida albicans*, *C. glabrata*, *C. parasilosis*, *C. tropicalis*, *C. krusei*, and *Cryptococcus neoformans* (*7*). Although the skin, mucosal surfaces, and immune system usually provide robust defenses, weakened immunodefenses dramatically increase susceptibility to debilitating and life-threatening opportunistic fungal infections. Fungal infections are normally treated with a modest repertoire of drugs derived from five antifungal classes that target DNA and RNA synthesis, ergosterol, the ergosterol biosynthetic pathway, or the biosynthesis of the cell-wall component 1,3-β-D-glucan (Table 1). Unfortunately, the prophylactic use of fungistatic azoles such as fluconazole has been associated with an increased frequency of innate or acquired drug resistance in clinical isolates and the selection of non-*albicans Candida*, non-*fumigatus Aspergillus*, opportunistic yeastlike fungi, zygomycetes, and hyaline molds. Despite the fact that broader-spectrum third-generation azole drugs and the more expensive echinocandin class of antifungals prevent an increased proportion of life-threatening infections, *Candida* species remain the fourth most common cause of hospital-acquired bloodstream infection and kill 40% of those patients, whereas disseminated *Aspergillus* infections kill up to 80% of affected patients.

Mechanisms of Multidrug Resistance

Because of its economic and clinical impact, a focus on multidrug resistance rather than resistance to specific antifungals in pathogenic fungi is timely. Multidrug resistance, called pleiotropic drug resistance (PDR) in *Saccharomyces cerevisiae*, is an ancient phenomenon that preceded the modern use of antifungals (*8*). The adenosine triphosphate (ATP)–binding cassette (ABC) and major facilitator superfamily (MFS) transporter families responsible for multidrug resistance operate in all fungi. We distinguish among the transporters that belong to different species by using the prefix Sc for *S. cerevisiae*, Cg for *C. glabrata*, or Ca for *C. albicans*.

Saccharomyces cerevisiae. PDR in *S. cerevisiae* is the best-understood multidrug resistance mechanism in fungi. Point mutations conferring resistance to chemically diverse drugs (including azoles) have been mapped in genes encoding the zinc-

finger transcription factors ScPdr1p or ScPdr3p (*9*, *10*). These gain-of-function mutations activate over 20 target genes, the major ones being either ATP-driven (ABC transporter genes *ScPDR5*, *ScSNQ2*, and *ScYOR1*) or proton motive force–driven (MFS transporter genes *ScTPO1* and *ScFLR1*) efflux pumps (*11*, *12*). Resistance to a wide spectrum of drugs is conferred via the activation of efflux-pump gene expression, which involves the binding of Pdr1p/Pdr3p to the consensus binding element PDRE (*13*, *14*). Mechanisms regulating PDR in *S. cerevisiae* include mutation of *PDR1/3*, plasma membrane sphingolipid homeostasis, ScPdr3p autoregulation, ScPdr3p-specific activation due to loss of mitochondrial respiration, chaperone-specific differential regulation of ScPdr1p and ScPdr3p (*15*), and ScPdr1p-dependent compensatory expression of efflux pumps (*16*). Yeast cells incubated with antifungals and other drugs transiently activate ScPdr1p/Pdr3p (*16*). Drugs such as itraconazole and progesterone bind to a 250–amino acid hydrophobic xenobiotic binding domain (XBD) of ScPdr1p/Pdr3p, enabling a specific association with the KIX domain of the Gal11p subunit of the mediator complex that recruits RNA polymerase II for expression of the ScPdr1p/Pdr3p-controlled genes (*17*) (Fig. 1A). Other transcription factors such as Yrr1p, Stb5p, Rdr1p, Yrm1p, and Yap1p also contribute to the expression of the various efflux transporter genes (*17*, *18*).

Pathogenic fungi. The human pathogen *C. glabrata* uses the transcription factor CgPdr1p to control expression of the ABC multidrug efflux pumps CgCdr1p and CgCdr2p through mechanisms very similar to those of its close relative *S. cerevisiae*. The pumps are induced by treatment with diverse drugs and are highly expressed in respiration-defective mutants. Antifungal binding to the CgPdr1p XBD induces multidrug resistance via a KIX domain from the *C. glabrata* mediator complex (*19*). Mutants overexpressing CgPdr1p coordinately regulate 11 genes homologous to ScPdr1/ScPdr3p targets (*20*). These similarities support the use of *S. cerevisiae* in developing tools that are directly applicable to antifungal resistance in *C. glabrata*.

Multiple azole resistance stemming from long-term prophylaxis is frequently found in clinical isolates of the more distant pathogen *C. albicans* (*21*). The resistance mechanisms of this diploid species are complex and include mutations in single genes, loss of heterozygosity, chromosomal rearrangements, and selective segregation of chromosomal fragments (*22*). About 85% of fluconazole-resistant clinical isolates show multidrug resistance due to overexpression of the ABC transporters CaCdr1p and CaCdr2p (homologs of the *S. cerevisiae* ScPdr5p) and the major facilitator superfamily (MFS) pump CaMdr1p (homolog of ScFlr1p). The efflux functions of these transporters can be cloned in *S. cerevisiae* (*23*, *24*). Expression of the CaCdr1p and CaCdr2p pumps is controlled by the transcription factor CaTac1p (*25*), which shares about 20% identity with ScPdr1/ScPdr3p. CaTac1p and ScPdr1p/ScPdr3p recognize substantially different PDREs (*25*), and CaTac1p causes more focused transcription than ScPdr1p/Pdr3p (*26*, *27*). High doses of the female steroid hormone progesterone transiently upregulate, via steroid-specific PDREs, the same core of ABC transporters induced by antifungal intervention or gain-of-function mutations in the transcription factors (*26*, *28*). Fluconazole-resistant clinical isolates often constitutively overexpress the MFS transporter CaMdr1p, either by itself or in combination with the azole target CaErg11p and/or the CaCdr1p and CaCdr2p ABC pumps. Although the MFS transporter CaMdr1p seems more efficient than its homolog ScFlr1p, it is overexpression of ABC transporters that confers clinically important, high-level azole resistance.

Preliminary data on non-*albicans Candida* species, *Cryptococcus neoformans*, and *A. fumigatus* (*29*) suggest that various resistance phenomena identified in *C. albicans* may operate in these pathogenic fungi and that PDR-related transcriptional mechanisms may contribute to their multidrug resistance.

Prospects

The long-awaited structural resolution of antifungal binding sites in the azole target ScErg11p as well as in the drug efflux pumps related to ScPdr5p would undoubtedly provide insight into multidrug resistance and guide strategies for impairing their activities. Meanwhile, and despite molecular mechanisms of differing complexity contributing to multidrug resistance in pathogenic fungi, a newly detected Achilles heel may be the transcriptional control of the antifungal efflux pumps. Of particular interest is the discovery that *PDR* transcriptional activators bind substrates of the efflux pumps they induce. We therefore may anticipate the development of novel multifunctional azole analogs. Erg11p would still be their primary target. Inclusion of a novel substituent would then enable inhibition of XBD-dependent coupling of Pdr1p/Pdr3p with its cognate mediator complex plus physical blockade of efflux via PDR transporters (Fig. 1B). The structures of itraconazole and fluconazole suggest that a fluconazole-like scaffold could be modified to antagonize not only Erg11p but also the transcriptional XBD and the active site from efflux pumps. Similarly, the dependence of the transient steroid response on interactions with the XBD domain of CgPdr1p, and possibly CaTac1p, indicates that a steroid hormone antagonist could increase the potency of azoles used against vaginal infections. Functional overexpression of Erg11p and both MFS and ABC drug efflux pumps from pathogenic fungi has been accomplished with an activated *PDR5* promoter in a *S. cerevisiae* host whose major *PDR* genes had been deleted (*24*). This approach has allowed the assessment of innate and overexpression-related resistance to antifungals and the discovery of efflux-pump inhibitors. Similarly, ScPdr1p-regulated overexpression of functional ScPdr5p-related pump homologs is expected to provide screens for the identification of the novel broad-spectrum azoles or narrower-spectrum steroid antagonists hypothesized above (*30*). By minimizing pump expression, drug pump activity, and the opportunity for stress responses, these drugs should transform the fungistatic azoles into potent fungicides.

A complementary strategy is the identification of new targets whose dysfunction kills fungi rapidly, thus avoiding the emergence of both drug

Table 1. Effects of antifungals.

Antifungal class	Drugs used in the clinic	Primary molecular target (mode of action)
Fluorinated pyrimidine analogs	5-Flucytosine	RNA and DNA biosynthesis (misincorporation of 5-fluorouridine)
Polyenes	Nystatin Amphotericin B	Cell-membrane ergosterol (increased permeability of plasma membrane and oxidative damage)
Allylamines and thiocarbamates	Terbinafine (for dermatophytes)	Ergosterol biosynthesis, squalene epoxidase, Erg1p (fungistatic inhibition of ergosterol biosynthesis)
Azoles and triazoles	Azoles Miconazole Triazoles Fluconazole Itraconazole Posaconazole Voriconazole Ravuconazole	Ergosterol biosynthesis, lanosterol 14α-demethylase, Erg11p (fungistatic inhibition of ergosterol biosynthesis and accumulation of toxic sterol intermediates)
Echinocandins	Caspofungin Micofungin Anidulofungin	Cell-wall biosynthesis, 1,3-β-D-glucan synthase, Fks1/2p (fungicidal or fungistatic inhibition of 1,3 β-D-glucan biosynthesis)

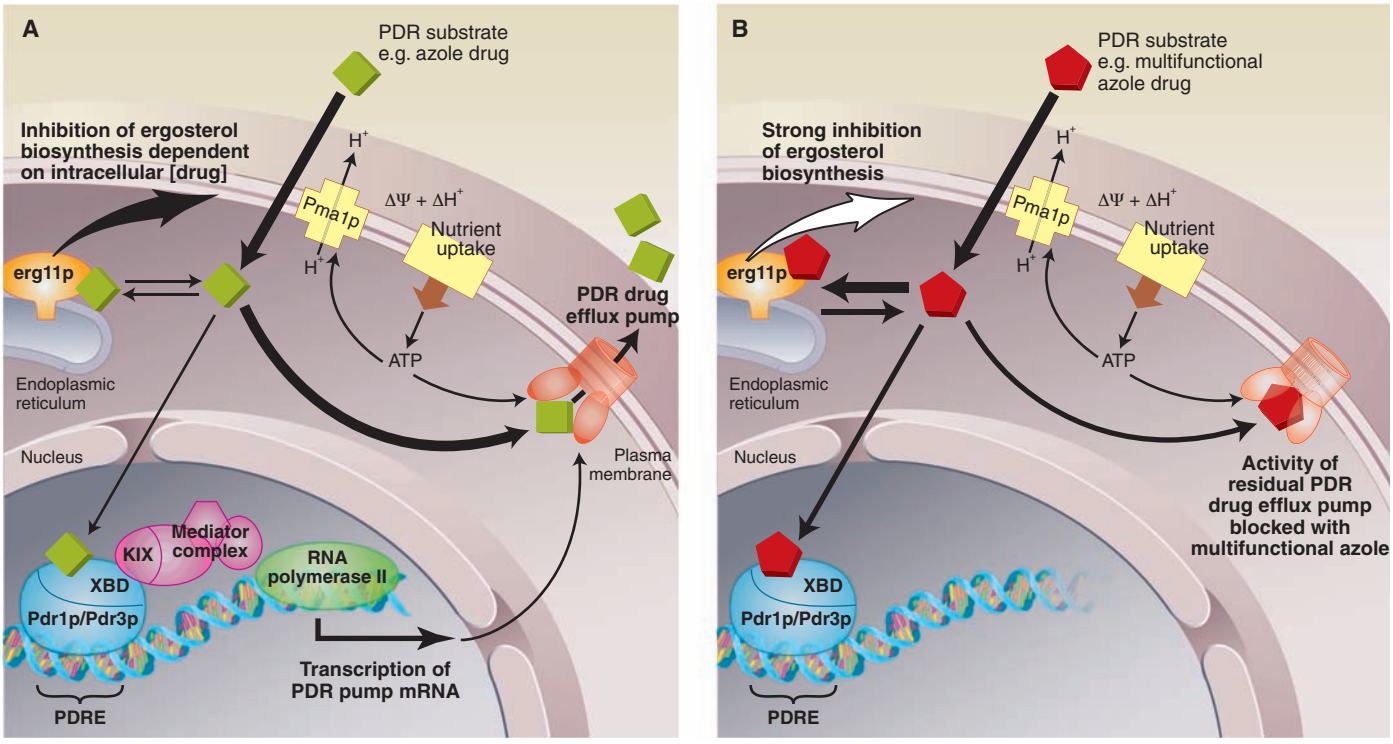

Fig. 1. Circumventing the PDR pathway in fungi. (**A**) In cells with clinically important resistance to azole drugs, high-level transcription of PDR efflux-pump genes involves the recruitment of RNA polymerase II, which depends on a drug-induced interaction between the ScPdr1p/Pdr3p and mediator complexes. The efflux pumps reduce the intracellular concentration of the drug below that required to inhibit the azole target Erg11p, allowing normal cell growth. (**B**) Binding of a multifunctional azole to the XBD domain of ScPdr1p/Pdr3p blocks expression of the drug pumps responsible for multidrug efflux and inhibits drug efflux by occupying a binding site in residual efflux pumps. The intracellular concentration of the drug is thus sufficient to block ergosterol biosynthesis in the endoplasmic reticulum. Reduced ergosterol content of membranes, production of toxic methylated sterols, and oxidative damage kill the fungal cell. Alternatively, other antifungals directly inhibit the electrogenic plasma membrane H^+-ATPase Pma1p, preventing the uptake of nutrients driven by the plasma membrane electrochemical gradient. The cells die rapidly because of a limited cellular energy supply and a loss of ion balance. Partial inhibition of Pma1p activity compromises the activity of both MFS and ABC transporters and increases the potency of azole drugs.

tolerance and efflux-mediated resistance. About 250 genes deemed essential in *S. cerevisiae* encode products that are at least 40% conserved across a broad range of fungi, including the fungal pathogens *C. glabrata*, *C. albicans*, *C. neoformans*, and *A. fumigatus* (*31*). Only about 50 of these gene products show less than 40% homology with human proteins. One of these is the plasma membrane proton pump (Pma1p), which generates the electrochemical gradient that fungi require for ion balance, nutrient uptake, and energy production. Pma1p inhibitors are fungicidal, indirectly block the activity of both ABC and MFS drug efflux pumps (*32*), and substantial resistance to them has yet to be detected.

Finally, diagnosis of disseminated fungal infections is too slow because conventional identification requires phenotypic examination of colonies grown for at least 48 hours on selective medium. The identification of drug resistance often requires a further step. Polymerase chain reaction amplification of ribosomal RNA intervening transcribed sequences followed by DNA pyrosequencing should halve the time needed for species-level fungal identification (*33*, *34*). Translation of this technology into the clinic will allow the early identification of fungal species, including innately resistant species or those susceptible to the development of multidrug resistance. The application of appropriate prophylaxis with existing and novel antifungals and of ongoing surveillance will save many lives.

References and Notes

1. L. H. Taylor, S. M. Latham, M. E. Woolhouse, *Philos. Trans. R. Soc. London Ser. B* **356**, 983 (2001).
2. D. Sanglard, J. Billie, in *Candidia and Candidiasis*, R. Calderone, Ed. (American Society for Microbiology Press, Washington, DC, 2002), pp. 349–383.
3. R. D. Cannon *et al.*, *Microbiology* **153**, 3211 (2007).
4. L. E. Cowen, *Nat. Rev. Microbiol.* **6**, 187 (2008).
5. A. Selmecki, M. Gerami-Nejad, C. Paulson, A. Forche, J. Berman, *Mol. Microbiol.* **68**, 624 (2008).
6. E. Balzi, M. Wang, S. Leterme, L. Van Dyck, A. Goffeau, *J. Biol. Chem.* **269**, 2206 (1994).
7. M. A. Pfaller, D. J. Diekema, *Clin. Microbiol. Rev.* **20**, 133 (2007).
8. Y. Gbelska, J. J. Krijger, K. D. Breunig, *FEMS Yeast Res.* **6**, 345 (2006).
9. E. Carvajal, H. B. van den Hazel, A. Cybularz-Kolaczkowska, E. Balzi, A. Goffeau, *Mol. Gen. Genet.* **256**, 406 (1997).
10. A. Nourani, D. Papajova, A. Delahodde, C. Jacq, J. Subik, *Mol. Gen. Genet.* **256**, 397 (1997).
11. J. DeRisi *et al.*, *FEBS Lett.* **470**, 156 (2000).
12. B. Rogers *et al.*, *J. Mol. Microbiol. Biotechnol.* **3**, 207 (2001).
13. D. J. Katzmann, T. C. Hallstrom, Y. Mahe, W. S. Moye-Rowley, *J. Biol. Chem.* **271**, 23049 (1996).
14. Y. M. Mamnun, R. Pandjaitan, Y. Mahe, A. Delahodde, K. Kuchler, *Mol. Microbiol.* **46**, 1429 (2002).
15. K. Gulshan, W. S. Moye-Rowley, *Eukaryot. Cell* **6**, 1933 (2007).
16. A. Kolaczkowska, M. Kolaczkowski, A. Goffeau, W. S. Moye-Rowley, *FEBS Lett.* **582**, 977 (2008).
17. B. Akache, S. MacPherson, M. A. Sylvain, B. Turcotte, *J. Biol. Chem.* **279**, 27855 (2004).
18. S. Le Crom *et al.*, *Mol. Cell. Biol.* **22**, 2642 (2002).
19. J. K. Thakur *et al.*, *Nature* **452**, 604 (2008).
20. J. P. Vermitsky *et al.*, *Mol. Microbiol.* **61**, 704 (2006).
21. T. C. White, K. A. Marr, R. A. Bowden, *Clin. Microbiol. Rev.* **11**, 382 (1998).
22. A. Coste *et al.*, *Eukaryot. Cell* **6**, 1889 (2007).
23. R. Prasad, P. De Wergifosse, A. Goffeau, E. Balzi, *Curr. Genet.* **27**, 320 (1995).
24. E. Lamping *et al.*, *Eukaryot. Cell* **6**, 1150 (2007).
25. A. T. Coste, M. Karababa, F. Ischer, J. Bille, D. Sanglard, *Eukaryot. Cell* **3**, 1639 (2004).
26. D. Banerjee *et al.*, *Eukaryot. Cell* **7**, 68 (2008).
27. T. T. Liu *et al.*, *Eukaryot. Cell* **6**, 2122 (2007).
28. B. Larsen, S. Anderson, A. Brockman, M. Essmann, M. Schmidt, *Yeast* **23**, 795 (2006).
29. A. M. Nascimento *et al.*, *Antimicrob. Agents Chemother.* **47**, 1719 (2003).
30. J. Cernicka *et al.*, *Int. J. Antimicrob. Agents* **29**, 170 (2007).
31. M. Liu *et al.*, *Eukaryot. Cell* **5**, 638 (2006).
32. B. C. Monk *et al.*, *Antimicrob. Agents Chemother.* **49**, 57 (2005).
33. A. M. Borman, C. J. Linton, S. J. Miles, E. M. Johnson, *J. Antimicrob. Chemother.* **61** (suppl. 1), i7 (2008).
34. B. L. Boyanton Jr., R. A. Luna, L. R. Fasciano, K. G. Menne, J. Versalovic, *Arch. Pathol. Lab. Med.* **132**, 667 (2008).

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