ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1999, p. 2798–2800 Vol. 43, No. 11
0066-4804/99/$04.00+0

Overexpression of Erg11p by the Regulatable *GAL1* Promoter Confers Fluconazole Resistance in *Saccharomyces cerevisiae*

DIMITRIOS P. KONTOYIANNIS,[1]* NAMITA SAGAR,[1] AND KENDAL D. HIRSCHI[2]

Department of Internal Medicine Specialties, Section of Infectious Diseases, The University of Texas M. D. Anderson Cancer Center,[1] and USDA Agricultural Research Service and Children's Nutrition Research Center and Department of Pediatrics, Baylor College of Medicine,[2] Houston, Texas

Received 1 June 1999/Returned for modification 17 August 1999/Accepted 7 September 1999

The contribution of the dosage of target enzyme P-450 14α-demethylase (14αDM) to fluconazole resistance in both *Candida albicans* and *Saccharomyces cerevisiae* remains unclear. Here, we show that overexpression of *Saccharomyces* P-450 14αDM in *S. cerevisiae*, under the control of the regulatable promoter *GAL1*, results in azole resistance.

Fluconazole, a useful azole antifungal, selectively inhibits the cytochrome P-450-dependent C-14 lanosterol α-demethylase or CYP51A1, encoded by the *ERG11* gene (13) in *Candida albicans*. Lanosterol 14α-demethylase (14αDM) is a key enzyme in ergosterol biosynthesis in both *C. albicans* and *Saccharomyces cerevisiae*, a genetically tractable fungus closely related to *C. albicans* (3, 10, 13).

Three mechanisms of fluconazole resistance that often operate simultaneously in *C. albicans* have been described: reduced accumulation of fluconazole, a defect in Δ5,6 desaturation, and target-site (CYP51A1) alterations (13). The last mechanism can be the result of either point mutations of *ERG11* (13) followed by loss of demethylation activity or high levels of CYP51A1 caused by the overexpression of *ERG11* (13). The role of point mutations of *ERG11* in fluconazole-resistant isolates of *C. albicans* has been documented (7, 11). However, the contribution of overexpression of *ERG11* to azole resistance has been less clear (13). In the few fluconazole-resistant isolates of *C. albicans* where *ERG11* is overexpressed that have been studied, the relatively low level of overexpression of *ERG11*, the concomitant presence of point mutations in *ERG11*, and the frequent overexpression of efflux pumps such as *CDR* or *MDR1* complicate the matter (9, 12, 13).

Even though the importance of point mutations in the *S. cerevisiae* gene *ERG11* which encodes for the P-450 14αDM or Erg11p (10) that results in the loss of 14αDM activity has been shown, the contribution of overexpression of P-450 14αDM in *Saccharomyces* is less clear (4). Heterologous overexpression and complementation with the closely related (5) *C. albicans* CYP51A1 in azole-sensitive *Saccharomyces* strains resulted in low and variable levels of overexpression; the effect of overexpression on azole resistance as measured by the MICs in liquid medium has been small (4, 5). As such, the effect of the level of P-450 14αDM on azole resistance in both *C. albicans* and *S. cerevisiae* remains unclear.

In the present study, we show that overexpression of Erg11p under the control of the *GAL1* promoter results in azole resistance. The regulation of the genes required to metabolize galactose in *S. cerevisiae* has been extensively studied (2). If cells are growing on glucose, the expression of these genes is repressed (2). The regulation of *GAL1* expression by carbon sources and the high expression of *GAL1* in galactose from low levels in glucose makes *GAL1* an effective regulatable promoter (2).

We transformed the *Saccharomyces* wild-type strain 10560-14C (*MAT***a** *ura3-52 leu2*::*hisG his3*::*hisG*) (Fink Laboratory, Whitehead Institute for Biomedical Research, Cambridge, Mass.) with a *Saccharomyces URA3*-based cDNA library (Fink Laboratory) under the control of the *GAL1* promoter (cloned to centromeric plasmid PRS 316 [2, 8]) and selected the Ura^+ transformants in synthetic complete medium lacking uracil (SC-uracil)-glucose plates. We used standard methods to prepare the yeast growth medium and to manipulate yeast (2). We then replica plated to SC-uracil–galactose plates, and we incubated these plates for 24 h at 30°C in order to allow the plasmid-dependent expression of cDNA in galactose medium. We then replica plated to SC-uracil–galactose–fluconazole (128 μg/ml) plates and looked for fluconazole-resistant colonies after 48 h of incubation. (Previous pilot experiments determined that the 10560-14C strain transformed by the *URA3* centromeric plasmid PRS 316 fails to grow in SC-uracil–galactose–fluconazole (32 μg/ml) medium. We then retested the purified candidates by streaking them on SC-uracil–glucose–fluconazole (128 μg/ml) plates and on SC-uracil–galactose–fluconazole (128 μg/ml) plates. The true positive candidates were fluconazole sensitive and fluconazole resistant, respectively. We then cloned and sequenced one of the cDNA clones with the primer *GAL1* (5′ TGGATAACCACTTTAACT 3′; position 690 to 707) that, when overexpressed, results in resistance to fluconazole. We found that the insert contained the *ERG11* sequence. We then subcloned the insert into pBluescript SK(−) (Stratagene, La Jolla, Calif.) (1). The clone contained the full-length *ERG11* cDNA and had no point mutations. This *ERG11* cDNA was then retransformed to the wild-type *S. cerevisiae* 10560-14C. The transformants were, as was the initial 10560-14C–*GAL1* cDNA clone, resistant to fluconazole in SC-uracil–galactose medium but not in SC-uracil–glucose medium. The following haploid strains (numbered 1 to 6) are shown in Fig. 1: 10560-14C transformed by PRS 316, a fluconazole-sensitive control (strain 1), 10560-14C–*ERG11* cDNA, two independent colonies (strains 3 and 4), and 10560-14C transformed by two additional random *GAL1* cDNAs that

* Corresponding author. Mailing address: Section of Infectious Diseases, Department of Internal Medicine Specialties, 1515 Holcombe Blvd., Box 47, Houston, TX 77030. Phone: (713) 792-6237. Fax: (713) 794-4351. E-mail: dkontoyi@notes.mdacc.tmc.edu.

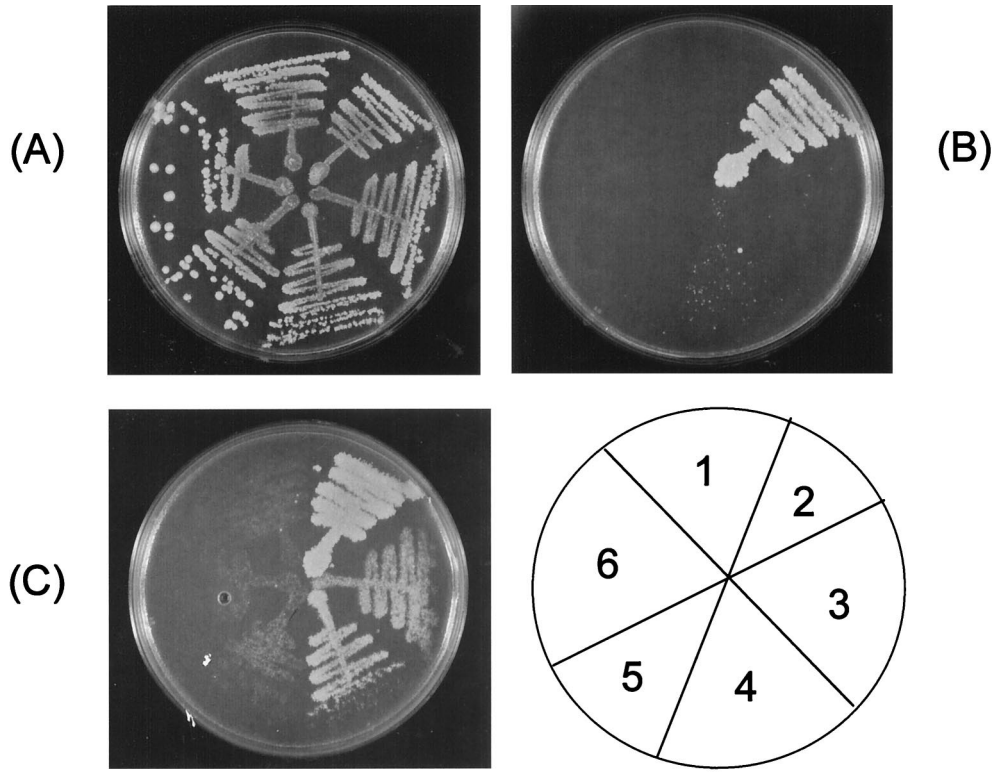

FIG. 1. *GAL1 ERG11* cDNA confers a galactose-dependent resistance to fluconazole. The growth responses of the *GAL1 ERG11* cDNA clone in the following media are shown: SC-uracil–glucose (A), SC-uracil–glucose–fluconazole (128 μg/ml) (B), and SC-uracil–galactose–fluconazole (128 μg/ml) (C). The schematic drawing of a plate (lower right) indicates the location of the plated strains (1 to 6). The strains are described in the text.

did not confer resistance to fluconazole, both controls (strains 5 and 6). *PDR1-100*, a fluconazole-resistant mutant transformed with the plasmid PRS 316, was used as the fluconazole-resistant control (strain 2). This mutant has a point mutation in the regulatory gene *PDR1*, and it overexpresses Pdr5p (6). *PDR5* encodes for an ATP-binding cassette transporter whose overexpression is well known to be involved in azole resistance (3, 4). As shown in Fig. 1B and C, the overexpression of *ERG11* cDNA in galactose results in fluconazole resistance. The *GAL1 ERG11* cDNA also exhibited slight growth in SC-uracil–glucose–fluconazole (128 μg/ml) (Fig. 1B). The microcolonies seen predominantly in strain 4 but also in strain 3 (both *ERG11* cDNA strains) could represent point mutations that result in *ERG11* overexpression in a *GAL1*-independent fraction. Since this growth was not seen in the controls, this phenomenon could alternatively imply a partial degree of expression of *GAL1 ERG11* cDNAs in glucose. The shadows seen in strains 1, 5, and 6 (fluconazole-sensitive controls) in the SC-uracil–galactose–fluconazole (128 μg/ml) plate (Fig. 1C) are a reflection of the density of the replica plating and do not constitute real growth. All growth was aerobic at 30°C for 2 days.

This work supports the concept that overexpression of Erg11p may result in azole resistance. This regulated system of Erg11p overexpression (galactose = on; glucose = off) may provide an additional tool for the dissection of the interrelated mechanisms of azole resistance in *Saccharomyces*. The implications of this study for overexpression of the *Candida* Erg11p in *C. albicans* need to be addressed with future work.

This work was supported by the Cancer Center (Core) Grant (CA16672) from The University of Texas M. D. Anderson Cancer Center to D.P.K. and by NIH1 RO1 GM 57427 to K.H. We thank NIH-CHRC 5P30 HD 27823 for sequence analysis.

We thank Denise Barrientos for excellent secretarial support.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1998. Current protocols in molecular biology. Greene Publishing Associates/Wiley Interscience, New York, N.Y.
2. **Guthrie, C., and G. R. Fink (ed.).** 1991. Guide to yeast genetics and molecular biology. Academic Press, New York, N.Y.
3. **Joseph-Horne, T., and D. W. Hollomon.** 1997. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol. Lett. **149:**141–149.
4. **Kelly, S. L., A. Arnoldi, and D. E. Kelly.** 1993. Molecular genetic analysis of azole antifungal mode of action. Biochem. Soc. Trans. **21:**1034–1038.
5. **Kirsh, D. R., M. H. Lai, and J. O'Sullivan.** 1988. Isolation of the gene for cytochrome P450 L1A1 (lanosterol 14α-demethylase) from *Candida albicans*. Gene **68:**229–237.
6. **Kontoyiannis, D. P.** Pdr5p could modulate sterol homeostasis in *Saccharomyces cerevisiae*. Submitted for publication.
7. **Lamb, D. C., D. E. Kelly, W.-H. Schunk, A. Z. Shyadehi, M. Akhtar, D. J. Lowe, B. C. Baldwin, and S. L. Kelly.** 1997. The mutation T315A in *Candida albicans* sterol 14α-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. J. Biol. Chem. **272:** 5682–5688.
8. **Liu, H., J. Krizek, and A. Bretscher.** 1992. Construction of a *GAL1*-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics **132:**665–673.
9. **Lopez-Ribot, J. L., R. K. McAtee, L. N. Lee, W. R. Kirkpatrick, T. C. White, D. Sanglard, and T. F. Patterson.** 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother. **42:**2932–2937.
10. **Paltauf, F., S. D. Kohlwein, and S. A. Henry.** 1992. Regulation and compartmentalization of lipid synthesis in yeast, p. 415–500. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast Saccharomyces. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

11. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. **42:**241–253.
12. **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.
13. **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.