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Azole Resistance in *Cryptococcus gattii* from the Pacific Northwest: Investigation of the Role of *ERG11*

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***Cryptococcus gattii* is responsible for an expanding epidemic of serious infections in Western Canada and the Northwestern United States (Pacific Northwest). Some patients with these infections respond poorly to azole antifungals, and high azole MICs have been reported in Pacific Northwest *C. gattii*. In this study, multiple azoles (but not amphotericin B) had higher MICs for 25 Pacific Northwest *C. gattii* than for 34 non-Pacific Northwest *C. gattii* or 20 *Cryptococcus neoformans* strains. We therefore examined the roles in azole resistance of overexpression of or mutations in the gene (*ERG11*) encoding the azole target enzyme. *ERG11*/*ACT1* mRNA ratios were higher in *C. gattii* than in *C. neoformans*, but these ratios did not differ in Pacific Northwest and non-Pacific Northwest *C. gattii* strains, nor did they correlate with fluconazole MICs within any group. Three Pacific Northwest *C. gattii* strains with low azole MICs and 2 with high azole MICs had deduced Erg11p sequences that differed at one or more positions from that of the fully sequenced Pacific Northwest *C. gattii* strain R265. However, the azole MICs for conditional *Saccharomyces cerevisiae erg11* mutants expressing the 5 variant *ERG11*s were within 2-fold of the azole MICs for *S. cerevisiae* expressing the *ERG11* gene from *C. gattii* R265, non-Pacific Northwest *C. gattii* strain WM276, or *C. neoformans* strains H99 or JEC21. We conclude that neither *ERG11* overexpression nor variations in *ERG11* coding sequences was responsible for the high azole MICs observed for the Pacific Northwest *C. gattii* strains we studied.**

Cryptococcus gattii is a basidiomycetous yeast-like fungus that infects both healthy and immunocompromised individuals (1–5). *C. gattii* infection is usually acquired by inhalation, which can be followed by pneumonia and/or dissemination to the central nervous system (6). *C. gattii* was once believed to cause serious infections only in tropical or subtropical regions of Africa, Asia, and Australia (4), but an outbreak of 59 human cases of *C. gattii* infection was recognized on Vancouver Island, British Columbia, Canada, in 2002 (5, 7). Since then, many more cases have been documented in the Pacific Northwest (8, 9), including 218 proven or probable cases in British Columbia through 2007 (10) and 83 proven cases in residents of Washington or Oregon between December 2004 and June 2011 (1). The growing importance of *C. gattii* infections in the northwestern United States is further shown by the fact that the number of cases per year rose each year from 2004 to 2010 (1) and that the overall mortality rate among the northwestern U.S. cases was 33% (1), which is much higher than the 13% mortality rate observed in people with *C. gattii* infections in Australia from 2000 to 2007 (11).

It was recognized in the 1990s that some patients infected with *C. gattii* respond slowly or incompletely to antifungal therapy (3, 12), and poor responses to azole therapy have also been observed among *C. gattii* patients in the northwestern United States (1). Although multiple factors probably contribute to unfavorable responses to therapy, several past studies suggest that resistance to antifungal drugs may play a role. For example, several groups have reported higher MICs of several azole antifungals for *C. gattii* than for *C. neoformans* (1, 13), and two recent studies reported higher azole MICs for *C. gattii* strains with genotypes prevalent in the Pacific Northwest than for *C. gattii* with other genotypes or *C. neoformans* (14, 15).

The major mechanisms by which medically important fungi acquire resistance to azole antifungals are (i) overexpression of the *ERG11* gene (also known as *CYP51*) encoding the azole target enzyme lanosterol 14-α demethylase (16, 17), (ii) mutations in *ERG11* that result in decreased susceptibility of lanosterol 14-α demethylase (Erg11p) to inhibition by azoles (17–20), and (iii) overexpression of one or more plasma membrane proteins that pump azoles out of the cell (21, 22). Most past studies of azole resistance mechanisms have been in *Candida albicans* or *Aspergillus* species, but a few groups have examined potential roles in azole resistance of *ERG11* (19, 23, 24) and of the ATP-binding cassette (ABC) transporters (25) in *C. neoformans*. We are not aware that mechanisms of azole resistance have previously been examined in *C. gattii* isolated from patients in the Pacific Northwest or elsewhere (non-Pacific Northwest). Therefore, the present study compared the MICs of several azole antifungals and the polyene antifungal amphotericin B for 25 *C. gattii* strains isolated from patients in the Pacific Northwest, 34 *C. gattii* strains from other geographic regions, and 20 *C. neoformans* strains. We also examined the role of *ERG11* in azole resistance by (i) comparing *ERG11* mRNA levels in the *C. gattii* and *C. neoformans* strains, (ii) sequencing the *ERG11* genes in the 25 Pacific Northwest *C. gattii* strains, and (iii) comparing the azole MICs of *Saccharomyces cerevisiae* strains that expressed wild-type and selected mutant *Cryptococcus ERG11* cDNAs.

MATERIALS AND METHODS

Strains and media. The tetracycline-repressible *erg11* mutant *Saccharomyces cerevisiae* strain DSY3961 (*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2* CMVp(*tetR′-SSN6*)::*LEU2trp1*::Tta *ERG11*::kanMX-*tetO*$_7$ *pdr5*Δ::HIS3kanMX)

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TABLE 1 Synthetic oligonucleotide primers used in this study

No.	Primer name	Sequence (5′→3′)
1	*S. cerevisiae* ERG11-5	CTTCTTGTCGACATGTCTGCTACCAAGTCAATCGTT
2	*S. cerevisiae* ERG11-3	CTTCTTGGATCCTTAGATCTTTTGTTCTGGATTTCTC
3	*C. albicans* ERG11-5	CTTCTTCTCGAGATGGCTATTGTTGAAACTGTCATTG
4	*C. albicans* ERG11-3	CTTCTTGGATCCTTAAAACATACAAGTTTCTCTTTTTTCCC
5	*Cryptococcus* ERG11-5	GCCACGCTCGAGATGTCGGCAATCATCCCCCA
6	H99 ERG11-3	GCCACGGGATCCTCAATTCATACTAAAACTCGCACCATC
7	JEC21 ERG11-3	GCCACGGGATCCTCATACCTCCTGCTTGACCTC
8	R265, WM276 and C71 ERG11-3	GCCACGGGATCCTTACACCTCCTGCTTGACCTC
9	ERG11 Northern Probe-5	AAGGGTAACAACCTTTCTTTG
10	ERG11 Northern Probe-3	TGAAGAAATCTTCGCATTCGC
13	RT-PCR ACT1-5	CCAAGCAGAACCGAGAGAAGATG
14	RT-PCR ACT1-3	GGACAGTGTGGGTGACACCGT
15	RT-PCR ERG11-5	CCATGTCCGAGCTCATCATTCTT
16	RT-PCR ERG11-3	ACTGGGAAGGGGCAAGTTGG

(18) was obtained from D. Sanglard (University of Lausanne, Lausanne, Switzerland), *C. albicans* SC5314 was obtained from W. Fonzi (Georgetown University), and *C. albicans* strain 17 (which bears the *erg11* R467K mutation and is resistant to fluconazole) (26) was obtained from T. White (University of Missouri, Kansas City, MO). *S. cerevisiae* DSY3961 transformants were grown on 0.67% yeast nitrogen base supplemented with Complete Supplement Mixture lacking histidine, leucine, and uracil (YNB −His, Leu, Ura) and containing either 2% glucose or both 3% galactose and 2% raffinose, with or without 2 μg doxycycline per ml. *C. gattii* R265 is a genotype VGIIa strain that was isolated from a patient on Vancouver Island, British Columbia, and *C. gattii* WM276 is an environmental strain from Australia. These are the two *C. gattii* strains for which complete genome sequences are available (*Cryptococcus gattii* Sequencing Project, Broad Institute of Harvard and MIT [http://www.broadinstitute.org/]). The *C. gattii* and *C. neoformans* clinical strains were provided by T. Mitchell (Duke University) or K. Datta (Oregon Health and Science University, Portland, OR [OHSU]), or they were collected by the Division of Infectious Diseases at OHSU. The molecular genotypes of Pacific Northwest *C. gattii* strains were provided by E. Debess (Oregon Health Division, Portland, OR) or S. Lockhart (Centers for Disease Control and Prevention, Atlanta, GA). As far as we know, all Pacific Northwest *C. gattii* strains in this study were initial isolates from unique patients in British Columbia, Washington, or Oregon. *Cryptococcus* strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) with or without fluconazole. Plasmids were amplified in *Escherichia coli* strain DH5α in Luria-Bertani medium with 100 μg ampicillin per ml.

Antifungal susceptibility testing. The CLSI M27-A3 broth microdilution method (27) was used to test *C. neoformans* and *C. gattii* for susceptibility to fluconazole, voriconazole, itraconazole, posaconazole, and amphotericin B. Due to poor growth of S. *cerevisiae* in RPMI medium, the CLSI M27-A3 broth microdilution method was modified to test the *S. cerevisiae* transformants by replacing RPMI medium with YNB plus His plus Leu with or without Ura containing either 2% glucose or 3% galactose and 2% raffinose, with or without doxycycline (2 μg/ml). The 2-tailed Mann-Whitney U test was used to compare MIC values between different groups.

Cloning and expression of *ERG11* cDNAs and ORFs. The synthetic oligonucleotide primers used in this study are listed in Table 1. The *ERG11* cDNAs from *C. neoformans* strains H99 and JEC21 and from *C. gattii* strains R265 and WM276 were amplified from total RNA by reverse transcription (RT)-PCR, and the resulting amplicons were ligated into the BamHI and SalI sites in plasmid YEp51 (28). BamHI- and EcoRI-digested fragments of YEp51 containing the *S. cerevisiae GAL10* promoter with or without the *Cryptococcus* cDNAs of interest were inserted into the low-copy-number, centromere-containing plasmid pRS316 (29). The *ERG11* open reading frames (ORFs) from *C. albicans* strains SC5314 and 17 or from *S. cerevisiae* DSY3961 were amplified from genomic DNA by PCR; the *ERG11* cDNAs from *C. gattii* strains C3, C16, C17, and C71 were amplified from total RNA by RT-PCR; and the resulting amplicons were ligated into the BamHI and SalI sites of plasmid pRS316 containing the *GAL10* promoter (pRS316GAL) and sequenced by standard methods. Proofreading DNA polymerases were used for all PCRs. Plasmids were introduced into *S. cerevisiae* using the Alkali-Cation Yeast Kit (MP Biomedicals).

ERG11 mRNA levels. RNA was extracted with the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad) from samples grown to mid-log phase in YPD medium. The sizes of the *ERG11* mRNAs were estimated by Northern hybridization (30), using a probe generated by (i) amplifying a portion of the *C. neoformans* H99 *ERG11* cDNA by PCR with primers 9 and 10 (Table 1) and (ii) labeling with [α-^{32}P]CTP using the random-primer method. *Cryptococcus ERG11* and *ACT1* mRNAs were quantified by RT-PCR using primers 13 to 16 (Table 1) and the iScript One-Step RT-PCR Kit with SYBR green (Bio-Rad). The 2-tailed Mann-Whitney U test was used to compare expression levels between populations, and re-

TABLE 2 Antifungal susceptibilities of *C. gattii* and *C. neoformans*

	Pacific Northwest *C. gattii* ($n = 25$)		Non-Pacific Northwest *C. gattii* ($n = 34$)		*C. neoformans* ($n = 20$)	
Drug	GM[a]	MIC_{50}/MIC_{90} (μg/ml)	GM	MIC_{50}/MIC_{90} (μg/ml)	GM	MIC_{50}/MIC_{90} (μg/ml)
Fluconazole	20	16/64	9.9[b]	8/32	8.6[b]	8/32
Voriconazole	0.53	0.5/1	0.28[b]	0.25/1	0.24[b]	0.25/0.5
Itraconazole	0.76	1/1	0.58[b]	0.5/1	0.57[b]	0.5/2
Posaconazole	0.95	1/2	0.48[b]	0.5/1	0.36[b]	0.25/1
Amphotericin B	0.69	0.5/1	0.72	0.5/1	0.68	0.5/1

[a] GM, geometric mean MIC from at least 3 independent experiments.
[b] $P < 0.05$ vs. Pacific Northwest *C. gattii*.

TABLE 3 Amino acid substitutions encoded by *C. gattii* or *C. neoformans ERG11* cDNAs[a]

Strain[b]	Genotype	Fluconazole MIC (μg/ml)	Q7R	V8A	V15L	Y18F	I19F	H21P	T24A	L30V	I32V	V33A	I37V	F42L	I43V	G45C	H50Q	R57K	R57T	R58K
R265	VGIIa	16																+		+
WM 276	VGI	4				+		+	+	+	+			+	+	+	+			+
H99		8				+	+	+	+		+	+	+	+			+	+		+
JEC21		4				+														
C1	VGIIb	64																		
C6	VGIIa	64																		
C8	VGIIa	64																		
C71	VGIIc	64																		
C2	VGIIa	32																		
C14	VGIIc	32																		
C59	VGIIa	32																		
C61	VGIIa	32																		
C4	VGIIa	16																		
C5	VGIIa	16																		
C7	VGIIa	16																		
C9	VGIIa	16																		
C11	VGIIa	16																		
C15	VGIIc	16																		
C16	VGI	16			+			+									+		+	+
C57	VGIIa	16																		
C60	VGIIa	16																		
C62	VGIIa	16																		
C63	VGIIa	16																		
C66	VGIIa	16																		
C67	VGIIa	16																		
C3	VGIII	8	+	+				+									+			+
C10	VGIIa	8																		
C17	VGI	8			+			+									+		+	+
C64	VGIIa	8																		

[a] +, substitution encoded. Amino acids that differ from *C. gattii* R265 Erg11p are listed.
[b] All except *C. neoformans* H99 and JEC21 are *C. gattii*.

gression analysis was performed to correlate mRNA levels with $\log_2$ fluconazole MICs.

ERG11 gene sequences. Genomic DNAs from 25 Pacific Northwest *C. gattii* clinical isolates, *C. neoformans* strains H99 and JEC21, and *C. gattii* strains R265 and WM276 were isolated using the MasterPure Yeast DNA Purification Kit (Epicentre). The *ERG11* coding sequences were amplified from genomic DNA by PCR with primers 5 to 8 (Table 1) and a proofreading DNA polymerase, and the amplicons were sequenced by standard methods. DNA and deduced peptide sequences were aligned using BioEdit Sequence Alignment Editor software.

Nucleotide sequence accession numbers. The *ERG11* cDNA sequences from *C. neoformans* H99 and JEC21 and from *C. gattii* R265 and WM276 have been deposited in GenBank under accession numbers JF965441, JF965442, JF965443, and JF965444, respectively.

RESULTS

Susceptibilities of *Cryptococcus* strains to azoles and amphotericin B. The geometric mean MICs, MIC_{50}s, and MIC_{90}s of all azole antifungals tested were approximately 2-fold higher for 25 Pacific Northwest *C. gattii* strains than for 34 non-Pacific Northwest *C. gattii* strains or for 20 *C. neoformans* strains, but there were no significant differences in the MICs of the polyene antifungal amphotericin B for the 3 groups (Table 2). Among all 25 Pacific Northwest *C. gattii* strains, the $\log_2$ MICs of fluconazole correlated with the $\log_2$ MICs of voriconazole (slope, 0.67; $r = 0.587$; $P = <0.05$), itraconazole (slope, 0.48; $r = 0.636$; $P < 0.001$), and posaconazole (slope, 0.40; $r = 0.502$; $P < 0.05$), but not with the $\log_2$ MICs of amphotericin B (slope, −0.39; $r = 0.073$; $P = 0.73$).

The 25 Pacific Northwest *C. gattii* strains included 18 genotype VGIIa strains, 3 genotype VGIIc strains, 2 genotype VG1 strains, 1 VGIIb strain, and 1 genotype VGIII strain. Fluconazole MICs ≥32 μg/ml were observed for 5 of 18 strains with genotype VGIIa, 2 of 3 with genotype VGIIc, 1 of 1 with genotype VGIIb, 0 of 2 with genotype VG1, and 0 of 1 with genotype VGIII (Table 3).

Identification and heterologous expression of *C. gattii ERG11*. A search of the genome sequences of *C. gattii* strains R265 and WM276 identified orthologs of the *ERG11* genes of *C. neoformans* and several other fungi. The corresponding cDNAs were cloned by RT-PCR from total RNA from *C. gattii* strains R265 and WM276 and from *C. neoformans* strains H99 and JEC21. The *C. gattii* R265 cDNA encoded a 550-amino-acid deduced peptide that was 98, 97, and 96% identical, respectively, to the corresponding deduced peptides from *C. gattii* WM276, *C. neoformans* H99, and *C. neoformans* JEC21. To verify that the *Cryptococcus* cDNAs of interest were derived from full-length mRNAs, Northern blots of total RNAs from the four strains of interest were hybridized with a probe derived from the *C. neoformans* H99 *ERG11* cDNA. All four mRNAs were approximately 1.65 kb in length, as predicted from the corresponding cDNAs (data not shown).

We used the *GAL10*-regulated expression plasmid pRS316GAL to determine if the *Cryptococcus* cDNAs of interest would complement *S. cerevisiae* DSY3961, in which *ERG11* expression is tightly repressed by tetracycline. The strain also lacks the *PDR5* gene, which makes it highly susceptible to azoles (18). When host cell *ERG11* expression was repressed with doxycycline, growth was observed only when heterologous expression of the *C. neoformans* or *C. gattii* cDNAs of interest or the *S. cerevisiae ERG11* ORF was induced with galactose, but not when expression of the *Cryptococcus* cDNAs or *S. cerevisiae ERG11* was repressed with glucose (Fig. 1). Since these results established that the *Cryptococcus* cDNAs of interest encode functional Erg11p proteins, the *ERG11* cDNA sequences from *C. neoformans* H99 and JEC21 and from *C. gattii* R265 and WM276 have been deposited in GenBank.

ERG11 mRNA levels. Whether azole resistance was associated with *ERG11* overexpression was examined by comparing the ratios of *ERG11* mRNA to *ACT1* mRNA in the 25 Pacific Northwest

TABLE 3 (Continued)

D59N	D80N	I99L	M101T	I105V	S191P	I196A	S203A	E223V	N249D	R256K	F268L	G293S	V307I	V340I	E349Q	K362R	A404S	A457V	T460S	N533T
+	+	+	+			+		+			+									
				+	+	+				+		+	+		+		+		+	
	+				+	+	+			+		+	+		+	+	+	+		
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C. gattii strains, 34 non-Pacific Northwest *C. gattii* strains, and 20 *C. neoformans* strains, all of which were grown to mid-log phase in liquid YPD medium. The *ERG11*/*ACT1* mRNA ratios (median [interquartile range]) in the 59 Pacific Northwest and non-Pacific Northwest *C. gattii* strains (0.94 [0.70 to 1.51]) were higher than in the 20 *C. neoformans* strains (0.48 [0.36 to 0.74]) ($P = 0.0002$), but the *ERG11*/*ACT1* mRNA ratios in the Pacific Northwest (0.82 [0.68 to 1.06]) and non-Pacific Northwest (1.09 [0.71 to 1.88]) *C. gattii* strains were not significantly different ($P = 0.08$) (data not shown). There also were no significant correlations between *ERG11*/*ACT1* mRNA ratios and fluconazole MICs within any of the 3 groups listed above (Fig. 2A) or within the subsets of Pacific Northwest *C. gattii* strains with genotype VGIIa ($n = 18$) or genotypes VGIIa, VGIIb, and VGIIc ($n = 22$) (data not shown).

Whether *ERG11* expression was induced by fluconazole exposure was examined by measuring the ratios of *ERG11* mRNA to *ACT1* mRNA when 8 strains each of Pacific Northwest *C. gattii*, non-Pacific Northwest *C. gattii*, and *C. neoformans* (4 with the highest and 4 with the lowest fluconazole MICs) were exposed to fluconazole. The *ERG11*/*ACT1* mRNA ratios rose in all strains tested following exposure to 8 μg fluconazole per ml for 2 h, and the magnitudes of *ERG11* induction did not differ significantly between the 3 groups of *Cryptococcus* strains or between the 4 strains within each group with the highest and lowest fluconazole MICs (Fig. 2B). Similar responses were observed when the *Cryptococcus* strains were exposed to 0.5 times each strain's fluconazole MIC for 2 h or 100 h (data not shown).

***C. gattii ERG11* sequences.** Since azole resistance was not associated with *ERG11* overexpression in the Pacific Northwest *C. gattii* strains we studied, we next examined whether azole resistance was associated with variations in Erg11p peptide sequences. We therefore (i) sequenced the *ERG11* genes in *C. gattii* strain R265, *C. gattii* strain WM276, and the 25 Pacific Northwest *C. gattii* clinical strains; (ii) aligned the *C. gattii* R265 and WM276 *ERG11* genes and cDNAs to identify the coding sequences; and (iii) deduced each strain's Erg11p peptide sequence. The deduced Erg11p sequences from *C. gattii* strains R265 and WM276 differed at 9 positions. Seven of these 9 varying amino acids in *C. gattii* R265 Erg11p and 4 of the 9 varying amino acids in *C. gattii* WM276 Erg11p were also present in the deduced Erg11p proteins of *C. neoformans* H99 and/or JEC21 (Table 3).

The deduced Erg11p sequences of 20 Pacific Northwest *C. gattii* clinical isolates (including all 18 VGIIa strains, the single VGIIb strain, and 1 of 3 VGIIc strains) were identical to that of *C. gattii* R265, and these 20 strains' fluconazole MICs ranged from 8 to 64 μg/ml. In contrast, the deduced Erg11p sequences of 5 Pacific Northwest *C. gattii* clinical isolates (both VG1 strains, 2 of 3 VGIIc strains, and the single VGIII strain) differed from that of *C. gattii* R265 at one or more positions. Three of these 5 strains (2 VGI and 1 VGIII) had low fluconazole MICs (8 to 16 μg/ml), and all of these 3 strains' deduced Erg11p sequences differed from that of *C. gattii* R265 at multiple positions, many of which (H21P, H50Q, R57K, R58K, and I196A) were also present in the deduced Erg11p sequences of *C. neoformans* H99, *C. neoformans* JEC21, and/or *C. gattii* WM276. In contrast, 2 VGIIc strains had high fluconazole MICs (32 to 64 μg/ml), and these strains' deduced Erg11p sequences contained a single amino acid substitution (N249D) that was not present in the deduced Erg11p sequence of any other *C. gattii* strain or in *C. neoformans* H99 or JEC21 (Table 3).

Azole inhibition of recombinant fungal Erg11p proteins. To determine if any of the observed differences in Erg11p sequences was responsible for differences in azole MICs, we used plasmid pRS316GAL to express *Cryptococcus* cDNAs of interest in *S. cerevisiae* DSY3961, and we measured the resulting transformants' azole MICs. To verify that this heterologous expression system could be used for this purpose, we compared the azole MICs of *S. cerevisiae* DSY3961 cells that expressed wild-type *C. albicans ERG11* or a mutant allele that encodes an amino acid substitution (R467K)

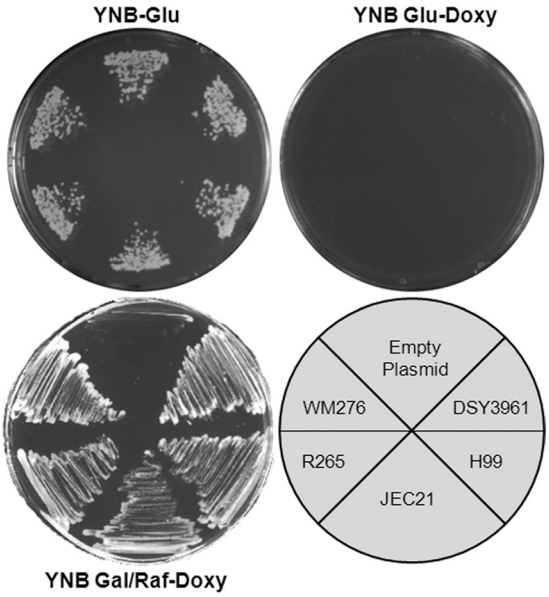

FIG 1 Complementation of an *S. cerevisiae erg11* mutant. *S. cerevisiae* DSY3961 cells expressing *ERG11* cDNAs from *C. neoformans* (H99 and JEC21) or *C. gattii* (R265 or WM276) or *ERG11* from *S. cerevisiae* (DSY3961) under the control of the *GAL10* promoter grew well when heterologous *ERG11* expression was repressed with 2% glucose (YNB-Glu), did not grow when heterologous *ERG11* expression was repressed with 2% glucose and when chromosomal *ERG11* expression was repressed with 2 μg/ml doxycycline (YNB Glu-Doxy), and grew well when chromosomal *ERG11* expression was repressed with doxycycline and when heterologous *ERG11* expression was induced with 3% galactose and 2% raffinose (YNB Gal/Raf-Doxy).

that has been shown to increase the fluconazole MIC by 4-fold (31). When chromosomal *ERG11* expression was repressed with doxycycline and heterologous *ERG11* expression was induced with galactose, the fluconazole MIC was 4-fold higher in *S. cerevisiae* transformants expressing the *C. albicans erg11*(R467K) allele than in controls expressing wild-type *C. albicans ERG11*.

The MICs of all azoles tested for *S. cerevisiae* expressing the *ERG11* cDNAs from *C. neoformans* H99 or JEC21 did not differ significantly from those for *S. cerevisiae* expressing the *ERG11* cDNAs from *C. gattii* R265 or WM276. The azole MICs for *S. cerevisiae* expressing the *C. gattii* R265 *ERG11* cDNA were approximately 2-fold higher than the MICs for *S. cerevisiae* expressing the *C. gattii* WM276 *ERG11* cDNA. However, the azole MICs for *S. cerevisiae* expressing cDNAs encoding all of the variant Erg11p sequences observed in this study differed very little from the azole MICs for *S. cerevisiae* expressing wild-type *C. gattii* R265 *ERG11* cDNA. Moreover, none of the *S. cerevisiae* transformants expressing a variant *ERG11* cDNA had a higher MIC of any azole than did *S. cerevisiae* expressing the *ERG11* cDNA from *C. gattii* R265. Lastly, the MICs of the polyene antibiotic amphotericin B were the same for all of the *S. cerevisiae* transformants we examined (Table 4).

DISCUSSION

The goals of the present study were (i) to compare the MICs of multiple azoles and of amphotericin B for 25 Pacific Northwest *C. gattii* strains, 34 non-Pacific Northwest *C. gattii* strains, and 20 *C. neoformans* strains and (ii) to determine if either *ERG11* overexpression or mutations in the *ERG11* coding sequences was associated with high azole MICs in the Pacific Northwest *C. gattii* strains. We found that the MICs of multiple azole antifungals for 25 Pacific Northwest *C. gattii* strains were approximately 2-fold-higher than for the 34 non-Pacific Northwest *C. gattii* strains or the 20 *C. neoformans* strains. Also, 8 of 25 (32%) Pacific Northwest *C. gatti* strains had fluconazole MICs of ≥32 μg/ml. Other groups have reported higher azole MICs for *C. gattii* than for *C. neoformans* (13, 32) and also higher azole MICs for *C. gattii* with molecular genotypes that are common in the Pacific Northwest than for *C. gattii* with other genotypes (14, 15), but we are unaware of prior studies that directly compared the azole MICs for *C. gattii* strains isolated from people infected in the Pacific Northwest to those for strains isolated elsewhere. Whether the higher azole MICs we observed for Pacific Northwest *C. gattii* than for non-Pacific Northwest *C. gattii* or *C. neoformans* play a causal role in clinical outcomes is not known, but the high azole MICs that we or others (14, 15) have found for Pacific Northwest *C. gattii* correlate with poor outcomes and high mortality rates in people with Pacific Northwest *C. gattii* infections (1). Lastly, our observation that high fluconazole MICs among Pacific Northwest *C. gattii* strains correlated with high MICs of other azoles suggests that using voriconazole, itraconazole, or posaconazole instead of fluconazole is unlikely to overcome the problem of azole resistance.

Since Pacific Northwest *C. gattii* strains were more resistant to multiple azoles than were other *C. gattii* strains or *C. neoformans*, we next examined whether *ERG11* overexpression was associated with azole resistance. The first step was to identify *C. gattii ERG11*, which was done by searching the *C. gattii* R265 and WM276 genome databases for orthologs of known fungal *ERG11* genes and then by showing that these genes' cDNAs complemented the conditional *erg11* mutation in *S. cerevisiae* DSY3961. When we tested the strains of interest for *ERG11* overexpression, the mean ratios of *ERG11* mRNA to *ACT1* mRNA were higher in *C. gattii* than in *C. neoformans*. However, the mean *ERG11* mRNA levels did not differ between the Pacific Northwest and non-Pacific Northwest *C. gattii* strains, nor did they correlate with fluconazole MICs within any group. Also, *ERG11* mRNA levels did not differ significantly when the Pacific Northwest *C. gattii* strains with the highest and lowest fluconazole MICs were exposed to fluconazole. *ERG11* overexpression is known to cause azole resistance in *C. albicans* (24), but not in *C. neoformans* (20). These results indicated that overexpression of *ERG11* was not responsible for azole resistance in the Pacific Northwest *C. gattii* strains we studied.

When we analyzed the 25 Pacific Northwest *C. gattii* strains' deduced Erg11p sequences, we found that variations in these proteins' sequences correlated better with the strains' molecular genotypes than with their azole MICs. For example, 18 of 18 genotype VGIIa, 1 of 1 VGIIb, and 1 of 3 VGIIc clinical isolates had deduced Erg11p sequences that were identical to that of VGIIa strain *C. gattii* R265, and these 20 strains' fluconazole MICs varied widely from 8 to 64 μg/ml. In contrast, 2 of 3 VGIIc, 1 of 1 VGIII, and 2 of 2 VGI strains had deduced Erg11p sequences that differed at one or more positions from that of *C. gattii* R265. Three of these strains had low fluconazole MICs, and multiple amino acid substitutions observed in these strains were also found in the deduced Erg11p sequences of the fluconazole-susceptible genotype I *C. gattii* strain WM276, *C. neoformans* H99, and *C. neoformans* JEC21 (33). The one finding that suggested a link between azole resistance and sequence variations in Erg11p was that 2 genotype VGIIc *C. gattii* strains with high fluconazole MICs had a single

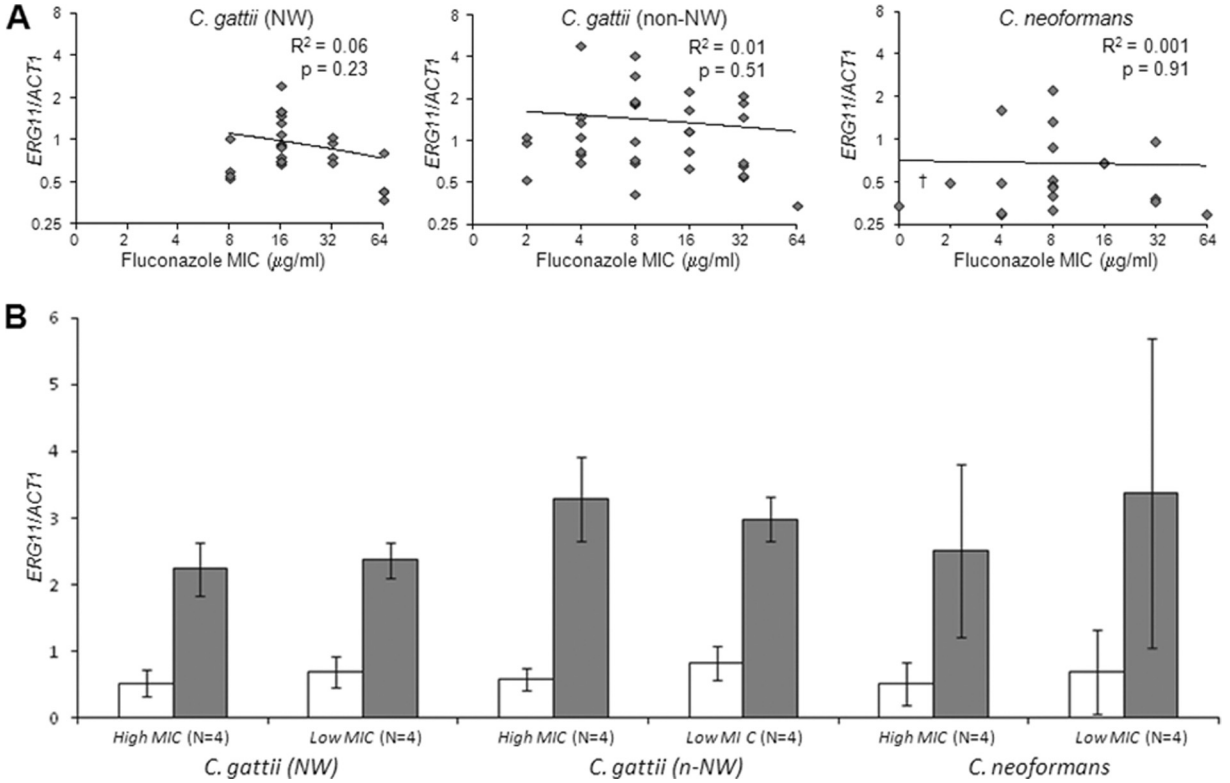

FIG 2 *ERG11* expression. (A) *ERG11/ACT1* mRNA ratios did not correlate with $\log_2$ fluconazole MICs in 25 Pacific Northwest *C. gattii*, 34 non-Pacific Northwest *C. gattii*, and 20 *C. neoformans* clinical isolates. (B) The *ERG11/ACT1* mRNA ratios were higher when 4 strains with the highest and the 4 strains of Pacific Northwest *C. gattii*, non-Pacific Northwest *C. gattii*, or *C. neoformans* with the lowest fluconazole MICs were incubated in the presence of 8 μg/ml fluconazole for 2 h (gray bars) than in cells incubated in the absence of fluconazole (white bars), but there were no significant differences in *ERG11/ACT1* mRNA ratios between the 3 groups or between the 4 strains with the high and low fluconazole MICs within each group. Shown are geometric means ± standard deviations from 3 experiments.

amino acid substitution (N249D) that was not observed in any other strain in this study and also had not been described previously. Notably, none of the Pacific Northwest *C. gattii* strains in this study had any of the Erg11p amino acid substitutions that had previously been described in azole-resistant *C. albicans* or *C. neoformans* (20, 24).

We used the tetracycline-repressible *erg11* mutant *S. cerevisiae* DSY3961 to compare the azole MICs of transformants expressing the *C. neoformans* and *C. gattii ERG11* cDNAs observed in this study. This heterologous expression system was similar to the system used by Alcazar-Fuoli et al. (18) to study the effects of specific amino acid substitutions on azole susceptibility in *C. albicans* Erg11p, except that we used a centromere-containing plasmid that replicates at a low copy number per cell instead of 2μ-containing

TABLE 4 Effects of heterologous *ERG11* expression on susceptibility of *S. cerevisiae* DSY3961 to antifungals

	Geometric mean of the MIC (μg/ml)[a]				
Source of ERG11	Fluconazole	Voriconazole	Itraconazole	Posaconazole	Amphotericin B
Empty plasmid[b]	1	0.032	0.032	0.128	2
S. cerevisiae DSY3961	8	0.25	0.5	0.5	2
C. albicans SC5314	8	0.25	0.25	0.5	2
C. albicans 17 (R467K)	32	0.125	0.25	0.5	2
C. neoformans H99	0.250	0.002	0.031	0.063	2
C. neoformans JEC21	0.125	0.001	0.015	0.063	2
C. gattii R265	0.250	0.002	0.036	0.055	2
C. gattii WM276	0.149	0.001	0.015	0.031	2
C. gattii C71 (N249D)	0.250	0.002	0.015	0.041	2
C. gattii C3	0.177	0.002	0.015	0.026	2
C. gattii C16	0.210	0.001	0.018	0.031	2
C. gattii C17	0.149	0.002	0.015	0.024	2

[a] MICs were measured in YNB − His, Leu, Ura containing 3% galactose, 2% raffinose, and 2 μg doxycycline/ml. $n = 3$ or more for all azoles; $n = 2$ for amphotericin B.
[b] Empty-plasmid controls were tested without doxycycline.

plasmids to minimize the effects of variable plasmid copy numbers on azole MICs. We verified that this method can be used for this purpose by confirming the earlier observation that *S. cerevisiae* cells expressing the *C. albicans erg11*(R467K) allele had 4-fold-higher fluconazole MICs than controls expressing wild-type *C. albicans ERG11* (30). The higher azole MICs we observed for *S. cerevisiae* DSY3961 cells expressing the *ERG11* genes from *S. cerevisiae* or *C. albicans* under the control of the *S. cerevisiae GAL10* promoter than for empty-vector controls incubated without tetracycline were not surprising because *ERG11* overexpression is a known cause of azole resistance (16, 17). The lower azole MICs observed for *S. cerevisiae* DSY3961 cells expressing *C. neoformans* or *C. gattii ERG11* cDNAs were likely due to differences in codon usage, translational efficiency, and/or mRNA stability when basidiomycete *ERG11* cDNAs were expressed in an ascomycete host. We did not examine these possibilities experimentally because the objective of these studies was to compare the effects on azole MICs of specific amino acid substitutions in the Erg11p proteins of closely related fungi.

When *S. cerevisiae* DSY3961 cells expressing the *ERG11* cDNAs from 2 wild-type strains each of *C. gattii* and *C. neoformans* were tested for susceptibility to multiple azoles, we found no significant differences between the cDNAs derived from *C. gattii* and *C. neoformans*. The MICs of multiple azoles were approximately 2-fold higher in *S. cerevisiae* expressing the *ERG11* cDNA from Pacific Northwest *C. gattii* strain R265 than in *S. cerevisiae* expressing the *ERG11* cDNA from non-Pacific Northwest *C. gattii* strain WM276, which corresponded to our observation that the MICs of several azoles for 25 Pacific Northwest *C. gattii* strains were approximately 2-fold higher than those for 34 non-Pacific Northwest *C. gattii* strains. However, we observed only minor differences in azole MICs when the *ERG11* cDNAs encoding all of the variant Erg11p sequences observed in this study were expressed in *S. cerevisiae*, and no transformant expressing a variant *ERG11* cDNA had a higher MIC of any azole than did *S. cerevisiae* expressing the *ERG11* cDNA from *C. gattii* R265. We concluded from these observations that the marked differences in azole MICs observed in the 25 Pacific Northwest *C. gattii* strains we studied cannot be explained either by inherent resistance of Pacific Northwest *C. gattii*'s Erg11p to inhibition by azoles or by the presence of any of the single or multiple Erg11p amino acid substitutions observed in this study.

In summary, we have shown that the MICs of multiple azole antifungals for 25 Pacific Northwest *C. gattii* clinical isolates were higher than those for 34 non-Pacific Northwest *C. gattii* or 20 *C. neoformans* isolates. Among these *C. gattii* and *C. neoformans* isolates, high azole MICs were not associated with *ERG11* mRNA levels in either the presence or absence of fluconazole induction. We also found that (i) *C. gattii* Erg11p was no more resistant to inhibition by azoles than was *C. neoformans* Erg11p, (ii) the Erg11p of Pacific Northwest *C. gattii* R265 was no more than 2.4-fold more resistant to inhibition by any azole tested than was Erg11p from non-Pacific Northwest *C. gattii* WM276, and (iii) the amino acid substitutions observed in the deduced Erg11p sequences of 5 Pacific Northwest *C. gattii* strains were not associated with differences in the susceptibilities of these proteins to inhibition by azoles. Since these results imply that *ERG11* does not play a significant role in azole resistance in Pacific Northwest *C. gattii*, the focus of our ongoing studies of mechanisms of azole resistance in Pacific Northwest *C. gattii* is the identification and characterization of plasma membrane azole efflux pumps. Plasma membrane azole efflux pumps are known to cause azole resistance in *C. albicans*, *C. neoformans*, and other fungi (21, 22, 25), but they have not yet been studied in *C. gattii*.

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