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Role of *AFR1*, an ABC Transporter-Encoding Gene, in the In Vivo Response to Fluconazole and Virulence of *Cryptococcus neoformans*†

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We have recently demonstrated that upregulation of the ATP binding cassette (ABC) transporter-encoding gene *AFR1* in *Cryptococcus neoformans* is involved in the in vitro resistance to fluconazole of this yeast. In the present study, we investigated the role of *AFR1* in the in vivo response to fluconazole in a mouse model of systemic cryptococcosis. Mice were infected with a wild-type fluconazole-susceptible strain of *C. neoformans*, strain BPY22; an *afr1* mutant, BPY444, which displayed hypersusceptibility to fluconazole in vitro; or an *AFR1*-overexpressing strain, BPY445, which exhibited in vitro resistance to the drug. In each of the three groups, infected animals were randomly assigned to fluconazole treatment or untreated-control subgroups. As expected, fluconazole prolonged survival and reduced fungal tissue burdens (compared with no treatment) in BPY22- and BPY444-infected mice, whereas it had no significant effects in mice infected with BPY445. When the pathogenicities of these strains in mice were investigated, strain BPY445 was significantly more virulent than BPY22 following inhalational or intravenous inoculation, but mice infected with BPY444 survived significantly longer than BPY22-infected animals only when infection was acquired via the respiratory tract. In in vitro macrophage infection studies, strain BPY445 also displayed enhanced intracellular survival compared with strains BPY22 and BPY444, suggesting that its increased virulence may be due to its reduced vulnerability to the antimicrobial factors produced by phagocytic cells. These findings indicate that the upregulation of the *AFR1* gene is an important factor in either determining the in vivo resistance to fluconazole or influencing the virulence of *C. neoformans*.

Fluconazole (FLC) and other azole antifungal drugs are the agents most widely used for prevention and treatment of infections with *Cryptococcus neoformans*. Their confirmed efficacy and safety combined with their excellent pharmacokinetic profiles make them extremely important therapeutic options for the management of cryptococcosis at various body sites (6). In spite of their extensive use, resistance to these drugs among *C. neoformans* strains is apparently uncommon, although it has been implicated in several cases of treatment failure or infection relapse (3–4, 6, 20). Some authors have suggested that the frequency of resistance may have been underestimated because azole-resistant mutants of *C. neoformans* are often less virulent than their wild-type counterparts (6, 22). However, findings with other pathogenic yeast species demonstrate that antifungal resistance is not necessarily associated with attenuated pathogenicity (2, 44). For example, in a study of *Candida albicans*, Becker et al. (2) demonstrated that virulence is reduced by the disruption of the efflux pump-encoding *MDR1* gene, whose overexpression leads to fluconazole resistance (36, 42). Graybill et al. (21) used a mouse model of systemic candidiasis to assess the virulences of a series of *C. albicans* isolates with increasing fluconazole MICs. In some cases, resistance to the azole drug was indeed associated with decreased virulence; for example, this was the case for two strains that overexpressed the *CDR* gene, which encodes an ATP binding cassette (ABC) drug efflux pump that has been strongly associated with azole resistance. In contrast, in other fluconazole-resistant isolates, which overexpressed the *MDR* gene, virulence was unchanged. These results highlight the complexities of the relationship between pathogen, host, and antifungal therapy.

In a recent study of *Brucella abortus*, a facultative intracellular pathogen that, like *C. neoformans* survives and proliferates in macrophages, Rosinha et al. (35) demonstrated that an ABC transporter, the polysaccharide-exporting ExsA protein, plays an essential role in the in vivo virulence of this bacterium. This finding is of particular interest in light of our recent demonstration (32) that the ABC transporter-encoding gene *AFR1* (*a*ntif*u*ngal *r*esistance *1*; formerly known as *CnAFR1*) is involved in the in vitro resistance of *C. neoformans* to fluconazole. *AFR1* expression in *C. neoformans* strain BPY22.17, a fluconazole-resistant mutant created by drug exposure, was significantly higher than that found in the fluconazole-susceptible parental strain, BPY22. Disruption of *AFR1* in strain BPY22.17 resulted in a deletion mutant that was fully susceptible to the azole, and reintroduction of the gene promptly restored the resistance phenotype (32). These findings clearly indicated that upregulation of *AFR1* is an important determinant of the increased in vitro resistance of *C. neoformans* to azoles, although other mechanisms may also be involved in this phenomenon (34).

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In the present study, we investigated the possible influence of *AFR1* expression on the in vivo response of *C. neoformans* to fluconazole therapy and on its in vivo virulence. For this purpose, we tested three *C. neoformans* strains in murine models of cryptococcosis: the wild-type BPY22 strain mentioned above, an *afr1* mutant (BPY444), and an *AFR1*-overexpressing mutant (BPY445).

MATERIALS AND METHODS

Strains, media, and drug susceptibility testing. The six *C. neoformans* strains used in this study are serotype D strains derived from the wild-type fluconazole-susceptible clinical isolate, strain BPY22 (32). Strain BPY22.17 is the fluconazole-resistant mutant created by exposing BPY22 to increasing concentrations of the antifungal drug (32). Strain BPY444 (an *afr1* mutant) and the three *AFR1*-overexpressing mutants, strains BPY445, BPY446, and BPY447, were created for the present study as described below.

Strains were stored in 20% glycerol stocks at −80°C and subcultured on YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates at 30°C when necessary. Unless otherwise stated, YPD liquid medium was used to support routine growth of the strains. YPD containing nourseothricin (clonNAT; Werner Bioagents, Jena, Germany) (final concentration, 100 μg/ml) was used to select transformants. D-Sorbitol (Sigma Aldrich, Milan, Italy) was present (final concentration, 1 M) in the YPD agar used for biolistic DNA delivery. Prior to being used in mouse studies, yeast strains were grown in YPD broth for 24 h at 30°C with shaking. They were then harvested by low-speed centrifugation, washed three times with sterile phosphate-buffered saline (PBS), counted with a hemocytometer, and adjusted to the appropriate inoculum density. Inoculum size was confirmed by plating dilutions on YPD agar. Strains were tested for azole susceptibility with the NCCLS M27-A2 broth microdilution method (28), and the lowest drug concentration that reduced growth to 80% of that of the control was recorded as the MIC.

Disruption and overexpression of the *AFR1* gene. To obtain the disruption construct, an 8.5-kb genomic fragment containing the *AFR1* gene in plasmid pCnAFR1gen (32) was digested with BglII and SacI to release an internal 3.1-kb fragment from the *AFR1* gene. This deleted fragment was replaced with a 1.7-kb insert containing a selectable nourseothricin resistance marker (26) whose ends had been modified to include BglII and SacI restriction endonuclease sites. The disruption construct was used to biolistically transform strain BPY22 to obtain the Δ*afr1* mutant strain BPY444. Transformants selected on YPD-nourseothricin medium were subjected to PCR with primers adjacent to the marker gene insert: AFR1ko1 (5′-CCCTGGAGCGCAGGTACTCT-3′) and AFR1ko2 (5′-GCCTC GAGACGCTCGGCAAG-3′). An alteration of the size of a single amplicon (from ~3.1 kb to ~1.7 kb) was considered presumptive evidence of disruption of the native locus. Southern blot hybridization with the C1H9 probe and real-time reverse transcription-PCR (RT-PCR) analysis were used to confirm gene disruption, as described in reference 32 and below.

The *AFR1*-overexpressing mutant strains were created with plasmid pRCD83, which was generously provided by J. Andrew Alspaugh from Duke University in Durham, N.C. (41). The *AFR1* gene was cloned in this vector under the control of the constitutively active promoter of *C. neoformans GPD1*. The *AFR1* gene was amplified from pCnAFR1gen plasmid DNA by use of primers AFR1exp1 (5′-AAGGATCCATGTCCGCTACGGGCGTTCCAGCG-3′) and AFR1exp2 (5′-AAAGGATCCGCCGGGTTGGTAAACCTGGA-3′), which contained BamHI restriction endonuclease sites at both the ends. The PCR fragment was ligated to a BamHI-cut pRCD83 vector to create plasmid pMS008 (p*GPD1-AFR1*). The nourseothricin resistance gene was then excised from plasmid pGMC300 (32) by digestion with XbaI and SpeI and cloned into the XbaI-cut pMS008 to create plasmid pMS012. By use of biolistic transformation, the new plasmid was integrated into the BPY22 strain to create the *AFR1*-overexpressing mutant strains BPY445, BPY446, and BPY447. Transformants were subjected to several passages on a nonselective medium followed by culturing on YPD-nourseothricin medium to assess the stability of the integration. Results were confirmed by PCR using primers specific for the *GPD1* promoter and for the *AFR1* gene: AFR1exp3 (5′-GGAAACGAACGCATGGGGAG-3′) and AFR1exp4 (5′-TTTTGCTTGCCATCGGCAGC-3′). Southern blot analysis was used to establish that the overexpression construct was inserted at different sites in the genome. Overexpression of *AFR1* in all three mutant strains was verified by real-time RT-PCR analysis, as described below.

Animal studies. Female BALB/c mice (4 to 6 weeks old; 20 to 25 g) purchased from Harlan Italy S.r.l. (San Pietro al Natisone, Udine, Italy) were housed in filter-top cages with free access to food and water. The mice were maintained in our Unit for Laboratory Animal Medicine according to protocols that had been approved by an institutional animal use committee.

C. neoformans infection was established via intravenous inoculation in accordance with a recently described protocol with some modifications (1). The inocula (5×10^4 yeast cells for tissue burden studies and 1×10^6 yeast cells for survival studies, each in a volume of 250 μl) were injected into the lateral tail veins. There were 10 mice per group for survival studies and 6 mice per group for tissue burden studies. Infection was also established via nasal inhalation, as described elsewhere (13). Groups of 10 mice were anesthetized with intraperitoneal injections of ketamine (1.25 mg per 25 g of body weight) and xylazine (0.2 mg per 25 g of body weight) and inoculated intranasally with 5×10^4 yeast cells in a 50-μl volume. The mice were monitored with twice-daily inspections. In survival studies, animals that appeared moribund or in pain were sacrificed by use of CO_2 inhalation. In tissue burden studies, infected mice were sacrificed in the same manner at a predetermined time point. Brains, lungs, and spleens were then removed aseptically, weighed, and homogenized in 5 ml of PBS by use of a Stomacher 80 device (Pbi International, Milan, Italy) for 120 s at high speed. Homogenates were plated onto YPD agar plates in 10-fold dilutions and incubated at 30°C. Colonies were counted 48 h later, and the numbers of CFU per gram of organ were calculated. An aliquot of each homogenate was stored at −80°C for RT-PCR analyses (see below).

For in vivo fluconazole susceptibility studies, mice infected with each cryptococcal strain were randomized to fluconazole treatment and untreated-control groups. A stock solution of fluconazole (2 mg/ml) (Pfizer Inc., New York, N.Y.) in sterile distilled water was used. The drug was administered intraperitoneally at a dose of 10 mg/kg/day (delivered in a 100-μl volume), which is the dose found to be most effective in the murine model being used (1). In survival experiments, treatment was initiated 24 h after infection and continued for 10 days (postinfection days 1 through 10). Morbidity and mortality were recorded daily through postinfection day 60. In tissue burden studies, treatment began 24 h postinfection and continued through postinfection day 7. Twenty-four hours after the end of therapy, mice were sacrificed and target organs were excised and examined as described above.

Quantitative real-time RT-PCR. Total RNA was extracted from exponential-phase yeast cultures or mouse organ homogenates with an RNeasy Protect Mini kit (QIAGEN, Hilden, Germany). The process involved mechanical disruption of the cells with glass beads and an RNase-free DNase treatment step as previously described (32). Expression of the *C. neoformans* genes *AFR1*, *LAC1*, *PLB1*, *URE1*, *SOD1*, *VPH1*, *CAP10*, *CAP59*, *CAP60*, and *CAP64* was quantitatively assessed with real-time RT-PCR in an i-Cycler iQ system (Bio-Rad Laboratories, Hercules, Calif.). All primers and probes (see Table S1 in the supplemental material) were designed with Beacon Designer 2 (version 2.06) software (Premier Biosoft International, Palo Alto, Calif.) and synthesized by MWG Biotech (Florence, Italy).

RT-PCRs were carried out as previously described (37). Each reaction was run in triplicate. For relative quantification of target gene expression, *ACT1* was selected as the normalizer gene. To assess the validity of *ACT1* as a reference gene for comparative studies of in vitro/in vivo expression, an absolute quantification of *ACT1* transcripts was performed. To this end, a standard curve was constructed by plotting serial dilutions of a cloned *ACT1* gene fragment (range, 10^6 to 10^1 copies/reaction) and used to quantify *ACT1* mRNA in samples of RNA extracted from cultured cryptococcal cells or from infected organ homogenates. The number of CFU was determined in each sample, and the results were expressed as the amount of *ACT1* transcript per CFU. Similar amounts of *ACT1* transcript were found in the cells grown in vitro and in those recovered from the infected brains (1.2×10^2 and 0.9×10^2 copies/CFU, respectively, for strain BPY22; 1.3×10^2 and 0.9×10^2, respectively, for strain BPY444; and 1.1×10^2 and 0.85×10^2, respectively, for strain BPY445). This finding, which is consistent with previous reports (18), demonstrates that this gene is expressed at nearly identical levels in *C. neoformans* cells grown in vivo and in vitro.

Changes (*n*-fold) in gene expression relative to that of the BPY22 strain were determined from mean *ACT1*-normalized expression levels, as previously described (31). A twofold increase in the expression level of each gene was considered significant.

Macrophage infection assay. Bone marrow-derived macrophages (BMMϕ) were collected by flushing the femurs of C57BL6/J mice. As described elsewhere (5, 33), collected cells were cultured in complete cell culture medium (cCM), i.e., Dulbecco's modified Eagle's medium (Invitrogen, San Giuliano Milanese, Milan, Italy) containing 10 mM HEPES, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 50 μg of gentamicin per ml, and 0.1 mM nonessential amino acids, and supplemented with 10% L-929 fibroblast-conditioned medium and 10% heat-inactivated fetal calf serum (Invitrogen). Briefly, cells were plated in 24-well

TABLE 1. Azole susceptibilities and *AFR1* expression levels of the *C. neoformans* strains evaluated in this study

Strain	Characteristic	*AFR1* expression[a]	MIC (μg/ml)[b] of:		
			Fluconazole	Ketoconazole	Itraconazole
BPY22	Parental strain	1.00	2	0.016	0.03
BPY444	*afr1*::*NAT*	None	0.25	0.008	0.008
BPY445[c]	*pGPD1-AFR1*	12.58 ± 0.25	64	0.125	0.25
BPY22.17	Fluconazole-resistant mutant[d]	16.02 ± 1.86	64	0.25	1

[a] Expression levels determined by real-time RT-PCR analysis (see the text for details) are expressed as increases (*n*-fold) over the level observed in the parental strain, expressed as 1.00.
[b] MICs were determined by the broth microdilution method, in accordance with NCCLS document M27-A2 (28).
[c] Two other *AFR1*-overexpressing strains, BPY446 and BPY447, used to confirm results obtained with strain BPY445, displayed *AFR1* expression levels of 13.02 ± 0.32 and 12.82 ± 0.23, respectively. Azole susceptibility was not determined for these strains.
[d] BPY22.17 is a fluconazole-resistant strain that had been generated by in vitro exposing the BPY22 strain to increasing drug concentrations (32).

tissue culture plates (Falcon, Becton Dickinson, Milan, Italy) at 10^5 per well in 1 ml of cCM. After 48 h of incubation at 37°C in 5% CO_2, nonadherent cells were removed, and the remaining cells, macrophages, were incubated for 6 more days. The cCM was replaced with incomplete cell culture medium (iCM), which lacked 2-mercaptoethanol, gentamicin, and L-929-conditioned medium. Cryptococci were washed twice with PBS, counted with a hemocytometer, and diluted to the appropriate concentration (10^5 cells/ml) in iCM. Immunoglobulin G1 anti-GXM monoclonal antibody (18B7) was added to the yeast inocula as an opsonin at a concentration of 10 μg/ml as previously described (15). Cryptococci (10^5 cells; multiplicity of infection, 1:1) were added to each well in a final volume of 2 ml. Control wells containing only macrophages or only yeast cells were included. Following incubation at 37°C in 5% CO_2 for 4 h, extracellular yeast cells were removed with three PBS washes, and a 2-ml volume of iCM was added to the infected monolayers. At 7 and 28 h postinfection, culture supernatants were aspirated, and the macrophages were lysed with 1 ml of 0.5% Triton X-100 in PBS. Aliquots of lysates were cultured on YPD agar containing chloramphenicol, and the number of CFU per ml was determined after 48 h of incubation at 30°C. All infected macrophage cultures were performed in triplicate, and all experiments were repeated three times.

Phenotypic virulence testing. Capsule formation, melanin production, growth capability at 37°C, and secretion of urease, phospholipase, and laccase were evaluated using previously described protocols (27, 43).

Statistics. Survival data from the mouse experiments were analyzed using the log-rank test. An unpaired *t* test was used in evaluating the CFU from the tissue burden studies. Macrophage survival data were analyzed by using one-way analysis of variance with a Bonferroni correction posttest. A *P* value of less than 0.05 was considered to be significant.

RESULTS

Generation of *AFR1*-deficient and -overexpressing mutant strains of *C. neoformans*. In the present study, we generated a set of isogenic strains of *C. neoformans* derived from strain BPY22, a fluconazole-susceptible clinical isolate of *C. neoformans* that has been described in detail elsewhere (32).

The *AFR1* gene was disrupted as described in Materials and Methods, and the resulting disruption allele, *afr1*::*NAT*, was introduced into the wild-type strain, BPY22. Of 60 transformants produced, 6 with presumptive evidence of native *AFR1* disruption were evaluated with Southern blotting and real-time RT-PCR analysis. These analyses confirmed the absence of *AFR1* transcripts in one of these transformants, and this Δ*afr1* mutant strain, designated BPY444, was used in subsequent experiments.

Next, the *AFR1* gene was cloned under the control of the constitutively active promoter of the glucose-6-phosphate dehydrogenase gene (*GPD1*) and integrated into the genome of the wild-type strain, BPY22, by use of biolistic transformation. Real-time RT-PCR analysis allowed us to select transformants whose expression of *AFR1* was increased over that of the wild-type strain. One of these *AFR1*-overexpressing mutant strains was designated BPY445 and used for further analyses. Two other strains (BPY446 and BPY447) were used as control strains in the virulence studies (see below).

We also compared the BPY22, BPY444, and BPY445 strains with BPY22.17 (32), a fluconazole-resistant mutant strain generated in vitro by exposing strain BPY22 to increasing concentrations of the drug. Table 1 summarizes the *AFR1* expression and azole susceptibility profiles of the *C. neoformans* strains evaluated in this study. *AFR1* expression levels in BPY445 (the *AFR1*-overexpressing strain) and BPY22.17 were over 10 times higher than those observed in the wild-type strain, BPY22. The similarities between these two strains also included identical high fluconazole MICs (64 μg per ml) (Table 1), both of which remained unchanged after serial passages on fluconazole-free media (data not shown). In contrast, no *AFR1* message was detected in BPY444, and this *afr1* strain displayed even greater susceptibility to fluconazole than BPY22 (Table 1). Otherwise, the disruption of the *AFR1* gene in BPY22.17 rendered the *afr1* mutant as susceptible to azoles as the parental strain BPY22 (32). Collectively, these findings strongly indicate that the *AFR1* gene is involved in the in vitro fluconazole resistance of *C. neoformans*.

***AFR1* and in vivo resistance to fluconazole.** To identify the possible role of *AFR1* in the in vivo response to fluconazole, we evaluated the effects of the drug in mice infected with *C. neoformans* strains BPY22, BPY444, and BPY445. For survival studies, three groups of mice were inoculated intravenously, the first with BPY22, the second with BPY444, and the third with BPY445. Twenty-four hours later, animals of each group were randomized to fluconazole treatment or untreated-control subgroups. The results of survival studies are summarized in Fig. 1. In the groups infected with BPY22 or BPY444, the survival times of fluconazole-treated mice were significantly longer than those of the untreated controls ($P < 0.0001$), but survival times among BPY445-infected mice were almost identical in treated and untreated subgroups (26 days versus 27 days; $P = 0.20$). Figure 2 shows the results of fungal tissue burden studies. In the group of animals infected with BPY445, there were no significant differences between treated and control subgroups in the fungal burdens found in the brain ($P = 0.12$), lung ($P = 0.11$), or spleen ($P = 0.46$). In contrast, in the groups infected with the BPY22 and BPY444 strains, fluconazole-treated animals presented fungal burdens significantly lower than those of controls (−2.2 and −3.1 logs in the brains,

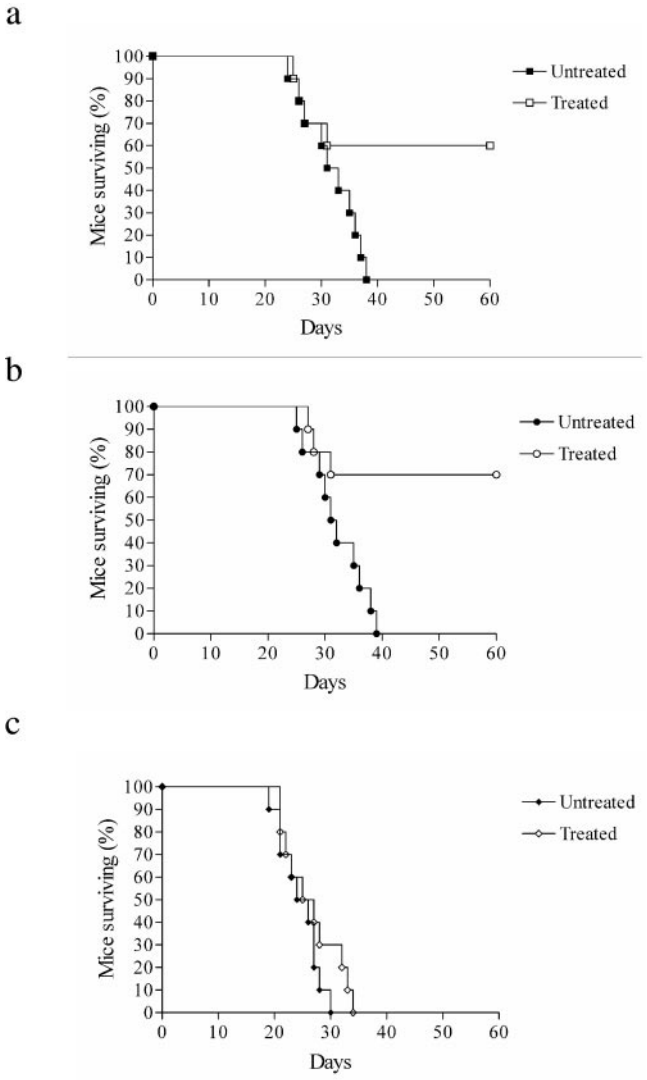

FIG. 1. Effects of FLC treatment on survival of mice infected intravenously with 1×10^6 viable cells of *C. neoformans* strain BPY22 (a), BPY444 (b), or BPY445 (c). FLC was administered intraperitoneally for 10 days at 10 mg/kg/day. Survival rates (percentages) for FLC-treated animals and untreated control animals are plotted against times after inoculation (days).

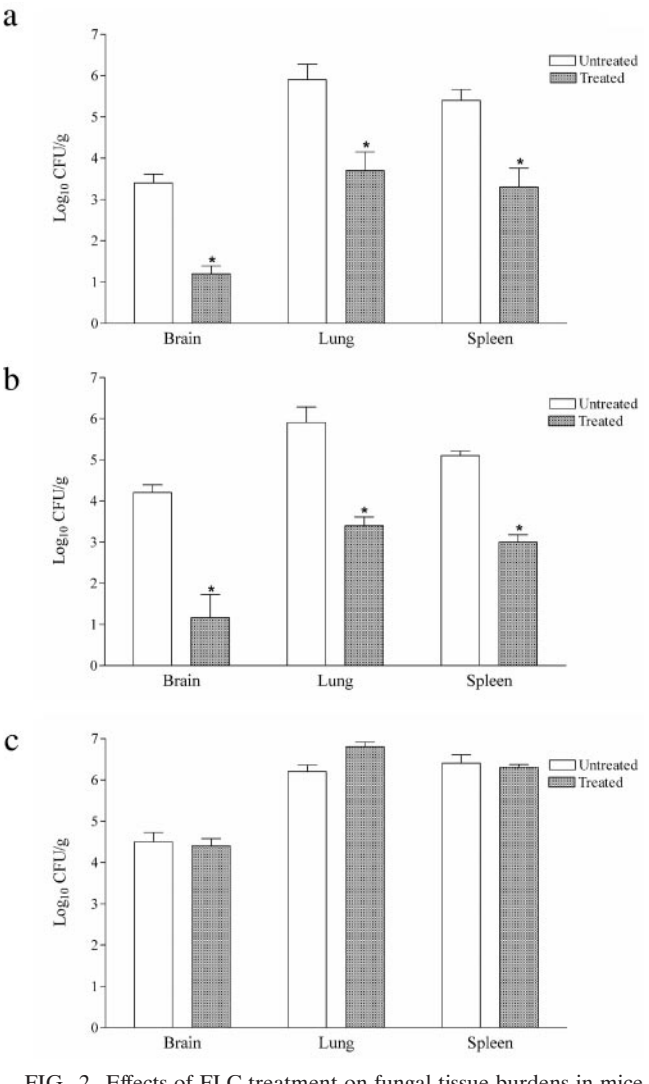

FIG. 2. Effects of FLC treatment on fungal tissue burdens in mice infected intravenously with 5×10^4 viable cells of *C. neoformans* strain BPY22 (a), BPY444 (b), or BPY445 (c). FLC (10 mg/kg/day) was administered by intraperitoneal injection. Treatment was initiated 24 h after inoculation (postinfection day 1) and continued through postinfection day 7. Organ homogenates were obtained from six mice per group that were sacrificed and necropsied on postinfection day 8. Results, which are expressed as CFU per gram of tissue, represent means of values recorded separately for each of the six mice. Error bars represent the standard deviation of the mean, and asterisks indicate statistically significant differences ($P < 0.0001$).

-2.2 and -2.5 logs in the lungs, and -2.1 and -2.1 logs in the spleens of BPY22- and BPY444-infected animals, respectively [$P < 0.0001$]). These results indicate that *AFR1* overexpression is essential for the in vivo fluconazole resistance we observed in *C. neoformans*.

***AFR1* and virulence.** Next, we investigated the possible involvement of *AFR1* in the virulence of *C. neoformans* by analysis of the behaviors of strains BPY22, BPY444, and BPY445 in two mouse infection models. As shown in Fig. 3a, in the intravenous model, BPY22-infected mice survived significantly longer than those infected with strain BPY445 (mean survival time, 32 days versus 26 days; $P < 0.05$), but the survival time of BPY444-infected animals was not significantly different from that of mice infected with the wild-type BPY22 strain ($P = 0.68$). In the inhalational model (Fig. 3b), the survival time of BPY445-infected mice was also significantly shorter than that of the BPY22-infected group (mean survival times, 30 days versus 40 days; $P < 0.0001$), and there was also a significant difference between mice infected with the BPY444 strain and those infected with the BPY22 strain (mean survival time, 47 days versus 40 days; $P < 0.05$).

These data, particularly those obtained in the inhalational model of infection, strongly suggested that the upregulation of *AFR1* also enhances the virulence of *C. neoformans*. However, to exclude the possibility that increased virulence of strain BPY445 was related to the genomic position of the overexpression construct we inserted, we repeated in vivo tests in

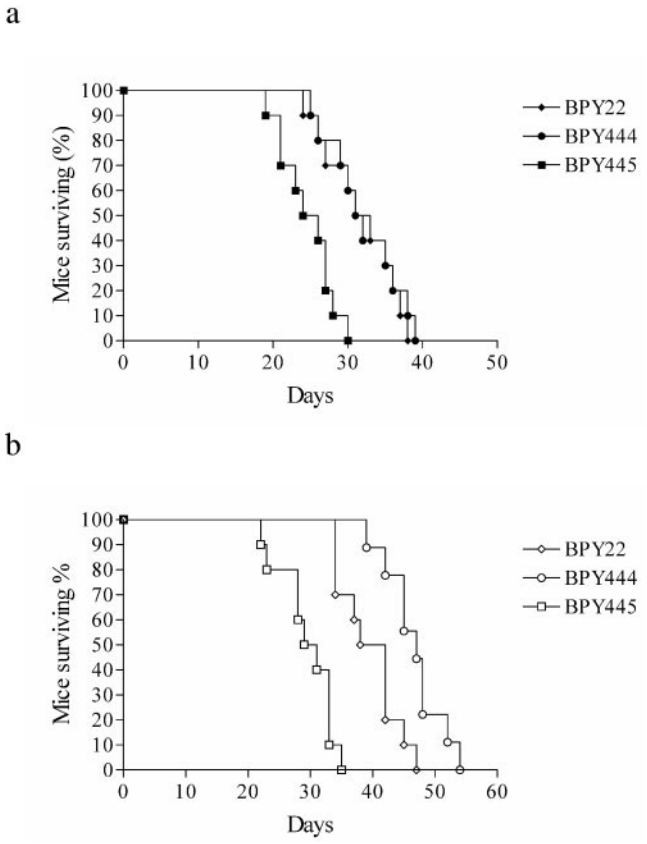

FIG. 3. Virulence of *C. neoformans* strains BPY22, BPY444, and BPY445 in two mouse models. (a) Model 1: intravenous infection. Mice were intravenously injected with 1×10^6 viable yeast cells. (b) Model 2: infection by inhalation. Anesthetized mice were intranasally inoculated with 5×10^4 viable yeast cells. Survival rates (percentages) are plotted against times after inoculation (days).

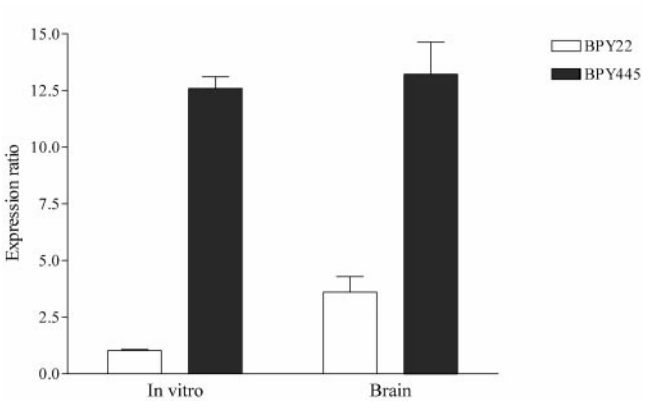

FIG. 4. Expression of *AFR1* in *C. neoformans* strains BPY22 and BPY445 grown in vitro and in those present in the brains of infected mice. Quantification of the relative transcript levels was performed by real-time RT-PCR analysis as described in Materials and Methods. Results are reported as increases (*n*-fold) in actin-normalized expression levels (with respect to that observed in BPY22 grown in vitro, which is represented as 1.0). Means are shown for three independent experiments. Error bars show standard deviations.

both models using two other independent transformants, BPY446 and BPY447, in which the overexpression construct was located at different sites in the *C. neoformans* genome, as assessed by Southern blot analysis (data not shown). Results obtained with these two strains were fully consistent with those reported for strain BPY445 (for the intravenous model, the mean survival time for BPY446 was 27.5 days versus 26 days for BPY455 [$P = 0.29$], and that for BPY447 was 27 days versus 26 days for BPY455 [$P = 0.36$]; for the inhalational model, the mean survival time of BPY446 was 31 days versus 30 days for BPY455 [$P = 0.55$], and that for BPY447 was 29.5 days versus 30 days for BPY455 [$P = 0.71$]). Strain BPY22.17 also proved to be significantly more virulent than strain BPY22 in both the intravenous model (mean survival time of 20 days versus 32 days [$P < 0.0001$]) and the inhalational model (mean survival time of 22 days versus 40 days [$P < 0.0001$]), adding further support to the concept that increased virulence can be associated with azole resistance in *C. neoformans*.

The next step was to verify that the *AFR1* gene was indeed being expressed in vivo. To this end, RNAs isolated from the brains of mice infected with each of the three strains were analyzed by real-time RT-PCR. Figure 4 shows the actin-normalized expression levels of the *AFR1* gene for the groups of BPY22- and BPY445-infected organs. As expected, there were no detectable *AFR1* transcripts in the brains of BPY444-infected mice (data not shown). The *AFR1* overexpression observed in strain BPY445 grown in vitro was also seen in yeast cells found in infected brain tissues (Fig. 4), and similar trends emerged from analysis of *AFR1* expression in the lungs and spleens of the three test groups (data not shown).

To determine whether the activity of known *C. neoformans* virulence-related genes might have contributed to the increased virulence of strain BPY445, we analyzed the expression of nine such genes, *LAC1*, *PLB1*, *URE1*, *SOD1*, *VPH1*, *CAP10*, *CAP59*, *CAP60*, and *CAP64* (8–11, 13–16, 43), in the brains of BPY445- and BPY22-infected mice. For all nine genes, RT-PCR revealed no significant differences between the expression levels observed in the two strains (data not shown). In parallel, we analyzed the classic phenotypic parameters of virulence, including growth at 37°C and the presence of polysaccharide capsule, melanin, urease, and phospholipase, and found no differences between strains BPY445 and BPY22 (data not shown).

***AFR1* and survival inside macrophages.** Cryptococci were tested in an in vitro macrophage infection model to explore the mechanisms underlying the increased virulence of strain BPY445. Strains BPY22, BPY444, and BPY445 were incubated with primary BMMϕ, and the survival of intracellular yeasts was determined by CFU assays. As shown in Fig. 5, the BPY445 strain exhibited a superior capacity for intracellular survival. Twenty-eight hours after infection, the number of viable yeast cells recovered from BPY445-infected macrophages (5.7×10^4 CFU/ml) was significantly higher than those for macrophages infected with strain BPY22 (3.5×10^4 CFU/ml) or BPY444 (3.1×10^4 CFU/ml) ($P < 0.05$). No significant differences in the colony counts were noted 7 h postinfection (Fig. 5). This observation suggests that the level of *AFR1* expression affects the capacity of *C. neoformans* to resist macrophage killing.

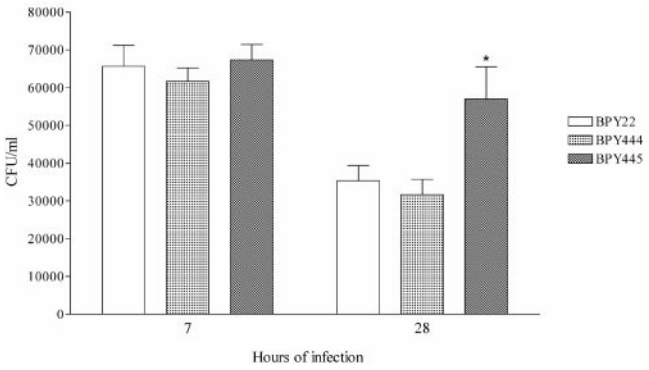

FIG. 5. Recovery of viable *C. neoformans* cells from BMMϕ infected with strains BPY22, BPY444, and BPY445. At the indicated hours after infection, serial dilutions of macrophage lysates were plated, and yeast colonies were counted 48 h later. Results, which are expressed as CFU per milliliter, represent the mean and standard error of three experiments. The asterisk indicates statistically significant difference with respect to results observed at the same time point for strain BPY22 ($P < 0.05$).

DISCUSSION

We have previously demonstrated that in vitro fluconazole resistance in *C. neoformans* can be related to a novel gene that encodes an ABC transporter, *AFR1*. The in vitro acquisition of fluconazole resistance by a laboratory-constructed strain of *C. neoformans*, BPY22.17, was associated with an increase in the expression of *AFR1* over that observed in the fluconazole-susceptible parental strain, BPY22. Furthermore, when this gene was deleted in strain BPY22.17, the strain's susceptibility to fluconazole was restored (32).

In the present study, we attempted to determine whether *AFR1* expression also influenced the in vivo response to fluconazole in murine models of cryptococcosis. For this purpose, we used a set of three isogenic cryptococcal strains which differed only in terms of *AFR1* expression: a wild-type parental strain, BPY22 (the same one used in our previous study), and two derivatives, an *afr1* mutant (BPY444) and one that overexpressed *AFR1* (BPY445). As shown in Table 1, strong positive correlation was observed between *AFR1* transcript levels and in vitro fluconazole MICs for the three strains.

Not surprisingly, fluconazole significantly prolonged survival and reduced fungal tissue burdens in mice intravenously infected with the BPY22 strain or the BPY444 strain. Mice that had been challenged with the BPY445 strain displayed significantly shorter survival times and higher fungal burdens in the brain, spleen, and lungs than the BPY22- and BPY444-infected animals, and there was no significant difference between the fluconazole treatment and untreated-control subgroups. These results confirm that the *AFR1* gene is directly implicated in the in vivo fluconazole resistance of *C. neoformans*, but the same gene was also shown to play a role in the enhancement of cryptococcal virulence. In two well-established murine models of cryptococcosis, the survival time among mice infected with strain BPY445 was significantly shorter than that observed following infection with the parental strain. The same increased virulence was displayed by two other independent *AFR1*-overexpressing mutant strains, BPY446 and BPY447, and by the fluconazole-resistant strain BPY22.17.

Our quantitative RT-PCR analyses of tissues from animals challenged with strains BPY22 and BPY445 provided clear evidence of the in vivo expression of *AFR1*, a fundamental requisite for proving that a given gene plays an important role in a process of microbial pathogenesis (17). The causal link between *AFR1* expression levels and the observed differences in in vivo pathogenicity is further supported by the absence in strain BPY445 of significant alterations at both the phenotypic and molecular levels in the expression of nine other *C. neoformans* genes that are known determinants of virulence.

The dual role of *AFR1* documented by our findings reflects a new scenario in the complex area of fungal virulence studies. Thus far, there has been no evidence to suggest that increased virulence in *C. neoformans* might be associated with azole resistance. On the contrary, some findings indicate that drug-resistant *C. neoformans* strains may actually be less virulent. Iwata et al. (22) reported that itraconazole- and ketoconazole-resistant mutants of virulent strains were avirulent in mice, although these observations cannot be considered conclusive in the absence of specific gene disruption and replacement experiments (7). In general, while research on the acquisition, regulation, and underlying mechanisms of drug resistance has been intense, much less attention has been focused on the relationships between drug resistance and virulence, at least for fungal pathogens.

At first glance, there is little reason to suspect that the two phenomena might be linked. Numerous studies conducted on bacteria have lent support to the "cost of resistance" theory, demonstrating that drug-resistant strains grow more slowly and are less virulent than their antibiotic-susceptible counterparts. However, there are several examples indicating that virulence determinants contribute to an antibiotic resistance phenotype, and vice versa (25). Host-pathogen interactions include the uptake and secretion of substances, which are facilitated by proteins termed transporters. Recent studies have shown that multidrug resistance efflux pumps (29) can also contribute to bacterial virulence by extruding compounds involved in the host defense (25). A good example is the multidrug resistance determinant *acrB* in *Salmonella enterica* serotype Typhimurium. The inactivation of this gene abolished the ability of the bacterium to extrude bile salts and consequently diminished its capacity for colonizing the intestinal tract in a murine infection model (23). The role of ABC transporters in bacterial pathogenesis has also been underscored in a report by Rosinha et al. (35), who demonstrated that the ABC transporter ExsA is required for full virulence during infection by the intracellular pathogen *B. abortus*.

Recent investigations have also revealed a complex link between fluconazole resistance and virulence in *C. albicans*. To better define this relationship, Wu et al. (44) examined a series of *C. albicans* isolates obtained from a single AIDS patient before and after the development of clinical and in vitro resistance to fluconazole. They measured the isolates' in vitro production of secreted aspartyl proteinase (Sap), a *C. albicans* virulence factor, and found that the enhanced Sap production induced by fluconazole was correlated with upregulation of the drug efflux pump gene *MDR1*. In an earlier study by this group (T. Wu, K. Wright, S. F. Hurst, and C. J. Morrison, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. F-81, p. 311, 1999), the same isolates were tested in a murine model of disseminated

candidiasis, and the one displaying the highest level of resistance to fluconazole was found to be innately more virulent than the most susceptible isolate in the series. Furthermore, unlike mice infected with susceptible isolates, those challenged with resistant isolates and treated with clinical doses of fluconazole were characterized by increased mortality and fungal tissue burdens compared with untreated controls.

The mechanism by which *AFR1* expression influences cryptococcal virulence is still unclear, but our findings indicate that the increased virulence of strain BPY445 might be due to its enhanced ability to survive inside macrophages. Alveolar macrophages are the primary host defense against inhaled cryptococci, and intracellular yeast cells are seen at all stages of infection produced by endotracheal inoculation (19). Viable cryptococci can be found in the peripheral blood, as free organisms, or internalized in mononuclear phagocytes (12). *C. neoformans*-infected macrophages and mononuclear phagocytes are vehicles of cryptococcal infection (38), and the ability of a strain to survive within these cells is thus an important determinant of its virulence. Using a primary BMMϕ model that has been validated for investigation of the interactions between host cells and intracellular pathogens, such as *Mycobacterium tuberculosis* (5, 33), we demonstrated that the overexpression of *AFR1* is indeed associated with a level of intramacrophage survival that is significantly enhanced compared with that of the wild-type strain.

Whether this association is causal remains to be seen. Nonetheless, it seems plausible that the ABC transporter encoded by *AFR1* might provide a form of defense against antimicrobial substances produced by the host phagocytic cells. The possible link between ABC transporters and fungal virulence is supported by other recent reports (39, 40). For instance, in the fungal plant pathogen *Magnaporthe grisea*, upregulation of the *ABC1* gene, which is similar to the ABC transporter genes implicated in multidrug resistance in yeasts, is necessary for host infection. Its contribution is probably related to the extrusion of plant-derived toxins (40). Furthermore, the role of the vacuolar ABC transporter Mlt1 in the virulence of *C. albicans* has been putatively attributed to its elimination of toxic substances produced by host phagocytes (39).

It is worth noting that the involvement of Mlt1 in *C. albicans* virulence was demonstrated in a mouse model of *Candida* peritonitis, but it was not observed in a model of systemic intravenous infection (39). Discrepancies of this type are not uncommon. In fact, the impact on virulence of certain *C. neoformans* genes (i.e., *URE1*, *CNLAC1*) is thought to be infection stage and/or infection site specific (13, 30). This might explain why, in our virulence experiments, the deletion of *AFR1* reduced cryptococcal virulence in the inhalational model of infection but had no significant effect in the intravenous model. It may be that the contribution of *AFR1* is limited to, or at least more important during, the early stages of infection (initiation and dissemination from the lungs). In other words, the efflux pump encoded by this gene might be a particularly important defense against microbicidal proteins produced by alveolar macrophages compared to those produced by peripheral blood monocytes.

It should also be recalled that the deletion of *AFR1* did not render the BPY444 strain avirulent, and this finding supports the view that cryptococcal pathogenicity (like that of other fungal pathogens) is multifactorial and regulated by more than one determinant. Indeed, Marchetti et al. (24) have reported similar levels of virulence in a fluconazole-susceptible *C. albicans* strain and in its hypersusceptible mutant derivative created by the deletion of four genes encoding multidrug efflux transporters (*CDR1*, *CDR2*, *CaMDR1*, and *FLU1*).

In conclusion, upregulation of the *AFR1* gene in *C. neoformans* is not only an important determinant of in vitro and in vivo resistance to fluconazole; it also enhances the virulence of this yeast and, specifically, its ability to survive within macrophages. The exact mechanisms underlying these last effects should be clarified in future studies.

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