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Genetic Dissection of Azole Resistance Mechanisms in *Candida albicans* and Their Validation in a Mouse Model of Disseminated Infection▽†

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Principal mechanisms of resistance to azole antifungals include the upregulation of multidrug transporters and the modification of the target enzyme, a cytochrome P450 (Erg11) involved in the 14α-demethylation of ergosterol. These mechanisms are often combined in azole-resistant *Candida albicans* isolates recovered from patients. However, the precise contributions of individual mechanisms to *C. albicans* resistance to specific azoles have been difficult to establish because of the technical difficulties in the genetic manipulation of this diploid species. Recent advances have made genetic manipulations easier, and we therefore undertook the genetic dissection of resistance mechanisms in an azole-resistant clinical isolate. This isolate (DSY296) upregulates the multidrug transporter genes *CDR1* and *CDR2* and has acquired a G464S substitution in both *ERG11* alleles. In DSY296, inactivation of *TAC1*, a transcription factor containing a gain-of-function mutation, followed by sequential replacement of *ERG11* mutant alleles with wild-type alleles, restored azole susceptibility to the levels measured for a parent azole-susceptible isolate (DSY294). These sequential genetic manipulations not only demonstrated that these two resistance mechanisms were those responsible for the development of resistance in DSY296 but also indicated that the quantitative level of resistance as measured *in vitro* by MIC determinations was a function of the number of genetic resistance mechanisms operating in any strain. The engineered strains were also tested for their responses to fluconazole treatment in a novel 3-day model of invasive *C. albicans* infection of mice. Fifty percent effective doses (ED_{50}s) of fluconazole were highest for DSY296 and decreased proportionally with the sequential removal of each resistance mechanism. However, while the fold differences in ED_{50} were proportional to the fold differences in MICs, their magnitude was lower than that measured *in vitro* and depended on the specific resistance mechanism operating.

Azole antifungal agents are widely used to treat fungal infections. Over the past 2 decades, several agents of this class, including fluconazole (FLC), itraconazole (ITR), voriconazole (VRC), and posaconazole (POS), have been made available for clinical use in life-threatening, invasive fungal disease. Each of these compounds has a specific clinical utility, which depends on its spectrum of activity, route of administration, and pharmacodynamic properties. Several fungal species have counteracted the action of azoles by developing a number of resistance mechanisms. The fungal target of azole antifungals is a cytochrome P450 (encoded by *ERG11*) involved in the 14α-demethylation of lanosterol, an essential step in the biosynthesis of ergosterol. One of the azole resistance mechanisms, therefore, involves alteration of the target enzyme by amino acid substitutions caused by nonsynonymous mutations in *ERG11*. Such modifications have been documented in *ERG11* from *Candida albicans*, *Candida tropicalis*, and *Cryptococcus neoformans* and in related *ERG11*-like genes, such as *Cyp51A* from *Aspergillus fumigatus* (reviewed by Sanglard et al. [24]).

In addition to target alterations, azole resistance mechanisms include transport phenomena. Azole drugs have been shown to be actively effluxed by transport systems. These include ABC transporters and major facilitator superfamily (MFS) members, which are overexpressed in azole-resistant isolates. In *C. albicans*, both the ABC transporter genes *CDR1* and *CDR2* and the MFS gene *MDR1* can be upregulated, although not simultaneously, indicating the existence of separate regulatory circuits for the two transporter classes. Regulators of these transporters, including *TAC1* and *MRR1*, which control the expression of *C. albicans CDR1*/*CDR2* and *MDR1*, respectively, have been identified (7, 17). Mutations in these transcription factors are responsible for the constitutively high expression of these transporters in clinical isolates (4–6, 9). Other azole resistance mechanisms have been reported; however, their occurrence is relatively rare. For example, loss-of-function mutations in the *ERG3* gene, encoding sterol $\Delta^{5,6}$ desaturase, have been reported in *C. albicans* and *Candida dubliniensis* as a cause of azole resistance (3, 22). Molecular epidemiology of azole resistance mechanisms has often demonstrated the existence of several resistance mechanisms in the same yeast isolate. In these studies, known mechanisms were probed either by testing the expression levels of multidrug transporter genes or by comparing *ERG11* nucleotide sequences between pairs of related azole-susceptible and azole-resistant isolates. In many cases, azole resistance correlated with the occurrence of transporter gene upregulation and nonsynonymous *ERG11* mutations (20, 21). Few attempts have

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been made to evaluate the role of individual mechanisms in strains with multiple azole resistance mechanisms. Wirsching et al. (30) inactivated *MDR1* in a *C. albicans* strain upregulating this transporter and also containing a G464S mutation in both *ERG11* alleles. The mutant strain exhibited lower azole MICs than the azole-resistant isolate, but these values were not as low as those measured for the related azole-susceptible strain. The remaining difference in the MIC was attributed to the G464S substitution still present in the *mdr1*Δ strain. Selmecki et al. (28) have evaluated the relative roles of *ERG11* and *TAC1*, which were present on an isochromosome originating from chromosome 5 (Chr. 5). *ERG11* contributed to azole resistance by the effect of gene copy number variation rather than by a mutation.

We previously investigated an azole-resistant clinical isolate, DSY296, and its azole-susceptible parent (DSY294) from the same patient. Coste et al. (7) showed that the transcription factor *TAC1* was involved in the regulation of the ABC transporters *CDR1* and *CDR2* when *C. albicans* was exposed to drugs, including fluphenazine and estradiol. *TAC1* in the azole-resistant isolate DSY296 is homozygous and contains a gain-of-function (GOF) mutation (N977D), resulting in constitutively high expression of *CDR1* and *CDR2* (6). We showed earlier that the mutation was homozygous at its Chr. 5 location, along with the mating type locus (*MAT***a**/**a**) (7). Deletion of *TAC1* in DSY296 decreased the expression of *CDR1* and *CDR2* to basal levels and consistently decreased azole resistance in the *tac1*Δ/Δ strain (DSY3083), thus demonstrating the relevance of *TAC1* in the development of azole resistance in *C. albicans* (6). No *MDR1* overexpression was noticed in DSY296 (27).

Genetic tools for *C. albicans* have improved greatly, and it is now possible to use almost any clinical strain for genetic manipulation. We have therefore further investigated the clinical isolate DSY296, with its dual azole resistance mechanisms (7, 27). The genetic mediators of these mechanisms (*TAC1* and *ERG11*) were sequentially removed by genetic manipulation or were replaced by wild-type genes, and the resulting azole susceptibilities were measured. Moreover, the azole susceptibilities of the different engineered strains were also determined in animal models with mice given fluconazole treatment.

MATERIALS AND METHODS

Strains and media. The *C. albicans* strains used in this study are listed in Table 1. Strains DSY294 and DSY296 were shown to be indistinguishable by several typing methods using a Ca3 repetitive probe (2) and by multilocus sequence typing (MLST) using recent standards (19). Both strains belong to clade 11 (D. Sanglard and F. Odds, unpublished data). Yeasts were grown in a complete medium, yeast extract-peptone-dextrose (YEPD) (1% Bacto peptone [Difco Laboratories, Basel, Switzerland], 0.5% yeast extract [Difco], and 2% glucose [Fluka, Buchs, Switzerland]), or in a defined medium, 0.67% yeast nitrogen base (YNB; Difco) with 2% glucose (Fluka). To prepare inocula for experimental infections, yeasts were grown in NGY medium (0.1% Neopeptone [Difco], 0.4% glucose, 0.1% yeast extract [Difco]). When strains were grown on a solid medium, 2% agar (Difco) was added. *Escherichia coli* DH5α was used as a host for plasmid constructions and propagation. DH5α was grown in Luria-Bertani (LB) broth or on LB plates, supplemented with ampicillin (0.1 mg/ml) when required.

Yeast transformation. *C. albicans* cells from 0.2 ml of stationary-phase cultures were resuspended in 0.1 ml of a solution containing 200 mM lithium acetate (LiAc) (pH 7.5), 40% (wt/vol) polyethylene glycol (PEG) 8000, 15 mg/ml dithiothreitol (DTT), and 250 μg/ml denatured salmon sperm DNA. Transforming DNA (1 to 5 μg) was added to the yeast suspension, which was incubated for 60 min at 44°C. Transformation mixtures were plated directly onto selective plates.

TABLE 1. Strains used in this study

Strain	Parent	Genotype	Reference
DSY294		*TAC1-3/TAC1-4* *ERG11-3/ERG11-4*	7
DSY296	DSY294	*TAC1-5/TAC1-5* *ERG11-5/ERG11-5*	7
DSY3040	DSY294	*ura3*Δ::*FRT*/*ura3*Δ::*FRT*	7
DSY3041	DSY296	*ura3*Δ::*FRT*/*ura3*Δ::*FRT*	7
DSY3083	DSY296	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG-URA3-hisG* *ERG11-5/ERG11-5*	7
DSY3090	DSY3083	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-5/ERG11-5*	7
DSY3586	DSY3090	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*CtURA3*/*ERG11-5*	This study
DSY3593	DSY3090	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-5*	This study
DSY3595	DSY3593	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-1*::*CtURA3*	This study
DSY3603	DSY3595	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-1*::*SAT1*	This study
DSY3604	DSY3593	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-5* *RPS1*::CIp10	This study
DSY3706	DSY3603	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-1*::*SAT1* *RPS1*::CIp10	This study
DSY3606	DSY3603	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-1*::*SAT1* *RPS1*::*TAC1-5*	This study
DSY3608	DSY3603	*tac1-5*Δ::*hisG*/*tac1-5*Δ::*hisG* *ERG11-1*::*SAT1*/*ERG11-1*::*SAT1* *RPS1*::*TAC1-1*	This study
DSY3671	DSY3041	*TAC1-5/TAC1-5* *ERG11-1*::*SAT1*/*ERG11-5*	This study
DSY3745	DSY3671	*TAC1-5/TAC1-5* *ERG11-1*::*SAT1*/*ERG11-1*::*CtURA3*	This study
DSY3750	DSY3745	*TAC1-5/TAC1-5* *ERG11-1*::*SAT1*/*ERG11-1*::*SAT1*	This study
DSY3752	DSY3750	*TAC1-5/TAC1-5* *ERG11-1*::*SAT1*/*ERG11-1*::*SAT1* *RPS1*::CIp10	This study
DSY294-CIp10	DSY3040	*RPS1*::CIp10	This study
DSY296-CIp10	DSY3041	*RPS1*::CIp10	This study
DSY3083-CIp10	DSY3090	*RPS1*::CIp10	This study

The *SAT1* marker, which is responsible for nourseothricin resistance, was selected with YEPD at a concentration of 200 μg/ml nourseothricin (Werner BioAgents, Germany).

Plasmid constructions. To replace *ERG11* from DSY296 with wild-type copies, two plasmids were constructed, one containing a *SAT1* marker (pDS1453) and the other containing a *URA3* marker from *C. tropicalis* (pDS1454). pDS1453 was obtained by inserting the *SAT1* marker, amplified from pSFS2 with primers SAT1-StuI (5′-ATAAGAATAGGCCTGTCAAAACTAGAGAATAATAAAG-3′) and SAT1-NruI (5′-GCGCAAATCGCGAGGACCACCTTTGATTGTAAATAGT-3′), into the PmeI site of pDS501. Plasmid pDS501 contains the entire *ERG11* open reading frame (ORF) and flanking regions from *C. albicans* strain SC5314, produced by cloning *ERG11* as a 3.5-kb EcoRV-ClaI blunt-ended fragment from pDS271 (26) into pMLT21, which had first been digested by SmaI and AccI followed by blunt ending. For *C. albicans* transformations, the *ERG11-SAT1* cassette was liberated from pDS1453 by XhoI and KpnI digestion. Plasmid pDS1454 was constructed by inserting the *C. tropicalis URA3* (*CtURA3*) gene as a 1.3-kb ApaI-BamHI fragment from pQDS62 in a PCR-amplified *ERG11* fragment from pDS501. Plasmid pQDS62 contains the *C. tropicalis URA3* gene as a 1.3-kb HindIII fragment cloned into the HindIII site of pBluescript KS(+) (D. Sanglard, unpublished data). The PCR fragment was obtained by using primers pSP2-Apa (5′-AATCCTGGGCCCATATTAGCAGATGATGC-3′) and pSP2-BamBI (5′-TTCATCGGATCCTCACCATGCCTTATT-3′) with pDS501 as a template. pDS1454 is similar to pDS1453 but contains *CtURA3* at the site of *SAT1* insertion. For *C. albicans* transformations, the *ERG11-CtURA3* cassette was liberated from pDS1454 by digestion with KpnI and StuI. pDS1663 and pDS1665 were obtained by subcloning *TAC1-1* and *TAC1-5* as BamHI-XhoI fragments from pDS1097 and pDS1099 (7) into the XhoI-BglII sites of CIp10 (18).

PCR and sequencing. *ERG11* alleles from *C. albicans* isolates were PCR amplified from genomic DNA. The primers used (CYPCB [5′-GCGGATCCTT AAAACATACAAGTTTCTCTTTT-3′] and CYPNS2 [5′-ACGCGTCGACAA TATGGCTATTGTTGAAACTGTC-3′]) amplified the entire *ERG11* ORF. Primer ERG11-3B (5′-CCCATTAAGAATCCCTGAA-3′) was used for sequencing in order to reveal the A1390G mutation, which leads to a G464S substitution. Sequencing was performed on a 3130XL genetic analyzer (Applied Biosystems). Geneious software (Biomatters Ltd., New Zealand) was used for sequence analysis.

SNP sequencing. To determine the single nucleotide polymorphisms (SNPs) along Chr. 5, several chromosomal positions were amplified by PCR and were subjected to sequencing. PCR fragments were obtained from primer pairs (listed in Table S1 in the supplemental material), each containing a forward or reverse sequence tag that was used in subsequent sequencing reactions. Geneious software (Biomatters Ltd., New Zealand) was used for SNP analysis.

Construction of DSY296-derivative strains. Plasmid pDS1453 was first used to transform the *ura3* derivative of DSY3083 (DSY3090), a *tac1*Δ/Δ strain from DSY296, to obtain nourseothricin-resistant colonies heterozygous for *ERG11-1* (DSY3593 [*ERG11-1*::*SAT1*/*ERG11-5*]). Sequencing of *ERG11* PCR products from DSY3593 showed an ambiguity at nucleotide 1390 in *ERG11*, consistent with the presence of the two different alleles (data not shown). Next, DSY3593 was transformed with pDS1454 to yield Ura^+ colonies. One of these colonies, DSY3595 (*ERG11-1*::*SAT1*/*ERG11-1*::*CtURA3*) was chosen for further work. To restore the *C. albicans URA3* gene, this strain was first plated onto 5-fluoroorotic acid (5-FOA), resulting in the loss of the *ERG11-1*::*CtURA3* allele and its replacement by the *ERG11-1*::*SAT1* allele, thus producing strain DSY3603 (*ERG11-1*::*SAT1*/*ERG11-1*::*SAT1*). DSY3603 was next transformed with CIp10 to introduce *URA3* at the neutral *RPS1* locus (18), resulting in DSY3706. The loss of *ERG11-1*::*CtURA3* in DSY3603 most probably occurred by mitotic recombination between the two homozygous left arms of Chr. 5 of this DSY296-derived strain. This conclusion was supported by the presence of SNPs (see above) in DSY3603 identical to those detected in DSY296 (data not shown) (see also Results). CIp10-derived plasmids containing the hyperactive *TAC1-5* allele (pDS1665) and the wild-type *TAC1-1* allele (pDS1663) were also used to transform DSY3603, yielding DSY3606 and DSY3608, respectively. This restored *TAC1* in these strain backgrounds. To restore *URA3* in the *ERG11-1*/*ERG11-5* background, DSY3593 was transformed with CIp10, yielding DSY3604.

Both *ERG11-5* alleles from strain DSY296 were also replaced by wild-type *ERG11-1* alleles in a similar fashion. The final isolate, in which *URA3* was introduced by CIp10, was named DSY3752 (*TAC1-5*/*TAC1-5 ERG11-1*::*SAT1*/*ERG11-1*::*SAT1*) (Table 1). This isolate, therefore, exhibits only one azole resistance mechanism mediated by both *TAC1-5* alleles.

Drug susceptibility testing. Azole MICs were measured by broth microdilution according to EUCAST standards with slight modifications (10, 11). Briefly, *C. albicans* strains were cultivated overnight at 30°C under constant agitation in YEPD. Cultures were diluted to a density of 5×10^4 cells per ml in RPMI 1640 medium (Sigma) with L-glutamine, without bicarbonate, and with phenol red as the pH indicator. RPMI 1640 medium was buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS) and was supplemented with glucose to a final concentration (wt/vol) of 2% and with 1% dimethyl sulfoxide (DMSO). Serial drug dilutions were made from stocks dissolved in DMSO for VRC, ITR, and POS and from stocks dissolved in H_2O for FLC. Drug dilutions were made for each azole in the corresponding solvent at 100-fold strength and were diluted 100-fold in RPMI 1640 medium. The inoculum was distributed in 100-μl volumes in 96-well flat-bottom microdilution plates; then 100-μl volumes of serially diluted drugs were added. Drug-free cultures and sterility controls were included in each 96-well plate. Plates were incubated at 35°C for 24 h, and then MICs were read with a spectrophotometer plate reader set at 450 nm. The MIC was defined as the drug concentration at which the optical density decreased more than 50% from that of the drug-free culture. Drug-free cultures contained 1% DMSO. Fluconazole was obtained from Sigma. Itraconazole, voriconazole, and posaconazole were gifts from Janssen, Pfizer, and Schering Plough, respectively.

RNA and DNA analysis. Expression of genes was analyzed by Northern blotting as described by Coste et al. (5). Labeled probes were generated by random priming with [α-^{32}P]dATP by using the Mega Labeling kit (GE Healthcare) according to the manufacturer's instructions. *CDR1*, *CDR2*, *ERG11*, and *ACT1* probes were obtained as described previously (7). Southern blotting was carried out as described previously (7). Signals resulting from Northern and Southern blotting were obtained by phosphorimaging on a Typhoon Trio system (GE Healthcare).

Animal experiments. The effect of fluconazole treatment was assessed in mice experimentally infected with five of the *C. albicans* isolates used in this study: DSY294-CIp10, DSY296-CIp10, DSY3083-CIp10, DSY3606, and DSY3706. All animal experimentation conformed to the requirements of United Kingdom Home Office legislation and of the Ethical Review Committee of the University of Aberdeen. We have previously shown a strong correlation between loss of mouse body weight over 3 days after intravenous (i.v.) challenge with *C. albicans* and survival times up to 28 days (14). Kidney fungal burdens at 3 days also showed an association with strain virulence (13). The present experiments were therefore designed with a 3-day end point to minimize their duration.

Female BALB/c mice (Harlan, United Kingdom) weighing 18 to 22 g, housed in groups of as many as 10 animals and individually marked for identification, were supplied with food and water *ad libitum*. Mice were infected i.v. with a saline suspension of *C. albicans* cells from cultures grown overnight in NGY medium (15). Fluconazole treatment was started by intraperitoneal injection 1 h after challenge and was repeated 24 h and 48 h after challenge. At 72 h postchallenge, animals were humanely terminated; the left kidneys were removed with aseptic precautions and were homogenized in saline; and *C. albicans* viable counts in the homogenates were determined as $\log_{10}$ CFU per g of kidney.

The 50% effective dose (ED_{50}) was defined as the fluconazole dose effective at reducing the pathological effects of i.v. *C. albicans* challenge significantly relative to those for saline-treated controls. The two parameters measured were the percentage of weight change over 3 days postchallenge, for which ED_{50} was the dose that reduced the mean weight change below 50% of that for controls, and log CFU/g, for which ED_{50} was the dose that reduced mean kidney burdens more than 1.5 log units below those of the controls. A third definition of ED_{50} was based on an "outcome score," devised to allow combination of the 3-day weight loss and kidney burden data. The outcome score is calculated as log CFU/g of kidney − (0.5 × percent weight change). This formula provided for approximately equal contributions of the two parameters to the score.

A pilot experiment with DSY294-CIp10, DSY296-CIp10, and DSY3083-CIp10 showed that all three strains had similar virulence for mice, and lower virulence than strains SC5314 and CAF2-1, which we have tested extensively previously (13, 15). We characterized the virulence of the five strains in terms of 3-day outcome parameters, with groups of 3 mice each receiving a test challenge dose of 5×10^4, 1×10^5, or 2×10^5 CFU/g of body weight. For these and several other *C. albicans* isolates, we have established a correlation between the logarithm of the challenge dose and the 3-day outcome score (D. M. MacCallum and F. C. Odds, unpublished data). The actual challenge doses used to investigate the efficacy of fluconazole treatment were based on the detailed results of these experiments. To minimize the numbers of mice involved in the experiments, fluconazole treatments with doses of 1.0, 3.5, 10, 35, 120, and 200 mg/kg of body weight were tailored by initial selection of two of these doses for groups of 6 mice according to the known *in vitro* susceptibility of the infecting strain. Further experiments were then carried out with other fluconazole doses when the pair of doses initially tested was insufficient to establish ED_{50}s.

RESULTS

Engineering DSY296-derivative strains. This study focused on two clinical isolates from the same patient, DSY294 and DSY296; the latter has been described as fluconazole resistant. In addition to the *TAC1* GOF mutation in DSY296, a mutation was also found in the *ERG11* gene of this isolate (G464S), which has been reported previously to be involved in azole resistance (25). The effect of this mutation was observed in strain DSY3083, which was *tac1*Δ/Δ and was directly derived from DSY296: the fluconazole (FLC) MIC for this mutant was 4 μg/ml, which was greater than the FLC MIC for the azole-susceptible parental isolate, DSY294 (0.25 μg/ml). Interestingly, *ERG11* is located at the left extremity of Chr. 5, and the G464S mutation was shown to be homozygous in DSY296. An SNP analysis of Chr. 5 performed in a previous study (6) indicated that the entire region spanning the telomere of Chr. 5 up to a region 100 kb downstream of *TAC1* was homozygous, thus suggesting that loss of heterozygosity occurred by mitotic recombination in this region. Further analysis of this region delimited the loss of heterozygosity within a minimal 348-bp sequence of orf19.3178, a gene situated 13 kb from *TAC1* (see Table S2 in the supplemental material).

The difference in azole susceptibility between DSY3083,

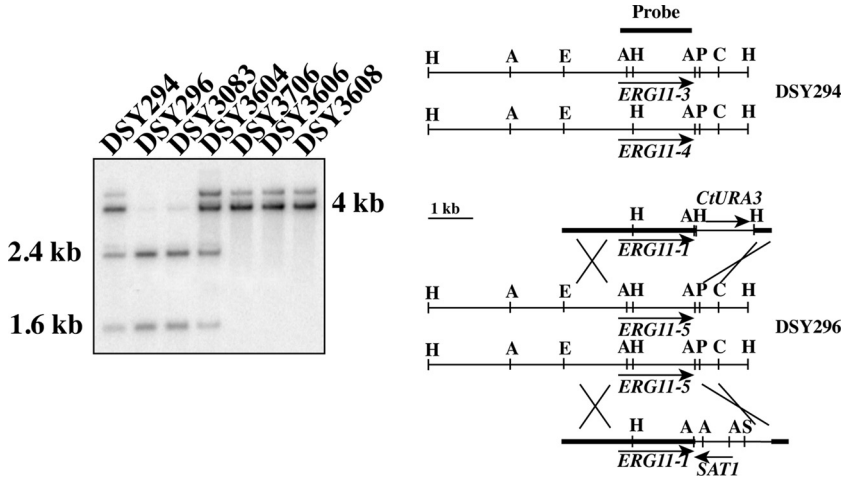

FIG. 1. Southern blot analysis of *ERG11* allele replacement. (Right) Restriction maps of genomic *ERG11* loci. Abbreviations: A, AciI; H, HindIII; P, PstI; C, ClaI; S, SalI; E, EcoRI. *ERG11* genomic elements from pDS1453 and pDS1454 are indicated by thick lines. (Left) Fragments expected from the hybridization with the labeled probe are 1.6 and 2.4 kb in *ERG11-3* and *ERG11-5* and 4.0 kb in *ERG11-4*. DSY294 shows the pattern of two different *ERG11* alleles, while DSY296 exhibits the pattern of only one allele. The weak signal above 4.0 kb is probably due to partial digestion of genomic DNA.

lacking *TAC1*, and DSY294 led us to replace the mutant *ERG11* alleles with wild-type alleles in DSY3083. To achieve this purpose, starting from a strain in which *TAC1* was inactivated by the use of the "Ura-blaster" method (DSY3083), two different genetic markers (*SAT1* and *CtURA3*) were used to selectively replace *ERG11-5* alleles with *ERG11* wild-type alleles. In order to achieve these replacements, we constructed two different plasmids (pDS1453 and pDS1454), each containing the wild-type *ERG11* allele from the *C. albicans* isolate SC5314 (orf19.922) but possessing *SAT1* or *CtURA3* as a genetic marker, respectively. For practical purposes, we will name this allele *ERG11-1*. The strategy adopted in this study (see also Fig. 1) was first to obtain Ura^+ and nourseothricin-resistant colonies (*ERG11-1*/*ERG11-1* genotype) and next to regenerate *ura3* by gene conversion of the *URA3*-containing allele with the *SAT1*-containing allele. This allowed the regeneration of the *ura3* auxotrophic marker and the subsequent reintroduction of the *C. albicans URA3* gene at the neutral *RPS1* locus using CIp10.

DSY294 possesses two distinct *ERG11* alleles (*ERG11-3* and *ERG11-4*), while DSY296 possesses a single *ERG11* allele (*ERG11-5*, derived from *ERG11-3*) (25). *ERG11-1* and *ERG11-3* differ by two nucleotides, resulting in the A129G and E266D substitutions. Since these two substitutions are not associated with azole resistance (25), we used *ERG11-1* for allele replacements. *ERG11-5* contains a single A1390G change compared to *ERG11-3*, resulting in a G464S substitution. The engineering of DSY296 derivatives in which *ERG11* alleles were replaced is detailed in Materials and Methods. Southern blot analysis of the *ERG11* loci demonstrated *ERG11* allele replacement starting from strain DSY3090 (*ERG11-5*/*ERG11-5*): while DSY3604 (*ERG11-5*/*ERG11-1*) exhibited AciI restriction profiles identical to those of DSY294, DSY3706 showed restriction profiles consistent with the presence of *ERG11-1* only (see Fig. 1 for details). Analysis of *ERG11* from this strain showed that nucleotide position 1390 was homozygous for "A," thus indicating that *ERG11* is, as expected, wild type at this site (data not shown).

We next verified the gene expression of major determinants of azole resistance, including *CDR1*, *CDR2*, and *ERG11*, in the engineered strains. As observed in Fig. 2, and as expected, *CDR1* and *CDR2* were not upregulated in the *tac1*Δ/Δ strain (DSY3083) or in other strains derived from this strain background (DSY3604 and DSY3706). *CDR1* and *CDR2* were, however, upregulated in strain DSY3606, into which the hyperactive *TAC1-5* allele had been reintroduced. In contrast, *CDR1* and *CDR2* levels in DSY3608, into which *TAC1-1* had been reintroduced, were equal to those in DSY294. *ERG11* expression in DSY296-derived isolates did not change significantly from that in the parent. This indicates that in the engineered strains, the presence of a genetic marker flanking *ERG11* did not influence the expression of *ERG11*. We also noticed that *ERG11* expression was approximately 2-fold higher in DSY296 than in DSY294, suggesting that some factor

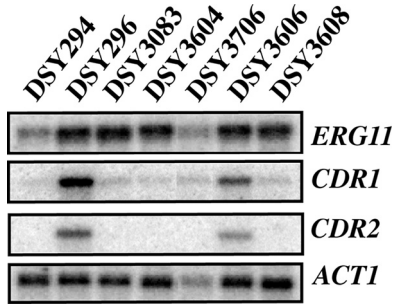

FIG. 2. Expression of azole resistance genes in DSY294, DSY296, and DSY296-derived strains. Signals were obtained by phosphorimaging and were quantified by ImageQuant software (GE Healthcare). *ERG11* expression was normalized to *ACT1* expression and to *ERG11* expression levels in DSY294. Differences in *ERG11* expression levels between DSY296 and derivative strains fluctuate from 1.7- to 2.4-fold.

TABLE 2. MICs of azole antifungals against clinical and engineered *C. albicans* strains

Strain	MIC (μg/ml) (fold increase in MIC relative to that for DSY294)			
	FLC	ITR	VRC	POS
DSY294	0.25 (1)	0.0156 (1)	0.0078 (1)	0.0312 (1)
DSY296	64 (256)	0.25 (16)	2 (256)	0.25 (8)
DSY3083 *tac1*Δ/Δ	4 (16)	0.031 (2)	0.125 (16)	0.031 (1)
DSY3604 *tac1*Δ/Δ *ERG11-1*/*ERG11-5*	2 (8)	0.031 (2)	0.0625 (8)	0.031 (1)
DSY3706 *tac1*Δ/Δ *ERG11-1*/*ERG11-1*	0.25 (1)	0.0156 (1)	0.0078 (1)	0.031 (1)
DSY3606 *tac1*Δ/Δ *ERG11-1*/*ERG11-1* (*TAC1-5*)	4 (16)	0.125 (8)	0.125 (16)	0.25 (8)
DSY3608 *tac1*Δ/Δ *ERG11-1*/*ERG11-1* (*TAC1-1*)	0.25 (1)	0.031 (2)	0.0078 (1)	0.031 (1)
DSY3752 (*TAC1-5*/*TAC1-5 ERG11-1*/*ERG11-1*)	4 (16)	0.25 (16)	0.125 (16)	0.25 (8)

in DSY296 may contribute to this property. No isochromosome formation has been detected in this strain background (5).

Drug susceptibility assays. Clinical strains and their derivatives were tested for susceptibilities to the four azole antifungals used in therapy of invasive fungal diseases (FLC, ITR, VRC, and POS), according to EUCAST protocols. FLC and VRC are close structural relatives, bearing structures different from those of ITR and POS, both of which have a series of heterocyclic rings attached to the azole pharmacophore via a dioxolane or furan ring. Table 2 summarizes the results obtained. Strikingly, the replacement of *ERG11-5* alleles by wild-type alleles in a *tac1*Δ/Δ mutant background increased azole susceptibility. When *ERG11* was in the heterozygous state, as in DSY3604 (*ERG11-5*/*ERG11-1*), the FLC MIC decreased 2-fold (to 2 μg/ml) from that for the *tac1*Δ/Δ parent strain (4 μg/ml). A further 8-fold MIC decrease (from 2 μg/ml to 0.25 μg/ml FLC) was obtained by restoring two wild-type *ERG11-1* alleles in the background of DSY3706. Interestingly, this MIC corresponded to that measured for DSY294, thus confirming by genetic evidence that only two azole resistance mechanisms contribute to the high azole MIC for DSY296, i.e., the G464S *ERG11* mutation and ABC transporter upregulation. FLC MICs did not change in this strain background when a wild-type *TAC1* allele was restored, consistent with previous observations (7). *TAC1* functionality was verified by *CDR1*/*CDR2* inducibility with fluphenazine exposure (data not shown). However, when *TAC1-5* was reintroduced in the DSY3603 background, FLC MICs were increased 16-fold (MIC, 4 μg/ml) over that for DSY294 or DSY3706. These values are consistent with the restoration of *CDR1*/*CDR2* upregulation, which was verified by Northern blot analysis. Besides confirming by genetic evidence the presence of two resistance mechanisms in DSY296, our results also highlight the fact that *C. albicans* requires the accumulation of several resistance mechanisms to obtain elevated levels of azole resistance. Both *TAC1-5*- and *ERG11-5*-mediated resistance mechanisms elevate the FLC MIC 16-fold, as deduced from the comparison between FLC MICs of 4 μg/ml and 0.25 μg/ml (DSY294), whereas the presence of both resistance mechanisms in DSY296 yields a 256-fold increase in the FLC MIC over that for DSY294 or DSY3706.

A similarly graded MIC hierarchy, related to the engineered presence and absence of one or two genes encoding azole resistance, was seen in the results with VRC (Table 2). The same was less true with ITR and POS, with which DSY296 and DSY3606 were inhibited at azole levels between 0.125 and 0.25 μg/ml, while all the other strains were inhibited at levels between 0.0156 and 0.0312 μg/ml. The MIC differences among strains thus ranged from 4- to 16-fold for ITR and POS, compared with 4- to 256-fold for FLC and VRC. In particular, replacement of *ERG11-5* by *ERG11-1* alleles in *tac1*Δ/Δ strains did not change ITR and POS MICs. Resistance to ITR and POS in DSY296 is mediated primarily by *CDR1* and *CDR2*, since the replacement of *ERG11-5* alleles with wild-type *ERG11* alleles did not increase the relative POS MIC. These data are confirmed by POS (and ITR) MIC testing of isolate DSY3752: the replacement of *ERG11-5* alleles by wild-type alleles in this strain did not decrease POS and ITR MIC values from those for DSY296, as opposed to FLC and VRC MIC values, thus confirming the differentiated effect of *ERG11*-mediated azole resistance on azoles.

Experimental infections and fluconazole efficacy. In order to evaluate the effects of azole resistance mechanisms on the efficacy of azole treatment *in vivo*, selected strains were used in a mouse model of systemic candidiasis, and the infected animals were treated with at least two fluconazole doses to allow determination of ED_{50}s. The selected strains contained CIp10 to restore *URA3* at the neutral *RPS1* locus: DSY294-CIp10 and DSY296-CIp10 (originating from the wild-type clinical strains DSY294 and DSY296), DSY3083-CIp10 (originating from the homozygous *tac1*Δ/Δ mutant DSY3083), DSY3706 (in which *ERG11-5* alleles were replaced by wild-type *ERG11-1* alleles), and finally DSY3606 (in which the *TAC1-5* allele was reintroduced into a background similar to that of DSY3706). The azole susceptibilities of CIp10-transformed derivatives of DSY294, DSY296, and DSY3083 were identical to those of their parents (data not shown). The results of the animal experiments are summarized in Tables 3 and 4. The challenge inocula were based on pilot dose-finding experiments and were intended to give a 3-day outcome score between 8 and 16.

For DSY294-CIp10 (FLC MIC, 0.25 μg/ml), the ED_{50} was 1.0 mg/kg based on weight change, 3.5 mg/kg based on the outcome score, and 10 mg/kg based on kidney burdens (Table 4), according to the ED_{50} criteria defined in Materials and Methods. The result, based on the median of the three ED_{50} calculations, was the same as that based on the outcome score. For DSY296-CIp10 (FLC MIC, 64 μg/ml), the ED_{50} was 120 mg/kg based on weight change, 200 mg/kg based on the outcome score, and >200 mg/kg based on kidney burdens (Tables 3 and 4). The median ED_{50} *in vivo* was thus 57-fold greater for

TABLE 3. Effect of FLC treatment on experimental infection outcome

Strain	Challenge inoculum (CFU/g body wt)	FLC Rx (mg/kg/day)	No. of animals	Infection outcome parameter on day 3 (mean ±SD)		
				Kidney fungal burden (log CFU/g kidney)	3-day wt change (%)	Outcome score[a]
DSY294-CIp10	1.2×10^5	Saline	12	5.7 ± 0.6	−7.0 ± 4.5	9.2 ± 2.6
		1.0	6	4.8 ± 0.1	−2.1 ± 3.0	5.9 ± 1.5
		3.5	6	4.7 ± 0.2	3.2 ± 3.7	3.1 ± 1.8
		10	6	4.3 ± 0.2	4.3 ± 2.7	2.2 ± 1.3
		35	6	4.3 ± 0.1	5.5 ± 0.9	1.5 ± 0.4
DSY296-CIp10	2.2×10^5	Saline	6	7.3 ± 0.2	−10.9 ± 2.6	12.7 ± 1.5
		120	6	6.3 ± 0.1	−4.4 ± 18	8.4 ± 1.0
		200	6	6.3 ± 0.1	0.2 ± 2.6	6.2 ± 1.2
DSY3083-CIp10	3.3×10^5	Saline	12	7.3 ± 0.7	−16.2 ± 2.6	15.4 ± 1.6
		3.5	6	6.3 ± 0.5	−14.2 ± 2.2	13.4 ± 1.4
		10	6	5.5 ± 0.3	−2.1 ± 2.8	6.6 ± 1.6
		35	6	5.3 ± 0.4	−0.7 ± 1.7	5.6 ± 1.1
		120	6	5.2 ± 0.2	0.7 ± 2.3	4.8 ± 1.2
DSY3606	5.6×10^4	Saline	6	6.1 ± 0.2	−9.3 ± 3.6	10.7 ± 1.9
		120	6	4.4 ± 0.2	−0.8 ± 2.8	4.8 ± 1.4
		200	6	4.5 ± 0.2	1.2 ± 2.2	3.9 ± 1.3
DSY3706	2.7×10^5	Saline	12	6.8 ± 0.6	−13.7 ± 3.0	13.6 ± 2.0
		1.0	6	5.7 ± 0.2	−5.9 ± 3.4	8.7 ± 1.9
		3.5	6	5.0 ± 0.3	−0.4 ± 2.9	5.2 ± 1.6
		10	6	4.7 ± 0.2	1.4 ± 5.7	4.0 ± 3.0
		35	6	4.5 ± 0.1	1.8 ± 2.6	3.6 ± 1.3

[a] Calculated as kidney burden − (0.5 × weight change).

DSY296-CIp10 than for DSY294-CIp10—less than the 1,024-fold measured *in vitro*, but substantial nevertheless.

With regard to the engineered strains, the FLC ED_{50} for DSY3083-CIp10 was 10 mg/kg by all three parameters, a 3-fold increase over that for DSY294-CIp10, compared with a 64-fold difference in the MIC (Tables 3 and 4). The ED_{50} for DSY3706 was 1.0 mg/kg by weight change and 3.5 mg/kg by kidney burden and outcome score; thus, it was the same as for DSY294, as was also found *in vitro*. Finally, the ED_{50} for DSY3606 was 120 mg/kg by all three parameters (Table 4), a 35-fold difference from the FLC ED_{50} for DSY294-CIp10, compared with a 64-fold MIC difference (Table 4).

DISCUSSION

This study undertook the sequential replacement of drug resistance genes by wild-type copies. Starting from the azole-resistant isolate DSY296, we produced isolate DSY3706 with *ERG11* wild-type alleles and with *TAC1* deleted. Using SNP analysis of markers along Chr. 5, we showed that this strain was indistinguishable from DSY296 in terms of the SNP profile (see Table S2 in the supplemental material). We showed that after the replacement of the *ERG11-5* allele by wild-type alleles, azole MICs for engineered strains reverted to those of the DSY294 isolate, the azole-susceptible parent of DSY296. MIC values were not different when *TAC1-1* was reintroduced into the DSY3706 background, a finding consistent with results obtained in previous studies (7). Until now, few other studies have dissected azole resistance mechanisms by sequential genetic manipulations. In one study, *MDR1* was inactivated in an azole-resistant isolate, but the resulting strains were not as susceptible as the azole-susceptible parent isolate, probably due to *ERG11* mutations (30). Another study inactivated *MRR1*, a regulator of *MDR1*, in the same isolates, which also resulted in strains with intermediate azole resistance (17).

TABLE 4. *In vivo* ED_{50} scores

Strain	FLC MIC (μg/ml)	ED_{50} (mg/kg) determined on the basis of:			Median ED_{50} (mg/kg)
		Kidney burden	Wt change	Outcome score	
DSY294-CIp10	0.25	10	1.0	3.5	3.5
DSY296-CIp10	64	>200	120	200	200
DSY3083-CIp10	4	10	10	10	10
DSY3606	4	120	120	120	120
DSY3706	0.25	3.5	1	3.5	3.5

In this study, we have utilized technologies now available to introduce specific alleles of the resistance-associated genes *TAC1* and *ERG11* in a stepwise fashion, and we have confirmed the relationship between the FLC MIC and the number of resistance genes being expressed in a *C. albicans* strain. It was previously self-evident that the level of fluconazole resistance of a *C. albicans* isolate was proportional to the number of molecular resistance mechanisms expressed. White et al. (29) investigated the resistance mechanisms expressed in 17 sequential isolates from a single HIV-positive patient with persistently relapsing oropharyngeal *C. albicans* infection managed clinically with increasing doses of fluconazole. As the fluconazole MIC for successive isolates rose progressively from

0.25 μg/ml against the pretreatment isolate to 64 μg/ml against isolate 17, the number of detectable resistance mechanisms increased from overexpression of *MDR1* through point mutations in *ERG11*, loss of *ERG11* allelic variation, and finally overexpression of *CDR1* (29). This isolate set thus represented a quantitative, stepwise rise in fluconazole resistance in which the FLC MIC depended on the number of different resistance mechanisms expressed in each isolate. The mechanisms detected in the previous publication were different from those in our pair of clinical isolates (DSY294 and DSY296), but the MIC difference measured was the same.

Our study shows that the impacts of resistance mechanisms on the MIC are dependent on the type of azole molecule investigated. These impacts differ for the two pairs of related azole antifungal agents, FLC and VRC versus ITR and POS. While ABC transporters confer cross-resistance to all the azoles investigated here, the *ERG11* mutations had a differential impact. Because of the absence of POS MIC variation when *ERG11-5* alleles were replaced with *ERG11-1* alleles (see Table 2), our data suggest that POS probably interacts with the Erg11 structure by mechanisms different from those of FLC and VRC. A mutation in Erg11, such as G464S, has no effect on POS, and probably this position interacts weakly with this azole. With regard to these properties, ITR has an intermediate position between POS and FLC/VRC. Our data are consistent with previous models of Erg11 interactions with azoles, where ITR and POS stabilize their binding to mutated Erg11 proteins more efficiently than FLC and VRC (31). The differential effects of mutations in other *ERG11*-like genes have been documented in other fungal species. For example, the *ERG11* ortholog in *Aspergillus fumigatus*, *cyp51A*, exhibits mutations as principal resistance mechanisms to azoles. However, the specific *cyp51A* mutations determine resistance to specific azoles (8, 16, 23). With the strains engineered in this study, it will be possible to test other azoles for their MIC profiles, allowing the impact of each resistance mechanism on specific azoles to be determined.

We have also shown here that the different levels of resistance, as measured by the FLC MIC *in vitro*, correlate with levels of resistance measured in the mouse i.v. challenge model of experimental *C. albicans* infection. Our *in vivo* data validate our experimental approach to antifungal treatment for mice: 48 h of treatment followed by assessment of kidney burdens and percentage of body weight change on day 3 postinfection. The ED_{50}s calculated from the kidney burden and weight change data were proportional to the MIC differences for the strains tested, although the magnitudes of the differences *in vivo* were smaller than those measured by FLC MICs. The mean ED_{50} values for strains DSY294-CIp10 and DSY3706, from which two resistance mechanisms were removed, were calculated by three different approaches and were identical (3.5 mg/kg). The absence of the hyperactive *TAC1-5* allele (strain DSY3083-CIp10) resulted in a 10-fold decrease in the mean ED_{50} value from that for DSY296-CIp10. This underscores the effect of the *ERG11* mutation (G464S), which is apparent by comparison of DSY3083-CIp10 with DSY3706, with a 3-fold difference between ED_{50} values. The effect of *TAC1-5* alone can be deduced from the comparison of mean ED_{50} values for DSY3606 and DSY3706 and is about 34-fold. This suggests that the relative contribution of azole resistance to these different ED_{50} values is much stronger for *TAC1* than for *ERG11*. The ED_{50} values obtained in the present work are in good agreement with those available from other animal experiment studies, with the major difference that immunocompetent mice were used and the length of therapy was longer (3 days versus 24 h) in this study. For example, Louie et al. (12) gave a value of 4.56 mg/kg based on decreased kidney burden for an azole-susceptible isolate. Andes and van Ogtrop (1) reported a value of 1.9 mg/kg for a *C. albicans* isolate with a FLC MIC of 0.5 μg/ml. The ED_{50} values were 61 and 114 mg/kg for two other isolates with MIC values of 16 and 32 μg/ml, respectively. We reported a mean ED_{50} value of 3.5 mg/kg for isolates with a FLC MIC of 0.25 μg/ml, which lies within the values reported by both studies. The ED_{50} values (10 and 120 mg/kg) for isolates with higher MICs (8 μg/ml) depend on the resistance mechanism in operation, as mentioned above. These values are, however, in the range of those given by Andes and van Ogtrop (1).

In conclusion, this study provides genetic evidence for the participation of different resistance mechanisms in the development of increasing azole resistance. The engineered strains not only were useful for distinguishing the effect of an individual resistance mechanism on specific azoles but also helped to estimate the impact of the mechanism on treatment efficacy. Since a variety of other azole resistance mechanisms exist, other clinical strains could be subjected to the same genetic dissections and *in vivo* validation; this work is under way in our laboratory.

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