Factors TAC1, MRR1, and UPC2 in Virulence

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Azoles are widely used in antifungal therapy in medicine. Resistance to azoles can occur in Candida albicans principally by overexpression of multidrug transporter gene CDR1, CDR2, or MDR1 or by overexpression of ERG11, which encodes the azole target. The expression of these genes is controlled by the transcription factors (TFs) TAC1 (involved in the control of CDR1 and CDR2), MRR1 (involved in the control of MDR1), and UPC2 (involved in the control of ERG11). Several gain-of-function (GOF) mutations are present in hyperactive alleles of these TFs, resulting in the overexpression of target genes. While these mutations are beneficial to C. albicans survival in the presence of the antifungal drugs, their effects could potentially alter the fitness and virulence of C. albicans in the absence of the selective drug pressure. In this work, the effect of GOF mutations on C. albicans virulence was addressed in a systemic model of intravenous infection by mouse survival and kidney fungal burden assays. We engineered a set of strains with identical genetic backgrounds in which hyperactive alleles were reintroduced in one or two copies at their genomic loci. The results obtained showed that neither TAC1 nor MRR1 GOF mutations had a significant effect on C. albicans virulence. In contrast, the presence of two hyperactive UPC2 alleles in C. albicans resulted in a significant decrease in virulence, correlating with diminished kidney colonization compared to that by the wild type. In agreement with the effect on virulence, the decreased fitness of an isolate with UPC2 hyperactive alleles was observed in competition experiments with the wild type in vivo but not in vitro. Interestingly, UPC2 hyperactivity delayed filamentation of C. albicans after phagocytosis by murine macrophages, which may at least partially explain the virulence defects. Combining the UPC2 GOF mutation with another hyperactive TF did not compensate for the negative effect of UPC2 on virulence. In conclusion, among the major TFs involved in azole resistance, only UPC2 had a negative impact on virulence and fitness, which may therefore have consequences for the epidemiology of antifungal resistance.

Candida albicans is a major fungal pathogen of humans. This species is commensal in healthy individuals and can be found on mucosal surfaces (oral cavity and genital tract) as well as in the gastrointestinal tract. As a pathogen in severely immunocompromised people, C. albicans can invade mucosal surfaces and internal organs via the bloodstream (1). The epidemiology of Candida infections in humans shows that C. albicans is the causative agent in 50 to 70% of the cases recorded. According to recent data, the proportion of non-C. albicans species seems to be increasing, while the occurrence of C. albicans in invasive infections is decreasing (2, 3).

The therapy of C. albicans infection is mainly based on treatment with antifungal agents. These belong to four distinct major classes (azoles, pyrimidine analogues, polyenes, and candins) (4). Because antifungal agents are administered to patients on a repeated basis, development of resistance is possible. Development of resistance is detected when the antifungal activity of a given agent against a specific fungal species is lower than that against a normal fungal population. This so-called in vitro resistance can often be correlated to the failure of treatment in the patient. Many studies have reported antifungal resistance in Candida species isolated from infected patients who do not respond to therapy (2, 5, 6). Azoles are still the most widely used agents, although novel antifungals (candins) are more frequently becoming employed (7).

The molecular basis of antifungal resistance has been resolved for diverse fungal pathogens and specific antifungals. Investigations of resistance to theazole class have generated a wide spectrum of mechanisms that can be applied to other agents. These resistance mechanisms fall into different categories according to molecular principles that include transport alterations, target alterations, utilization of compensatory and catabolic pathways, and the presence of complex multicellular structures (biofilms) (8). Several genes encoding mediators of antifungal resistance have been characterized, and their involvement in the drug resistance of clinical isolates has been demonstrated by either their inactivation or the presence of mutations (8). A specific category of drug resistance genes encodes transcriptional activators, which themselves are responsible for the control of expression of target genes, some of them as direct mediators of resistance.

For example, alterations of azole transport are mediated by two transporter families, the ABC transporters and the major facilitator superfamily (MFS). When azoles accumulate in the cell, Erg11 (the key enzyme of the ergosterol biosynthesis pathway and the target of azoles) is inhibited, leading to growth arrest of the cells. Upregulation of one of the genes encoding the transporter leads to enhanced efflux and therefore to azole resistance in clinical isolates (9–11). In C. albicans, the ABC transporters Cdr1 and Cdr2 and their upregulation are controlled by the transcription factor
(TF) TAC1 (for transcriptional activator of CDR genes). So-called gain-of-function (GOF) mutations in TAC1 confer hyperactivity to this transcription factor and result in high expression of CDR1 and CDR2 (12–14). Mdr1 is a member of the major facilitator family and is a known mediator of azole resistance in C. albicans when upregulated (9). Control of MDR1 expression was shown to involve the transcriptional activator MRR1 (for multidrug resistance regulator) (15). As for TAC1, GOF mutations in MRR1 are associated with MDR1 upregulation (16). Upregulation of ERG11 can mediate azole resistance, especially when this gene contains mutations modifying azole affinity (11, 17, 18). ERG11 expression is mainly controlled by the TF UPC2, and GOF mutations in this gene result in ERG11 upregulation (19–21). While TAC1, MRR1, and UPC2 control specific target genes involved in drug resistance, they also express the regulation of other genes.

The operons of these TF genes have been investigated, and each of them controls a wide range of targets (22–24). Therefore, one can expect that GOF mutations in these transcriptional activators may have a significant fitness cost for the overall cellular metabolism of C. albicans. Fitness can be defined as the ability of a microbe to propagate and evolve within a given environment in competition with another microbe. Antifungal resistance may have a negative impact on fitness and may therefore reduce the competitiveness of strains compared to that of wild-type isolates, especially when there is no selection for drug resistance. The decrease in fitness of drug-resistant strains may indeed compromise their virulence. A few studies have addressed this question by testing the virulence of azole-resistant C. albicans isolates compared to that of their azole-susceptible parents; however, no direct relationship between the development of azole resistance and virulence could be established (25). The trajectory of azole resistance and the associated genome changes had rather an unpredictable effect on fitness (26). In vitro studies have also addressed the relationship between the development of azole resistance and changes in fitness. Individual C. albicans colonies were subcultured in fluconazole-containing medium, and each developed individual trajectories in their development of azole resistance (27). These in vitro studies showed no direct relationship between the cost of developing azole resistance and changes in fitness, as measured by competition assays between susceptible and resistant isolates. Remarkably, an in-depth analysis of genome changes of azole-resistant isolates produced by Cowen et al. (27) revealed several alterations, among which were aneuploidies (for example, the formation of an isochromosome from the chromosome 5 left arm, the so-called 5IL alteration). However, these alterations did not affect the in vitro fitness of C. albicans (28). The notion of a neutral fitness cost upon azole exposure in vitro has been confirmed by others (29).

The idea that the development of drug resistance can negatively impact fitness was recently challenged by our laboratory when addressing the effect of azole resistance on virulence in C. glabrata. Azole resistance in this yeast species is mediated almost exclusively by upregulation of ABC transporters, including C. glabrata CDR1 (CgCDR1), C. glabrata PDH1 (CgPDH1), and C. glabrata SNQ2 (CgSNQ2) (30–34). C. glabrata possesses PDR1 as a transcriptional activator of these genes (35–38). We have described a wide variety of GOF mutations in CgPDR1 (39). Surprisingly, we found that these mutations could enhance the virulence of C. glabrata compared to that of wild-type isogenic isolates in an intravenous infection model in mice (39). Enhanced virulence could be associated with a gain of fitness of strains carrying CgPDR1 GOF mutations. Therefore, our results with C. glabrata have so far challenged the dogma existing between the development of drug resistance and fitness costs in vivo.

As mentioned above, the in vivo fitness cost of azole resistance in C. albicans was investigated using clinical isolates of multiple origins which themselves were carrying several resistance mechanisms, rendering it difficult to estimate the individual impact of a particular mechanism on fitness and virulence. Recently, this problem was circumvented by constructing isogenic C. albicans strains carrying combined sets of GOF mutations in TAC1, MRR1, and UPC2, thus enabling a better assessment of fitness in comparison with that of a wild-type parent in a gastrointestinal model of infection (40). In this work, we established an isogenic collection of C. albicans isolates carrying a specific transcriptional activator of azole resistance as either a wild-type allele or a variant allele with a GOF mutation. The engineered strains were next used individually in a mouse model of intravenous systemic infection and compared with the wild type. Our results show that the GOF mutations in some transcriptional activators (TAC1, MRR1) have a neutral effect on virulence and fitness, while other GOF mutations (UPC2) have a negative impact.

**MATERIALS AND METHODS**

**Strains and media.** Escherichia coli DH5α (41) was used as a host for plasmid construction and propagation. To grow DH5α, LB broth medium was used, and when necessary, it was supplemented with ampicillin (0.1 mg/ml) (42). Cultures of E. coli were incubated at 37°C under constant agitation (220 rpm) for 16 to 20 h. For growth on plates, 2% Bacto agar was added to the medium. For deletion of genes in C. albicans, YEPD containing 50 μg/ml of nourseothricin (Werner Bioagents, Germany) was added to YEED agar plates. To obtain nourseothricin-sensitive (Nours) derivatives, transformants were grown for 6 to 8 h in YEPD medium with 2% maltose. One hundred to 200 cells were then spread onto YEPD plates containing 15 μg/ml of nourseothricin and grown for 2 days at 34°C. The number of CFU required for colonization of the kidneys was counted on YEED containing 50 μg/ml chloramphenicol. The C. albicans strains used in this study are listed in Table 1.

**Plasmid and strain constructions.** For deletion of TAC1, we used the plasmid pDS1196 (12), in which the SAT1 flipper cassette (43) was inserted as a marker, resulting in a deletion of 1,924 bp between nucleotides (nt) + 501 and +2425 with respect to the TAC1 ATG. The plasmid was linearized with SphI and SacI, and the fragment obtained was transformed into SC5314 to yield the heterozygotes DSY4214 (TAC1::FRT::SAT1, where FRT is the gene for the FLP recombination target) and DSY4217 (TAC1::FRT::FRT::SAT1) after removal of the SAT1 cassette. Transformation with the restricted pDS1196 yielded the homozygote mutants DSY4220 (tac1Δ::FRT::FRT::FRT::SAT1) and DSY4241 (tac1Δ::FRT::tac1Δ::FRT::FRT::FRT::FRT::). To reintroduce the TAC1-4 wild-type and TAC1-5 GOF alleles in the background of DSY4241, the genes were amplified from strains DSY294 and DSY296, respectively, with primers TAC1-SPH and TAC1-APA containing Sphl and Apal restriction sites, respectively. The PCR fragments were next cloned into pDS1196 with the compatible restriction sites to yield pDS1747-37 (TAC1-4) and pDS1746-28 (TAC1-5). The plasmids were linearized with Sphl and SacI and transformed into strain DSY4241 to yield revertant strains.

For deletion of MRR1, the MPA flipper cassette in pFSU4 (44) was replaced by the SAT1 flipper cassette, yielding plasmid pFSU1, as de-
scribed by Coste et al. (12). To replace the URA3-flanking regions of the SAT1 flipper cassette in plasmid pSFIU4, the amplification of 471 bp of the MRR1 3’ untranslated region (UTR) was performed with primers MRR-Xho and MRR-Sacl from SC5314 genomic DNA, and the ampiclon was cloned after restriction with XhoI and SacI into pSFIU4 to yield plasmid pDS1568. Next, 561 bp of the MRR1 5’ UTR was amplified from C. albicans SC5314 genomic DNA with primers MRR-SphI and MRR-SacII and cloned after restriction with SphI and SacI into pDS1568 to yield plasmid pDS1569. This plasmid was linearized with SphI and SacI, and the fragment obtained was transformed into SC5314 to yield the heterozygotes DSY4216 (MRR1/mrr1::FRT-SAT1) and DSY4219 (MRR1/ mrr1::FRT) after removal of the SAT1 cassette. Transformation with the restricted pDS1569 yielded the homozygote mutants DSY4221 (mrr1:: FRT/mrr1::FRT-SAT1) and DSY4278 (mrr1::FRT/mrr1::FRT). For reinsertion of MRR1 wild-type and GOF alleles, the genes were amplified from strains DSY3534 and DSY3548, respectively, with the primers MRR-SphI and MRR-SacII containing the restriction sites SphI and SacI. The amplified genes were cloned into pDS1581 to yield pAL1 (wild-type allele MRR1-1) and pAL2 (GOF allele MRR1-3). The plasmids were linearized with SphI and SacI and transformed into the deletion mutant DSY4278.

To delete UPC2, the entire UPC2 open reading frame (ORF), the 500-bp 5’ UTR, and the 380-bp 3’ UTR were amplified from C. albicans SC5314 genomic DNA with primers UPC2-BamHI and UPC2-XhoI, introducing BamHI and XhoI restriction sites, respectively. This PCR product was cloned into pMTL21 to obtain pDS1565. An inverse PCR with primers UPC2-Apal and UPC2-SacII was performed using this plasmid as the template, deleting 2,210 bp starting 90 nt upstream of the ATG and terminating 20 nt upstream of the UPC2 stop codon. This product was digested by Apal and SacI and inserted into pSFS2 (43) to yield pDS1567-8. To delete UPC2 in SC5314, this plasmid was linearized with SphI and SacI, and the fragment obtained was transformed into SC5314 to yield the heterozygotes DSY4248 (UPC2/ups2::FRT-SAT1) and DSY4353 (UPC2/ups2::FRT) after removal of the SAT1 cassette. Transformation with the restricted pDS1568 yielded the homozygote mutants DSY3545 (ups2::FRT/ups2::FRT-SAT1) and DSY4357 (ups2:: FRT/ups2::FRT). To introduce UPC2-1 and UPC2-3, the genes were amplified from clinical isolates DSY2321 and DSY2323, respectively, with

| Strain     | Description                                      | Parental strain | Reference or source |
|------------|--------------------------------------------------|-----------------|---------------------|
| SC5314     | Wild type                                        |                 | 69                  |
| DSY294     | Azole-susceptible clinical isolate (TAC1-3/TAC1-4) | DSY296          | 9                   |
| DSY296     | Azole-resistant clinical strain (TAC1-3/TAC1-5)   | DSY294          | 9                   |
| DSY4214    | TAC1/tac1::FRT-SAT1                               | SC5314          | This study          |
| DSY4217    | TAC1/tac1::FRT                                    | DSY4214         | This study          |
| DSY4220    | tac1::FRT/tac1::FRT-SAT1                          | DSY4217         | This study          |
| DSY4241    | tac1::FRT/tac1::FRT                              | DSY4220         | This study          |
| ALY5       | tac1::FRT/tac1::TAC1-4-FRT                        | ALY5            | This study          |
| ALY12      | tac1::FRT/tac1::TAC1-5-FRT                        | ALY12           | This study          |
| ALY21      | tac1::TAC1-4-FRT/tac1::TAC1-5-FRT                 | ALY5            | This study          |
| ALY22      | tac1::TAC1-5-FRT/tac1::TAC1-5-FRT                 | ALY12           | This study          |
| DSY3534    | Azole-susceptible clinical isolate (MRR1-1/MRR1-2)   | DSY3534         | 13                  |
| DSY3548    | Azole-resistant clinical isolate (MRR1-3/MRR1-3)   | SC5314          | This study          |
| DSY4216    | MRR1/mrr1::FRT-SAT1                               | DSY4216         | This study          |
| DSY4219    | MRR1/mrr1::FRT                                    | DSY4119         | This study          |
| DSY4221    | mrr1::FRT/mrr1::FRT-SAT1                           | DSY4221         | This study          |
| DSY4278    | mrr1::FRT/mrr1::FRT                               | DSY4278         | This study          |
| ALY32      | mrr1::FRT/mrr1::MRR1-1-FRT                        | DSY4278         | This study          |
| ALY33      | mrr1::FRT/mrr1::MRR1-3-FRT                        | DSY4278         | This study          |
| ALY37      | mrr1::MRR1-3-FRT/mrr1::MRR1-3-FRT                 | ALY33           | This study          |
| DSY2321    | Azole-susceptible clinical isolate (UPC2-1/UPC2-2) | DSY2321         | 13                  |
| DSY2323    | Azole-resistant clinical isolate (UPC2-1/UPC2-3)  | SC5314          | This study          |
| DSY4348    | UPC2/ups2::FRT-SAT1                               | DSY4348         | This study          |
| DSY4353    | UPC2/ups2::FRT                                    | DSY4353         | This study          |
| DSY4357    | ups2::FRT/ups2::FRT-SAT1                          | DSY4357         | This study          |
| ALY17      | ups2::FRT/ups2::UPC2-1-FRT                        | ALY17           | This study          |
| ALY18      | ups2::FRT/ups2::UPC2-3-FRT                        | ALY18           | This study          |
| ALY30      | ups2::UPC2-1-FRT/ups2::UPC2-1-FRT                 | ALY17           | This study          |
| ALY25      | ups2::UPC2-2-FRT/ups2::UPC2-2-FRT-SAT1            | ALY18           | This study          |
| ALY31      | ups2::UPC2-3-FRT/ups2::UPC2-3-FRT                 | ALY25           | This study          |
| ALY39      | mrr1::MRR1-3-FRT/mrr1::MRR1-3-FRT/UPC2/UPC2-3-FRT-SAT1 | ALY37          | This study          |
| ALY41      | mrr1::MRR1-3-FRT/mrr1::MRR1-3-FRT/UPC2/UPC2-3-FRT  | ALY39           | This study          |
| ALY42      | mrr1::MRR1-3-FRT/mrr1::MRR1-3-FRT/UPC2-3-FRT/UPC2-3-FRT-SAT1 | ALY41        | This study          |
| ALY44      | mrr1::MRR1-3-FRT/mrr1::MRR1-3-FRT/UPC2-3-FRT       | ALY42           | This study          |
| ALY46      | ADH1::pADH-RRP-SAT1                               | SC5314          | This study          |
| ALY47      | ADH1::pADH-GFP-SAT1                               | SC5314          | This study          |
| ALY48      | ADH1::pADH-RRP-SAT1                               | ALY31           | This study          |
| ALY49      | ADH1::pADH-GFP-SAT1                               | ALY31           | This study          |
TABLE 2 Primers used for plasmid construction and deletion and insertion of genes in this study

| Primer purpose, gene, primer or probe labeling | Sequence |
|-----------------------------------------------|----------|
| **Plasmid construction, gene deletions and insertion** | |
| **Primer purpose, gene, and primer or probe labeling** | |
| TACI | |
| CaZinc2-XHO | CCGCAAGACTGATGTCAGATCAGATACTTCAGTCAC |
| CaZinc2-PST | TTCTGTGAGCTCGTGGACATTTGTCAGGTTCTG |
| TAC1-SPH | CCGATGCCGGCCGAAATGATTCGTTGTTGGAAA |
| TAC1-APA | CGGATGGCCGGGAAATGATTCGTTGTTGGAAA |
| **MRR1** | |
| MRR-SPH | CTGTTGAGCTCGTGGACATTTGTCAGGTTCTG |
| MRR-SacII | CTATAGCGCGGGGGTTGTTTATCTGGTTT |
| MRR-Xho | GATTGTGAGCTCGTGGACATTTGTCAGGTTCTG |
| MRR-SacI | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| MRR-SacIIC | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| **UPC2** | |
| UPC2-BamHI | CCGGAAAGATGCTTCTAGTATCTAACAAA |
| UPC2-XhoI | GCGCAAGACTGATGTCAGATCAGATACTTCAGTCAC |
| **SEQING** | |
| Zinc2-604 | ATAAAGATTGGCTGATGTA |
| Zinc2-1708 | CAGAATTCGTTGGAAGAT |
| Zinc2-2242 | GCCGTTGACATCACTGAG |
| Zinc2-2683 | GCAGATGTTTTAGTATG |
| Zinc2-3224 | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| Zinc2-3087C | GGGTGTGAGCTCGTGGACATTTGTCAGGTTCTG |
| Zinc2-1491C | GCGAAAGCTGCTTATAGT |
| Zinc2-1491C | GCGAAAGCTGCTTATAGT |
| Zinc2-1247C | TCTTCAGCTGTCGTT |
| Zinc2-3635C | AACGTGCGTCTTTT |
| Zinc2-2422 | GGGTGTGAGCTCGTGGACATTTGTCAGGTTCTG |
| Zinc2-3224 | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| Zinc2-1491C | GCGAAAGCTGCTTATAGT |
| Zinc2-1247C | TCTTCAGCTGTCGTT |
| Zinc2-3635C | AACGTGCGTCTTTT |
| Zinc2-1491C | GCGAAAGCTGCTTATAGT |
| Zinc2-1247C | TCTTCAGCTGTCGTT |
| Zinc2-3635C | AACGTGCGTCTTTT |
| Zinc2-1491C | GCGAAAGCTGCTTATAGT |
| **MRR1** | |
| MRR1-1P | CCAACCTTGCTTGTAGTTAC |
| MRR1-1 | TGTATGAGCTGCTTATAGT |
| MRR1-9 | GGTTGAGCTGCTTATAGT |
| ZCF63EQ2 | CAGTTGTTGAGCTGCTTATAGT |
| ZCF63EQ3 | AGTTCATTTTTAGAAGGG |
| ZCF63EQ4 | GGTTGTTGAGCTGCTTATAGT |
| ZCF63EQ5 | CTGTTGTTGAGCTGCTTATAGT |
| ZCF63EQ6 | CTTATGTTGAGCTGCTTATAGT |
| UPC2 | CTTATGTTGAGCTGCTTATAGT |
| ORF19.391-P2 | CCTATGTTGAGCTGCTTATAGT |
| ORF19.391-P3 | CACATGTTGAGCTGCTTATAGT |
| ORF19.391-Bam | CTTATGTTGAGCTGCTTATAGT |
| Primers and probes used for qRT-PCR | |
| CDR1-ORF-R | ATGACTGCTGATGTCAGATCAGATACTTCAGTCAC |
| CDR1-ORF-F | TTAAAGATTGGCTGATGTA |
| CDR2-ORF-R | TTAAAGATTGGCTGATGTA |
| CDR2-ORF-F | TTAAAGATTGGCTGATGTA |
| CDR3-ORF-R | GAAAGATGCTGCTTATAGT |
| CDR3-ORF-F | TTAAAGATTGGCTGATGTA |
| ERG11-ORF-R | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| ERG11-ORF-F | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| ACT-ORF-R | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |
| ACT-ORF-F | ATGGAGCAAGTTGGAGGCTCTCATCGTTGA |

a Primers were labeled with 6-carboxyfluorescein (6-FAM) at the 5’ end and tetramethylrhodamine (TAMRA) at the 3’ end and were used as TaqMan probes in qRT-PCR.

Primers Upc2SacIrevet and Upc2SacIrevet containing SacI and SacII restriction sites, respectively. This product was restricted with SacI and SacII and cloned into plasmid 1567-8, yielding pAL3 and pAL4, respectively. The plasmids were linearized with SacI and SpII and transformed into the deletion mutant DSY4357.

The construction of a strain with two different MRR1 and UPC2 GOF mutations was performed using isolate AY39 (MRR1-1/MRR1-3) as the parental strain. Introduction of UPC2-3 was performed by sequential transformation of pAL2 (see above) linearized with SpII and SacI, yielding strains AY39 (UPC2-3/UPC2-3) and AY41 (UPC2/UPC2-3) after removal of the SAT1 cassette. A second round of transformation resulted in strains AY42 (UPC2-3/UPC2-3) and AY44 (UPC2-3/UPC2-3).

**DNA sequencing.** To obtain the DNA sequences of UPC2 alleles, PCR products were amplified from strains DSY2321 and DSY2323 with primers UPC2-BamHI and UPC2-XhoI and introduced into pMTL21. From DSY2321, two alleles were obtained (UPC2-1 and UPC2-2). From DSY2323, two alleles were recovered (UPC2-1 and UPC2-3). To obtain the DNA sequences of MRR1 alleles, PCR products were amplified from strains DSY3534 and DSY3548 with primers MRR-SacI and MRR-SacII and cloned into pBluescript SK (+). From DSY3534, two MRR1 alleles were obtained (MRR1-1 and MRR1-2). From DSY3548, one allele was recovered (MRR1-3). Cloned fragments were sequenced with the primers listed in Table 2 using an ABI Prism 3130 XL automated DNA sequencer (PerkinElmer, Applied Biosystems, Foster City, CA) with a BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems) according to the manufacturer’s protocol.

**Transformation of yeast strains.** All strains were transformed as previously described (10), with slight modifications. After addition of DNA and heat shock steps, the cells were resuspended in 1 ml of YEPD medium and incubated for 4 h with agitation at 30°C. The cells were harvested in 200 µL of YEPD and spread onto YEPD plates containing 200 µg/mL nourseothricin.

**Southern blotting.** Southern blotting and probe labeling with [α-32P]dATP were performed as described elsewhere (9). The signal detections were performed with a Typhoon Trio variable-mode imager scanner (GE Healthcare GmbH, Germany). The 1,168-bp probe for detection of TAC1 deletion and insertion was generated with PCR by primers CaZinc2-XHO and CaZinc2-PST. To detect MRR1 deletion or insertion, a 565-bp probe was amplified with primers MRR-SacI and MRR-SacII. The 413-bp probe for detection of UPC2 deletion and insertion was generated with primers UPC2-BamHI and UPC2-SacII. For Southern analysis of GOF allele combinations, the UPC2 probe was generated with primers UPC2-BamHI and UPC2-XhoI. All PCRs were performed with GC3514 genomic DNA as the template.

**Drug susceptibility assays.** The yeast cells were grown in YEPD medium overnight and were then diluted to a density of 1.5 × 10^6 cells/mL. From this dilution, five 10-fold serial dilutions were prepared. A quantity of 4 µL of each yeast dilution was spotted onto YEPD rich medium and incubated for 4 h at agitation at 30°C. The cells were harvested in 200 µL of YEPD and spread onto YEPD plates containing 200 µg/mL nourseothricin.

**Growth.** Yeast cells were grown in YEPD medium overnight and were then diluted to a density of 1.5 × 10^6 cells/mL. From this dilution, five 10-fold serial dilutions were prepared. A quantity of 4 µL of each yeast dilution was spotted onto YEPD rich medium and incubated for 4 h at agitation at 30°C. The cells were harvested in 200 µL of YEPD and spread onto YEPD plates containing 200 µg/mL nourseothricin.

**GFP expression.** To detect GFP expression, the yeast cells were grown in YEPD medium overnight and were then diluted to a density of 1.5 × 10^6 cells/mL. From this dilution, five 10-fold serial dilutions were prepared. A quantity of 4 µL of each yeast dilution was spotted onto YEPD rich medium and incubated for 4 h at agitation at 30°C. The cells were harvested in 200 µL of YEPD and spread onto YEPD plates containing 200 µg/mL nourseothricin.

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umes of each well up to the last well of the microplate row. Finally, 150 µl of the yeast inoculum was added to each well. 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 microtiter plate reader set at 450 nm. The MIC was defined as the drug concentration at which the optical density was equal to or decreased more than 50% from that of the drug-free culture.

For drug susceptibility assays using serial dilutions on agar plates, several drug concentrations (fluconazole with cyclosporine and fluphenazine) were added to YEPD agar plates, as mentioned in the corresponding figure legends.

**Immunoblotting.** For protein extractions, an alkaline extraction procedure was used as described before (46). Cells were grown in YEPD medium overnight. Five milliliters of YEPD medium was inoculated with 100 µl of 10^7 cells/ml, and cells were grown to a concentration of 1.5 × 10^7 cells/ml. Cells were exposed or not exposed to the specific drugs (as mentioned in each case). The samples were separated in a 10% (wt/vol) polyacrylamide gel. Electrophoresis was performed in running buffer (1.5 g/liter Tris, 7.2 g/liter glycine, 0.5 g/liter SDS) at room temperature with a constant voltage of 100 V for the stacking gel and 150 V for the resolution of polypeptides. The gel was transferred onto a nitrocellulose membrane by Western blotting. Cdr1, Cdr2, and Mdr1 were then detected by chemiluminescence (ECL kit; Amersham, Bioscience) using polyclonal rabbit anti-Cdr1, anti-Cdr2, and anti-Mdr1 antibodies, respectively, and an antit-rabbit horseradish peroxidase-labeled secondary antibody (47).

**qRT-PCR.** Total RNA was extracted with a phenol-chloroform-lithium chloride procedure as previously described (48). Biological triplicates from strains were prepared by growing the strains in 10 ml of YEPD medium until mid-exponential growth phase (1.5 × 10^7 cells/ml). Five milliliters of solution was exposed or not to specific drugs. Contaminating genomic DNA was removed by DNase treatment (DNA free; Ambion). The expression of CDR1, CDR2, MDR1, and ERG11 was quantitatively determined by quantitative real-time reverse transcription-PCR (qRT-PCR) in a StepOne real-time PCR system (Applied Biosystems). The expression levels were normalized by ACT1 expression. Changes (n fold) in gene expression relative to that of wild-type strain SC5314 were determined from CDR1-normalized expression levels. The primers and probes used for this assay are described in Table 2.

**Competition assays in vitro.** Strains were thawed from −80°C stocks and plated onto YEPD medium plates. A single colony of each strain was grown in biological duplicate for 24 h at 30°C under constant agitation. This culture was diluted 200-fold in 20 ml YEPD medium and grown for 15 h at 30°C. The cells were washed in phosphate-buffered saline (PBS) solution buffer and resuspended in YEPD medium to a final concentration of 3.7 × 10^6 cells/ml. In order to control the inocula, samples from dilution assays were plated onto YEPD agar plates and the numbers of CFU were counted. Equal volumes of each culture were mixed together, and each single strain was incubated at 30°C for 24 h under constant agitation. The growth of the coculture was determined directly and after 2, 4, 6, 8, and 24 h by measuring the absorbance at 540 nm and by plating diluted samples onto YEPD agar plates. After 2 days of incubation at 34°C, colonies were replicated onto 200 µg/ml nourseothricin to obtain the relative proportion of both strains over time.

**BMDMs.** Female BALB/c mice (Charles River Laboratories, France) were housed in filter-top cages with free access to food and water. Mice were treated under the surveillance of the local governmental veterinarian office (Affaires Vétérinaires du Canton de Vaud, Switzerland; authorization number 2240). Eight- to 10-week-old mice were euthanized by inhalation of CO₂, and bone marrow was extracted from both femurs and tibiae. The extracted bone marrow cell suspension was filtered through a 40-μm-mesh-size cell strainer filter and suspended in culture medium (high-glucose Iscove’s modified Dulbecco’s medium with GlutaMAX [IMDM]; Life Technologies) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies), 10% fetal bovine serum (FBS; Life Technologies), and 20% L-cell-conditioned medium (as a source of macrophage colony-stimulating factor [M-CSF]). Cells were seeded into petri dishes 150 mm by 20 mm high (Sarstedt) at 4 × 10^5 cells/plate and incubated at 37°C in the presence of 5% CO₂. Fresh medium was added after 2 and 4 days of incubation to feed the cells. Nondifferentiated cells do not adhere to the plastic surface of petri dishes and are therefore washed away by aspiration when fresh medium is added. On day 6 of incubation, the majority of adherent cells were bone marrow-derived macrophages (BMDMs) and were harvested with EDTA (Life Technologies), a nonenzymatic solution, and used for further experimentation.

**Phagocytosis and filamentation assays.** BMDMs were transferred to 24-well plates containing round cover slides inside the wells at a density of 3 × 10^6 cells/well in 1 ml of medium (IMDM with antibiotics and 10% FBS) and incubated at 37°C with 5% CO₂. After an additional 20- to 24-h incubation, preconfluent BMDM monolayers were used to perform fungal coinfection phagocytosis assays. Test yeast strains were AY46 to AYL49 containing pADH-GFP-SAT1 and pADH-RFP-SAT1 (see Table 1 for details; plasmids were kindly supplied by N. Dean, Stony Brook University, Stony Brook, NY) integrated by BamHI digestion at the ADHI locus. To prepare the yeast suspensions for infection, overnight cultures of fluorescent strains in YEPD medium were diluted in fresh medium and grown for a minimum of 2 generations to mid-log phase. These cells were washed and resuspended in PBS. The inoculum concentration was confirmed by plating serial dilutions in YEPD agar plates. Macrophage cultures were infected with yeast strain suspensions, which were mixed with the macrophages at a ratio of 1:1, at a multiplicity of infection (MOI) of 1 (according to the macrophage seeding density), and the plates were centrifuged at 200 × g for 1 min. These cocultures were incubated at 37°C in 5% CO₂, and at selected time points, non-macrophage-associated yeasts were removed by washing twice with PBS. Yeast-macrophage cocultures established on top of round cover slides were mounted onto microscopy slides. The quantification was undertaken by using a Zeiss Axiosplan 2 epifluorescence microscope (images were recorded using a Visirion Systems HistoScope camera and VisiView imaging software). The numbers of phagocytosed yeast cells within 100 to 120 macrophages were counted. The same experiment was performed by swapping fluorescent proteins in each of the investigated genetic backgrounds (strains SC5314 and ALY31).

Filamentation of cells was estimated by visual observation of green fluorescent protein (GFP)- and red fluorescent protein (RFP)-fluorescent cells during phagocytosis at different time points. Filamentation was considered positive when a germination tube could be detected on each type of cell.

**Survival assay in BMDMs.** BMDMs were prepared and incubated as described for the phagocytosis assay. They were seeded at a concentration of 3 × 10^7 cells/ml in flat-bottom 96-well plates (Costar) in 150 µl IMDM. The strains were also prepared as described above but were washed in IMDM instead of PBS. Serial 4-fold dilutions of separate yeast strain suspensions (SC5314 and ALY31) were carried out starting from suspensions corresponding to an MOI of 1 in a volume of 200 µl. The plates were centrifuged at 200 × g for 1 min, and the plates were incubated at 37°C in 5% CO₂ for 48 h. Yeast colonies were visualized and counted by light microscopy, and the numbers were compared to those obtained with the same dilutions with noninfected macrophages. Two technical replicates per strain were used.

**Animal experiments.** The animal experiments were performed at the University Hospital Center of Lausanne under authorization number 2240 (Affaires Vétérinaires du Canton de Vaud, Switzerland), respecting Decree 18 of the federal law on animal protection. Strains were prepared as described above to a final concentration of 4 × 10^6 cells/ml. The numbers of CFU were controlled by plating onto YEPD agar plates. PBS (250 µl) containing 10^6 CFU was injected into the lateral tail vein of the mice. For all experiments, BALB/c mice (female; age, 6 to 8 weeks; weight, 20 to 25 g; Charles River, France) were housed in ventilated cages. All mice had free access to food and water. Mice were monitored daily and according to the federal law on animal protection were sacrificed by inhalation of CO₂, when necessary.
Survival assays. Mice were monitored for a maximal period of 21 days. A mouse was declared moribund and then sacrificed when a loss of a maximum of 30% of its initial weight was measured and when prolonged suffering of the mice would no longer be in accordance with the rules of humane endpoints of animal treatment. Survival rates were compared among groups by use of the log-rank test with the software GraphPad Prism (version 5.0). Differences were considered statistically significant when P values were \( \leq 0.05 \).

Kidney tissue burden. To assess fungal burdens, mice were euthanized at 3 days postinjection (dpi) and 7 dpi with CO2 inhalation. Both kidneys were excised aseptically, weighed, and homogenized in PBS. Homogenized kidneys were plated directly, and two to three serial dilutions (10-fold) were plated onto YEPD agar containing 50 \( \mu \)g/ml chloramphenicol. CFU numbers were determined as the number of CFU/g of kidney. Analysis was performed with the nonparametric Kruskal-Wallis test, followed by pairwise comparisons between groups, using the software PASW Statistics 18. Differences were considered statistically significant when P values were \( \leq 0.05 \).

Competition assays in vivo. One group of four mice was injected with a 1:1 mix ratio of strains SC5314 and ALY25, and two other groups of mice were infected with each strain separately. Kidneys were collected, homogenized, and diluted, and the numbers of CFU per gram of kidneys were obtained as mentioned above. Kidneys were collected, homogenized, and diluted as described above for counting of the number of CFU on YEPD medium and YEPD medium with 200 \( \mu \)g/ml nourseothricin.

Nucleotide sequence accession numbers. Sequences were deposited in the GenBank database under accession numbers KF429710 to KF429715.

RESULTS

Construction of isogenic mutant strains. In order to address the role in virulence of each GOF mutation contained in the Tfs Tac1, Mrr1, and Upc2, isogenic sets of strains were engineered. The general strategy was to first delete the genes of interest using the regenerable dominant marker SATI. After flip out of the SATI marker, TF alleles were reintroduced at their genomic sites as a GOF mutation, as published (see below). Drug-inducing conditions were used as positive controls for the increase of expression of the target proteins. As observed in Fig. 1, Cdr1/Cdr2 (the targets of Tac1) and Mdr1 (the target of MRR1) signals were increased, as expected, in SC5314 under conditions with drug induction. Accordingly, no signals corresponding to these proteins were detected in TF mutant strains (DSY4241, DSY4278), thus confirming correct gene deletions. Insertion of wild-type TAC1 (TAC1-4 in ALY5) and MRR1 (MRR1-1 in ALY32) alleles restored the ability of C. albicans to respond to drugs, as in the parent wild type, since Cdr1/Cdr2 and Mdr1 levels were increased by drug exposure.

Phenotypic verifications of engineered strains. The engineered strains were next subjected to different assays that aimed to verify that TF target genes and the resulting proteins exhibit changes in expression that depend on the status of the introduced alleles.

The reintroduction of wild-type alleles as well as TAC1 and MRR1 GOF alleles in the respective mutant backgrounds was assessed by immunodetection of target proteins (Cdr1/Cdr2, Mdr1) and by qRT-PCR. Targets of UPC2 were verified only by qRT-PCR (see below). Drug-inducing conditions were used as positive controls for the increase of expression of the target proteins. As observed in Fig. 1, Cdr1/Cdr2 (the targets of Tac1) and Mdr1 (the target of MRR1) signals were increased, as expected, in SC5314 under conditions with drug induction. Accordingly, no signals corresponding to these proteins were detected in TF mutant strains (DSY4241, DSY4278), thus confirming correct gene deletions. Insertion of wild-type TAC1 (TAC1-4 in ALY5) and MRR1 (MRR1-1 in ALY32) alleles restored the ability of C. albicans to respond to drugs, as in the parent wild type, since Cdr1/Cdr2 and Mdr1 levels were increased by drug exposure.

The introduction of GOF alleles TAC1-5 and MRR1-3 in one or two copies resulted in Cdr1/Cdr2 and Mdr1 expression levels comparable to those obtained under conditions with drug induction. These results are consistent with hyperactive states of TAC1 and MRR1 yielding constitutive high expression of target genes. The results also confirmed that the previously unreported substitution G963S was a GOF mutation since the target protein Mdr1 was found to be constitutively highly expressed in ALY33 (one MRR1-3 copy) and not in ALY32. We did, however, observe some gene copy number effects at this stage. We noticed that the signals for Cdr2 in ALY22 (with two TAC1-5 copies) were increased compared to those in ALY12 (with one TAC1-5 copy) in the absence of drugs. However, this increase in signal intensity could not be observed for Cdr1. Likewise, in the case of MRR1, the intensity of the signal of Mdr1 in ALY33 (with one MRR1-3 copy) was approximately half the intensity of Mdr1 in ALY37 (with two MRR1-3 copies) in the absence of drugs. Interestingly, drug exposure could still increase the Mdr1 signal intensity proportionally to the MRR1-3 copy number.
Given that GOF alleles were reintroduced in one or two copies, we also verified this dosage effect by measuring corresponding mRNA levels with qRT-PCR. Figure 2 shows the results obtained for TAC1, MRR1, and UPC2 target genes expressed in the engineered strains. Drug-inducing conditions were used as controls for target gene upregulation. As observed in Fig. 2, the addition of fluphenazine, benomyl, and fluconazole resulted, as expected, in the upregulation of the respective target genes. CDR1 and CDR2, which are part of the TAC1 regulon, were upregulated 10- and 300-fold, respectively, upon fluphenazine addition. ERG11 (UPC2 regulon) and MDR1 (MRR1 regulon) were upregulated 4-fold and about 600-fold, respectively, upon fluconazole and benomyl exposure. The increase in the amount of CDR1 mRNA in ALY12 (with one TAC1-5 copy) and ALY22 (with two TAC1-5 copies) was 5-fold greater than that in SC5314 for both strains. The levels of CDR2 mRNA in ALY12 were about half of those measured in ALY22, thus correlating with the copy number of TAC1-5 in these strains. When measuring the MRR1 target, strain ALY37 (with two MRR1-3 copies) also exhibited 2-fold higher levels of MDR1 mRNA than ALY33 (with one MRR1-3 copy), thus correlating with the MRR1 copy number. ERG11 was taken as a target of UPC2. As shown in Fig. 2, the levels of ERG11 mRNA were elevated about 3- and 4-fold compared to those in the wild type in ALY18 and ALY31, containing one and two copies of the UPC2-3 GOF allele, respectively. In this case, the amount of ERG11 mRNA does not necessarily correlate with the UPC2-3 allele copy number. Measuring other UPC2 targets did not alter the interpretation (data not shown).

The last phenotypic verification of the strains constructed was performed by drug susceptibility assays. We reasoned that since fluconazole has only a fungistatic effect on *C. albicans*, our observations could be perturbed by the trailing phenomenon due toazole tolerance. To circumvent this phenomenon, cyclosporine (a calcineurin inhibitor) was used together with fluconazole (49, 50). Strains were also spotted onto YEPD medium with fluphenazine, which helped to distinguish between deletion mutants and the insertion of wild-type alleles. Fluphenazine is a drug interfering with the calmodulin functions in yeast and is also an efficient inductor of Cdr1 and Cdr2 in *C. albicans*. This drug helped to identity in susceptibility assays the effect of TFs involved in the regulation of these transporters (14, 51).

As shown in Fig. 3A, the wild-type strain SC5314, DSY4241 (tac1Δ/tac1Δ), and ALY5 (TAC1-4) did not grow on fluconazole and cyclosporine medium, which were findings opposite those obtained for ALY12 and ALY22 (with one and two TAC1-5 copies, respectively). The magnitude of growth on fluconazole-containing medium correlates with the insertion of one and two hyperactive alleles in these strains. The absence of growth of DSY4241 on fluphenazine compared to the growth of SC5314 is consistent with the homozygous deletion of TAC1. The effect of the TAC1 inser-
tion was best revealed on fluphenazine medium. ALY22 showed enhanced growth compared to that of ALY12 on this medium, with that finding thus correlating with the insertion of a second hyperactive allele in the latter strain.

Strains SC5314, DSY4278 (mrr1Δ/mrr1Δ), and ALY32 (with one MRR1-1 copy) all showed fluphenazole susceptibility, whereas the other spotted strains, including ALY33 and ALY37 (with one and two MRR1-3 copies, respectively), did not (Fig. 3B). DSY4357 (upc2Δ/upc2Δ) and ALY17 (with one UPC2-1 copy) did not grow on fluphenazole, as in the case of SC5314. With the ability to grow on fluphenazine, we confirmed the insertion of wild-type allele UPC2-1 in strain ALY17. ALY18 and ALY31 (with one and two UPC2-3 copies, respectively) were able to grow on fluphenazole; however, ALY31 showed enhanced growth compared to that of ALY18, indicating the expected gene dosage effect.

Finally, flucanazole MIC measurements were performed with the engineered strains according to the EUCAST protocol (Table 3). In general, flucanazole MICs increased proportionally to the number of GOF alleles introduced in C. albicans. For example, while the MIC for a strain with one TAC1-5 copy was between 1 and 2 μg/ml (8- to 16-fold higher than that for the wild type), this value increased to 8 μg/ml when two TAC1-5 alleles were present. The highest MIC values were obtained with TAC1-5 and MRR1-3 strains, while the MIC values obtained for the strain with UPC2-3 were decreased by 8- to 16-fold compared to those for strains with other GOF alleles.

In conclusion, our results demonstrate that the engineered strains exhibited the expected genotypes and phenotypes. To carry out animal experiments, it is noteworthy that only nourseothricin-susceptible isolates were used (see Discussion for an explanation of this choice).

Kidney tissue burden in mice infected with C. albicans strains. Next we addressed the effect of GOF mutations on virulence with each of the TFs involved in azole resistance. The mouse model of disseminated candidiasis was used to assess the ability of the above-described strains to colonize (kidney fungal burden assay) and to kill (survival assay) the host. The numbers of CFU in kidneys were measured at 3 and 7 dpi. Each experiment contained a group of mice infected with SC5314, a TF deletion mutant, and revertant strains containing a wild-type or a GOF allele in one or two copies. The results are summarized in Table 4. The results showed that only the presence of two TAC1-5 copies in C. albicans significantly affected the tissue burdens. It is noteworthy that TAC1-5 had no negative impact on tissue colonization, since the numbers of CFU in the kidney were higher for strain ALY22 (with two TAC1-5 copies) than for strain SC5314. The MRR1-3 GOF allele had no impact on kidney colonization compared to that obtained with the wild-type allele. The data in Table 4 indicate that the deletion of UPC2 results in a decreased capacity to colonize kidney tissues compared to the capacity of the wild type. Surprisingly, the presence of two UPC2-3 alleles also had a negative impact on fungal burdens from kidneys compared to the impact of the wild-type allele. Taken together, these data show that not only the absence of UPC2 and but also UPC2 hyperactivity are critical for kidney colonization.

Mouse survival assays. Mouse survival assays were next performed with the engineered strains. Survival was observed after strain injection for a period of 21 days. For technical reasons, experiments were carried out in two distinct groups of experiments with each TF type investigated. One group of experiments compared the virulence of the wild type, the TF mutant, and the revertant strains, each containing a wild-type and a GOF allele. A second group compared the wild type, the TF mutant, and revertant strains with one and two GOF alleles.
As shown in Fig. 4A, all TAC1-derived strains and the wild type behaved similarly, except for strain ALY22. This strain, which contains two TAC1-5 GOF alleles, exhibited a significant attenuation of virulence compared to the virulence of the other strains; however, it was at the limit of significance (P = 0.0492 versus SC5314). Graybill et al. (25) investigated the effect of azole resistance on the virulence of two C. albicans isolates exhibiting overexpression of CDR1, which was probably caused by the presence of TAC1 GOF alleles in the clinical strains. These authors showed that azole resistance in these isolates was responsible for decreased virulence in systemic infections compared to the virulence of susceptible isolates, a finding which seems to contradict our assumption. Given that no survival differences existed between mice infected with the different MRR1-derived isolates (Fig. 4B), our data suggest that MRR1 and the associated MRR1-3 GOF allele have no negative impact on virulence.

With regard to the effect of UPC2 on C. albicans virulence, the results in Fig. 4C indicate that the deletion of UPC2 (strain DSY4357) results in the significant attenuation of virulence compared to the virulence of the wild type. Likewise, only when a second UPC2-3 allele is present in C. albicans, the virulence decreased significantly compared to that of the other strains. Therefore, the presence of two hyperactive UPC2-3 copies in C. albicans not only increases fluconazole resistance but also results in decreased virulence.

**In vitro/in vivo competition assays.** Competition assays were undertaken to assess whether a decrease of virulence may be associated with fitness costs in *in vitro* or *in vivo*. As mentioned above, the presence of UPC2-3 in *C. albicans* resulted in decreased virulence, and we therefore addressed only this TF in competition assays. The assays were performed with strains SC5314 and ALY31 (UPC2-3/UPC2-3). To distinguish between the two cell types (SC5314 and ALY31), we used a parental strain of ALY31 (ALY25) still containing the SAT1 flipper cassette at the UPC2 genomic locus. This allowed us to distinguish between Nours’ (ALY25) and Nour+ (SC5314) cells. It is also the reason behind the choice of using Nour+ cells after SAT1 flipping in strains used for single infections (Fig. 4A to C). For the fitness assay *in vivo*, 8 mice per group were used. After 3 and 7 dpi, half of the group was euthanized to monitor the percentage of Nour+ cells in the population.

**TABLE 4 Kidney burdens in infected mice.**

| Strain | 3 dpi | 7 dpi |
|--------|-------|-------|
|        | Mean no. | SEM | Significance | Mean no. | SEM | Significance |
| SC5314 | 12 | 1.52E+04 | 6.12E+03 | 1.25E+05 | 2.62E+04 |  |
| DSY4241 (tac1Δ/tac1Δ) | 12 | 1.92E+04 | 5.08E+03 | 1.95E+05 | 4.61E+04 |  |
| ALY5 (tac1Δ/TAC1-4) | 6 | 1.18E+04 | 4.55E+03 | 2.35E+05 | 5.32E+04 |  |
| ALY12 (tac1Δ/TAC1-5) | 12 | 1.51E+04 | 4.19E+03 | 6.02E+04 | 1.05E+04 |  |
| ALY22 (TAC1-5/TAC1-5) | 6 | 2.02E+04 | 6.11E+03 | 5.82E+05 | 2.10E+05 |  |
| SC5314 | 12 | 3.81E+04 | 4.43E+03 | 4.77E+05 | 8.09E+04 |  |
| DSY4278 (mrr1Δ/mrr1Δ) | 12 | 2.73E+04 | 3.10E+03 | 2.32E+05 | 1.23E+05 |  |
| ALY32 (mrr1Δ/MRR1-1) | 6 | 3.90E+04 | 7.06E+03 | 5.71E+05 | 2.44E+05 |  |
| ALY33 (mrr1Δ/MRR1-3) | 12 | 2.73E+04 | 3.87E+03 | 2.82E+05 | 8.33E+04 |  |
| ALY37 (MRR1-3/MRR1-3) | 6 | 1.98E+04 | 1.88E+03 | 3.87E+05 | 9.61E+04 |  |
| SC5314 | 12 | 5.76E+04 | 2.01E+04 | 6.53E+05 | 9.26E+04 |  |
| DSY4357 (upc2Δ/upc2Δ) | 12 | 6.41E+03 | 1.78E+03 | 8.04E+04 | 1.61E+04 |  |
| ALY17 (upc2Δ/UPC2-1) | 6 | 5.25E+04 | 3.39E+04 | 2.00E+05 | 1.01E+05 |  |
| ALY18 (upc2Δ/UPC2-3) | 12 | 2.23E+04 | 5.06E+03 | 3.88E+05 | 7.10E+04 |  |
| ALY31 (UPC2-3/UPC2-3) | 6 | 2.43E+04 | 5.43E+03 | 1.31E+05 | 4.62E+04 |  |

*Value significantly different from that for SC5314. Value significantly different from those for SC5314, ALY18, and ALY31. Value significantly different from that for SC5314. C. albicans inocula varied from 2 × 10⁴ to 4 × 10⁵ in all experiments. Number of infected mice. Significance was measured by the Kruskal-Wallis one-way analysis of variance test using GraphPad Prism (version 5.0) software. Only significant differences are reported.*

Taken together, our results indicate that virulence and fitness defects in *C. albicans* were associated only with UPC2 and, more specifically, only when UPC2 GOF mutations were present on both alleles.

**Combining two GOF mutations in *C. albicans*.** It is not uncommon for GOF mutations of specific transcriptional activators to combine in the same isolate to increase its level of azole resistance. For example, Hoot et al. (19) reported the occurrence of a
substitution (A643V) in UPC2 from parent isolates that already upregulated MDR1 compared to its level of regulation in wild-type isolates. We show here that this MDR1 upregulation was due to the MRR1-3 GOF allele (see above). Flowers et al. (52) described the occurrence of UPC2 GOF mutations together with CDR1/CDR2 or MDR1 upregulation. To our knowledge, a single case of a clinical isolate with a combination of MRR1 and TAC1 GOF mutations has been identified (DSY3548 or TW17) (11, 13). This motivated us to associate two GOF mutations from separate TFs in the same strain. Here we chose to associate UPC2-3 and MRR1-3 in isolate ALY44. As summarized in Table 3, the combination of both UPC2 and MRR1 GOF mutations in ALY44 resulted in fluconazole MICs ranging from 8 to 16 μg/ml, while the values were 0.5 μg/ml for the UPC2-3/UPC2-3 strain and 2 μg/ml for the MRR1-3/MRR1-3 strain. This suggests that the effect of this particular combination of GOF mutations is not additive but synergistic in terms of the gain of fluconazole resistance. MDR1 and ERG11 expression was tested in strains with single and combined GOF mutations. As shown in Fig. 6, isolate ALY44 did not exhibit significant changes in expression of the two typical MRR1 and UPC2 target genes compared to their expression in strains with single GOF alleles. Therefore, the gain of azole resistance by the combination of GOF mutations must be due to other factors.

When tested for virulence in animal assays, while the costs of
resistance on virulence were reproduced in isolates ALY37 (MRR1-3/MRR1-3; neutral effect) and ALY31 (UPC2-3/UPC2-3; fitness cost), strain ALY44 exhibited the most attenuated virulence compared to that of the wild type (P < 0.003; Fig. 7). However, this difference was not statistically meaningful between ALY31 (UPC2-3/UPC2-3) and ALY44 (UPC2-3/UPC2-3 MRR1-3/MRR1-3). Therefore, our data indicate that the fitness costs of the UPC2-3 allele cannot be compensated for but are, rather, enhanced by the presence of another GOF mutation from an additional TF involved in drug resistance. Similar data were obtained by the combination of TAC1-5 with UPC2-3 (data not shown).

Interaction with host cells. We next tried to address the reasons behind the specific in vivo decreased fitness of strain ALY31 (UPC2-3/UPC2-3) coinfected with SC5314. As a model of the interaction of yeast cells with the innate immune system, we designed a coculture phagocytosis assay with primary BMDMs (extracted from the bone marrow of mice) to ask whether the UPC2-3 allele had an influence on phagocytosis by BMDMs. We used a competitive phagocytosis assay with strains SC5314 and ALY31 labeled with GFP and RFP, respectively, as published by Vale-Silva et al. (53). For this experiment, preconfluent BMDMs on cover slides were cocultured with SC5314 and ALY31 mixed in equal amounts at an MOI of 1 for 0.5 h. The percentage of internalization ratio (PIR) is displayed in Fig. 8A. The results show that SC5314 and ALY31 are indistinguishable in their internalization by BMDMs. In addition, the survival of SC5314 and ALY31 in BMDMs was similar (Fig. 8B). Thus, the difference in fitness and virulence between the two cell types is based on parameters other than defects in adherence/phagocytosis.

The capacity to filament in C. albicans is one of the criteria contributing to virulence (54). Interestingly, during our experiments with BMDMs, we observed that ALY31, containing two UPC2-3 alleles, was delayed in its capacity to filament in BMDMs compared to SC5314. We undertook a phagocytosis assay with BMDMs over time to display the kinetics of filamentation of strains SC5314 and ALY31 (Fig. 9). While in the wild-type strain approximately 80% of cells started to germinate 30 min after phagocytosis, only 8.5% of ALY31 cells showed this phenotype. After 2 h of incubation, only 64% of ALY31 cells formed filaments (Table 5). Additional experiments performed under hypoxic conditions in the cell culture medium (IMDM) used for the phagocytosis assays did not reveal filamentation defects due to the presence of UPC2-3 (Fig. S3 in the supplemental material). These results suggest that the GOF allele UPC2-3 interferes at some step with a normal filamentation process within host cells. This defect of ALY31 could negatively affect virulence and fitness compared to those of SC5314, as documented in the present work.

DISCUSSION

The costs of drug resistance. In this work, we showed that only a specific azole resistance mechanism related to the GOF mutation in UPC2 induces fitness costs in C. albicans. In bacterial systems, the association between resistance and virulence and fitness has been investigated in great detail. For example, in vitro and in vivo fitness studies have been performed on the forearms of human volunteers with a fusidic acid-resistant clinical isolate of Staphylo-
coccus epidermidis. The resistance to fusidic acid, due to fusA mutations, resulted in a decreased growth rate in vitro and a considerable loss of fitness in competition assays in vivo (55). A set of in vitro stepwise-selected teicoplanin-resistant mutants which were derived from susceptible Staphylococcus aureus SA113 showed slower growth in vitro, thus likely correlating with in vivo fitness costs (56). Mutation of the parE fluoroquinolone resistance gene of a Streptococcus pneumoniae clinical isolate was tested in an isogenic background and led to reduced fitness in vitro and in vivo (57). In these bacterial studies, most of the antibiotic resistance mechanisms were associated with fitness costs, which were mostly due to reduced growth rates (58). Mutations of genes which are carried on plasmids or transposons and which lead to antibiotic resistance result in fitness costs, monitored by attenuation in growth, survival, or virulence (59, 60). Even if the association between resistance and fitness costs in bacteria is well described, other cases where resistance did not lead to fitness costs were also reported, such as in clinical isolates of Streptococcus pneumoniae and Mycobacterium smegmatis resistant to several antibiotics (57, 61).

The resistance of fungi to antifungal drugs is a much more recently observed phenomenon than the resistance of bacteria to antibiotics, and therefore, it has not been as well investigated. Resistance to antifungal drugs is currently rising among pathogenic fungi, and therefore, it is important for the efficacy of future antifungal therapy to identify the genes involved in resistance. These factors could constitute key steps for virulence and can be used as possible targets for novel antifungal treatments. Drug resistance in fungal pathogens has different outcomes on virulence and fitness in vitro and in vivo. One general issue in addressing this problem is the lack of isogenic sets of strains with different phenotypes, which was resolved here by making targeted strain constructions. The need to engineer isogenic strains for such studies is illustrated by a study of Vale Silva et al. (62) in which targeted mutations in ERG3 (a sterol desaturase) leading to azole resistance appeared to result in decreased virulence in animal models. The same mutation engineered in the context of the originalazole-resistant clinical strain did not result in virulence deficits. Caspofungin resistance in C. albicans as a result of mutations in the FKS1 gene, encoding one of the subunits of β-1,6-glucan synthase, caused decreased virulence in an animal model of infection (63, 64). FKS1 mutations cause cell wall stress and therefore altered fungal cell wall presentation to the immune cells at the expense of C. albicans survival. Interestingly, caspofungin resistance in C. glabrata is not associated with fitness costs, as measured in the minihost model Galleria mellonella; however, this effect has not been yet addressed in animal models (65). As a comparison, one of the few other studies in which engineered strains were used was on the effect of CgPDR1 GOF mutations involved in azole resistance on virulence. Contrary to what was expected from studies of PDR1 in other species of Candida, this study found that the GOF mutations resulted in enhanced virulence and fitness in C. glabrata (39).

FIG 7 Effect of the combination of two GOF alleles on C. albicans virulence. Survival curves of mice infected with SC5314, ALY31 (UPC2-3/UPC2-3), ALY37 (MRR1-3/MRR1-3), and ALY44 (UPC2-3/UPC2-3 MRR1-3/MRR1-3). Statistical analysis was carried out by the log-rank Mantel-Cox test using GraphPad Prism (version 5.0) software. Only significant comparisons (P ≤ 0.05) are given. Inocula ranged from 2.9 × 10⁵ to 3.8 × 10⁵ cells.

FIG 8 Phagocytosis and survival assays of C. albicans with BMDMs. (A) Phagocytosis of coinfected C. albicans strains. The internalization ratio is displayed for GFP-labeled SC5314 and RFP-labeled ALY31 (UPC2-3/UPC2-3). Cells were added to preconfluent BMDM monolayers (established on top of round cover-slides in 24-well plates) with a 1:1 mix of the two strains (MOI, 1), as described in Materials and Methods. Cocultures were incubated in IMDM at 37°C in 5% CO₂, and after 0.5 h of coincubation, the cultures were washed to remove noninternalized yeast cells. Cover slides were mounted onto microscopy slides for quantification. The difference between the two strains was not statistically significant (P > 0.05, unpaired Student’s t test). (B) Endpoint dilution survival assay with C. albicans strains. Strains SC5314 and ALY31 (UPC2-3/UPC2-3) were added to the first well of preconfluent BMDM monolayers in 96-well plates with a 1:1 mix of each strain (MOI, 1), as described in Materials and Methods. A 4-fold dilution was carried out on each following well. Cocultures were incubated in IMDM at 37°C in 5% CO₂ for 48 h, and visible yeast colonies were counted and the numbers were compared to those of a control yeast culture without BMDMs.
have essentially a neutral impact on virulence. The hyperactivity of UPC2, due to the presence of the G648D substitution in both UPC2 alleles, leads to a significant decrease of virulence and a strong decrease of fitness in vivo. These fitness costs in vivo are likely not due to in vitro growth deficiency (see Fig. S2 in the supplemental material) or fitness costs in vitro, as tested with competition assays. The fitness costs were observed only in in vivo assays, a finding which therefore suggests that changes in fitness are due to specific host-pathogen interactions. The results presented in Table 5 and Fig. 9 suggest that a UPC2 GOF mutation can delay filamentation in C. albicans. This defect may contribute to the decrease of virulence and fitness; however, it does not exclude the possibility that other factors could also be involved in this phenotype.

Some remarks concerning the impact of each hyperactive TF on virulence can be made. No significant difference in the results of kidney colonization and survival assays could be obtained between the MRR1-derived strains and the wild type (Table 4; Fig. 4B). Therefore, neither the deletion of MRR1 nor the presence of the GOF mutation on either one or two MRR1 alleles showed any impact on virulence in the model of systemic infection. In apparent contrast to this observation, it has been reported by Becker et al. (66) that the disruption of MDR1, a major target of MRR1, led to the attenuation of virulence in immunocompetent and immunocompromised mice. It should be noted that the genetic construction of the strains employed at that time led to positional artifacts of the genetic markers. This is in contrast to the present study, where only neutral genetic markers were used for strain constructions. Another study concluded that a clinical isolate with high MDR1 mRNA levels exhibited decreased virulence (67). The finding of this study corroborated that of a separate one obtained with two fluconazole-resistant clinical isolates of C. albicans with MDR1 overexpression which exhibited low virulence in a murine model of systemic infection (25). In the last two studies, only sequential clinical isolates from different patients were tested; therefore, some additional genomic alterations with a potential impact on virulence may have also occurred in these isolates.

In the present study, no change in virulence of the TAC1-derived strains compared to that of the wild type could be obtained. When mice were infected with strains containing two TAC1 hyperactive alleles, a slight increase of mouse survival compared to that for mice infected with one hyperactive allele was visible (Fig. 4A). Colonization of the kidneys by the same strains at 7 dpi was not consistent with the observed decreased virulence (Table 4). Given that the effect of TAC1 hyperactivity on virulence reached the limits of significance (P = 0.049), we suggest that TAC1 could not be considered to play a major role in virulence.
With regard to UPC2-derived strains, the isolate with two UPC2 hyperactive alleles failed to colonize kidneys, unlike the wild type (Table 4), and showed significantly attenuated virulence in the survival assay (Fig. 4C). In this case, a gene dosage effect was required to fully exhibit attenuated virulence. These results are also mirrored in those of the kidney colonization assays. Therefore, the only clear association between the deletion or hyperactivity of a TF regulating azole resistance genes and virulence was observed for UPC2. To verify whether or not the decrease of virulence associated with the GOF mutation on the UPC2 allele was a cause of the fitness costs, competition assays were carried out in vitro and in vivo. The in vitro competition assay did not reveal fitness costs associated with the presence of UPC2 hyperactive alleles compared to the results obtained with the wild-type strain (Fig. 5). In contrast, when the competition was performed in vivo, a strong attenuation of fitness related to the presence of two UPC2-3 alleles was apparent (Fig. 5).

In a recent study published by Sasse et al. (40), the same UPC2 GOF mutation (G648D) was inserted on two copies at the genomic loci. In agreement with the findings of the present study, the authors concluded that UPC2 hyperactivity showed a tendency to decrease in vivo fitness compared to that for the wild type, although the difference was not statistically significant. The same conclusion was made for a strain containing MRR1 hyperactive alleles but not for a strain containing TAC1 hyperactive alleles. While our conclusions reinforce the notion of a negative role of UPC2-3 in fitness and virulence, a negative impact of MRR1 could not be highlighted here. Several reasons might explain these differences. For example, a mouse model of gastrointestinal colonization with antibiotic administration was used in the study of Sasse et al. (40) and represents a pathogenesis model different from the systemic model used here. Another difference is that the strains with single hyperactive alleles constructed in the study of Sasse et al. (40) showed fitness costs in vitro in YEPD rich medium but not in minimal medium when their fitness was compared to that of the wild type. Combining TAC1, UPC2, and MRR1 hyperactive alleles contributed to in vitro fitness defects in both media. Unsurprisingly, the combination of hyperactive alleles resulted in fitness defects in vivo (40). In the present study, no fitness costs in YEPD medium in vitro were measured with the strain homozygous for UPC2-3 (Fig. 5). The growth curves of strains constructed in this study were essentially similar to the growth curve of the wild type (see Fig. S2 in the supplemental material); thus, it is unlikely that they exhibited fitness defects. The reasons behind these inconsistencies between data from different studies are not clear but might be explained by differences in the engineering of the strain constructions. As a matter of fact, the wild type used for in vitro/in vivo fitness assays in the study of Sasse et al. (40) contained a constitutively expressed RFP gene and the SAT1 cassette in the genome. In the present study, the wild-type strain SC5314 was used, and only the nourseothricin resistance marker SAT1 was employed in engineered strains.

**UPC2 and C. albicans-host cell interactions.** We attempted to correlate the attenuated virulence as a consequence of UPC2 hyperactivity using simple interaction assays (phagocytosis, survival) with host cells; however, the findings of these assays were not instructive. On the other hand, we repeatedly observed during prolonged phagocytosis assays that the germination process of C. albicans, which yields hyphal structures, was delayed in the C. albicans strain with UPC2-3 hyperactive alleles (Fig. 9). Whether or not this phenotype is sufficient to cause an attenuation of virulence is difficult to address. Together with still unknown factors, it might certainly contribute to decreased virulence. One prominent category of genes upregulated by UPC2 hyperactivity are the ERG genes and those involved in lipid metabolism (23, 68). It is possible that altered sterol and lipid metabolism could indirectly impact the dynamic of morphogenesis, a hypothesis that remains to be addressed in the future.

**Hyperactive allele combinations.** The combination of TFs with GOF alleles was tested here with MRR1 and UPC2 hyperactive alleles. This experiment was motivated by the fact that azole resistance mechanisms involving gene upregulation are often found in combination in clinical strains. By probing the combination of MRR1 (which has a neutral effect on virulence) with a UPC2 GOF mutation (which has a negative effect on virulence), it was of interest to address whether the decreased virulence phenotype due to UPC2 would be altered. Our data showed, rather, the opposite trend, i.e., an increase of attenuated virulence by this combination of GOF mutations. This suggests that the combination of resistance mechanisms may have a negative effect on C. albicans fitness. Given that the occurrence of UPC2 GOF mutations in clinical isolates acquiring azole resistance is not uncommon (68), it is likely that the negative effect of the UPC2 GOF could be corrected by other compensatory mutations to allow propagation under host conditions. Until now, no studies have attempted to identify such compensatory mutations. With the recent progress in whole-genome sequencing, comparison of the genomes of several clinical strains and those of their parents may help with their identification.

In conclusion, the present study highlighted that a GOF mutation in UPC2 has a negative impact on virulence that could be correlated to high fitness costs. The presence of other GOF mutations in TAC1 and MRR1 does not result in significant fitness costs.

**ACKNOWLEDGMENTS**

We are thankful to Françoise Ischer for technical assistance, to Luis Vale Silva for help and advice in cell culture experiments, and to Sara Martins Amorim Vaz for critical reading. We thank N. Dean (Stony Brook University, Stony Brook, NY) for providing plasmids.

This work was supported by a grant to D.S. from the Swiss National Research Foundation (31003A-127378).

**REFERENCES**

1. Calderone RA (ed). 2002. *Candida* and candidiasis. ASM Press, Washington, DC.
2. Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ, Chiller T. 2012. Species identification and antifungal susceptibility testing of Candida bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. J. Clin. Microbiol. 50:3435–3442. http://dx.doi.org/10.1128/JCM.01283-12.
3. Arendrup MC. 2010. Epidemiology of invasive candidiasis. Curr. Opin. Crit. Care 16:445–452. http://dx.doi.org/10.1097/MCC.0b013e3283e4d2d.
4. Sanglard D, Odds FC. 2002. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. 2:73–85. http://dx.doi.org/10.1016/S1473-3099(02)00181-0.
5. Kontoyiannis DP, Lewis RE. 2002. Antifungal drug resistance of pathogenic fungi. Lancet 359:1135–1144. http://dx.doi.org/10.1016/S0140-6736(02)08162-X.
6. Gulshan K, Meye-Rowley WS. 2007. Multidrug resistance in fungi. Eukaryot. Cell 6:1933–1942. http://dx.doi.org/10.1083/EC.00254-07.
7. Diekema D, Abarbeville S, Boyken L, Kroege J, Pfaffer M. 2012. The changing epidemiology of healthcare-associated candidemia over three
Dunkel N, Barker KS, Znaidi S, Schneider D, Dierolf F, Dunkel N, Aid M, Boucher G, Rodgers PD, Raymond M, Morschhauser J. 2010. Regulation of the efflux pump expression and drug resistance by the transcription factors Mrr1, Upc2, and Cap1 in Candida albicans. Antimicrob. Agents Chemother. 55:2212–2223. http://dx.doi.org/10.1128/AAC.00134-10.

Graybill JR, Montalbo E, Kirkpatrick WR, Luther MF, Revankar SG, Patterson TF. 1998. Fluconazole versus Candida albicans: a complex relationship. Antimicrob. Agents Chemother. 42:2938–2942.

Covene LE. 2008. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. Nat. Rev. Microbiol. 6:167–198. http://dx.doi.org/10.1038/nrmicro1835.

Covene LE, Kohn LM, Anderson JB. 2001. Divergence in fitness and evolution of drug resistance in experimental populations of Candida albicans. J. Bacteriol. 183:2971–2978. http://dx.doi.org/10.1128/JB.183.2797-2978.2001.

Selmecki AM, Dulmage K, Covene LE, Anderson JB, Morschhauser J. 2009. Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. PLoS Genet. 5:e1000705. http://dx.doi.org/10.1371/journal.pgen.1000705.

Huang M, McClellan M, Berman J, Kao KC. 2011. Evolutionary dynamics of Candida albicans during in vitro evolution. Eukaryot. Cell 10:1413–1421. http://dx.doi.org/10.1128/EC.00168-11.

Sanglard D, Ischer F, Calabrese D, Majerczyc PA, Bille J. 1999. The ATP-binding cassette transporter CgCDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob. Agents Chemother. 43:2753–2765.

Sanglard D, Ischer F, Bille J. 2001. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in Candida glabrata. Antimicrob. Agents Chemother. 45:1174–1183. http://dx.doi.org/10.1128/AAC.45.4.1174-1183.2001.

Torelli R, Posteraro B, Ferrari S, La Soleda M, Fadda G, Sanglard D, Sanguinetti M. 2008. The ATP-binding cassette transporter-encoding gene CgSNQ2 is contributing to the CgPDR1-dependent azole resistance of Candida glabrata. Mol. Microbiol. 68:186–201. http://dx.doi.org/10.1111/j.1365-2958.2008.06143.x.

Izumikawa K, Kaeya H, Tsai H, Grimerb G, Bennett J. 2003. Function of Candida glabrata ABC transporter gene, PdH1. Yeast 20:249–261. http://dx.doi.org/10.1002/yea.962.

Bennett JE, Izumikawa K, Marr KA. 2004. Mechanism of increased fluconazole resistance in Candida glabrata during prophylaxis. Antimicrob. Agents Chemother. 48:1773–1777. http://dx.doi.org/10.1128/AAC.48.5.1773-1777.2004.

Vermithys F-J, Edlin TD. 2004. Azole resistance in Candida glabrata: correlate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. Antimicrob. Agents Chemother. 48:3771–3781. http://dx.doi.org/10.1128/AAC.48.5.3771-3781.2004.

Tsai H, Krol A, Sarti K, Bennett J. 2006. Candida glabrata Pdr1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrob. Agents Chemother. 50:1384–1392. 2006.

Caudle KE, Barker KS, Wiederhold NP, Xu L, Homayouni R, Rogers PD. 2011. Genomewide expression profile analysis of the Candida glabrata Pdr1 regulon. Eukaryot. Cell 10:373–383. http://dx.doi.org/10.1128/EC.00073-10.

Paul S, Schmidt JA, Moye-Rowley WS. 2011. Regulation of the CgPdr1 transcription factor from the pathogen Candida glabrata. Eukaryot. Cell 10:187–197. http://dx.doi.org/10.1128/EC.00277-10.

Ferrari S, Ischer F, Calabrese D, Posteraro B, Sanguinetti M, Fadda G, Rohde B, Bausa C, Bader O, Sanglard D. 2009. Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence. PLoS Pathog. 5:e1000268. http://dx.doi.org/10.1371/journal.ppat.1000268.

Sasse C, Dunkel N, Schaefer T, Schneider D, Dierolf F, Oehlsen K, Morschhauser J. 2012. The stepwise acquisition of fluconazole resistance mechanisms causes a gradual loss of fitness in Candida albicans. Mol. Microbiol. 86:539–556. http://dx.doi.org/10.1111/j.1365-2958.2012.08210.x.

Hanahan D. 1985. Techniques for transformation of E. coli, p 109–135. In Glover D (ed), DNA cloning. A practical approach. IRL, Oxford, United Kingdom.
Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. 2006. Overexpression of the MDR1 gene is sufficient to confer increased resistance to toxic compounds in Candida albicans. Antimicrob. Agents Chemother. 50:1365–1371. http://dx.doi.org/10.1128/AAC.50.4.1365-1371.2006.

de Micheli M, Bille J, Schueler C, Sanglard D. 2002. A common drug-responsive element mediates the upregulation of the Candida albicans ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Mol. Microbiol. 43:1197–1214. http://dx.doi.org/10.1046/j.1365-2958.2002.02841.x.

Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. 2004. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eur. J. Cell Biol. 3:1639–1652. http://dx.doi.org/10.1128/EC.3.6.1639-1652.2004.

Marchetti O, Moreillon P, Glauser MP, Bille J, Sanglard D. 2000. Potent synergism of the combination of fluconazole and cyclopamiporine in Candida albicans. Antimicrob. Agents Chemother. 44:2373–2381. http://dx.doi.org/10.1128/AAC.44.9.2373-2381.2000.

Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J. 2003. Calcineurin A of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. 48:959–976. http://dx.doi.org/10.1046/j.1365-2958.2003.03495.x.

Fardeau V, Lelansais G, Oldfield A, Salin H, Lemoine S, Garcia M, Tanty V, Le Croom S, Jaqc C, Devaux F. 2007. The central role of PDR1 in the foundation of yeast drug resistance. J. Biol. Chem. 282:5063–5074. http://dx.doi.org/10.1074/jbc.M610197200.

Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gyge SE, Morschhauser J, Rogers PD. 2012. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation inazole-resistant clinical isolates of Candida albicans. Eukaryot. Cell 11:1289–1299. http://dx.doi.org/10.1128/EC.00015-12.

Valle-Silva L, Ischer F, Leibundgut-Landmann S, Sanglard D. 2013. Gain of function mutations in CgPDR1, a regulator of antifungal drug resistance. Mol. Microbiol. 2013. Gain-of-function mutations in CgPDR1, a regulator of antifungal drug resistance. Mol. Microbiol. 82:258–263. http://dx.doi.org/10.1093/mrc/dkg331.

McCallum N, Karauzum H, Getzmann R, Bischoff M, Majcherczyk P, Berger-Bachi B, Landmann R. 2006. In vivo survival of teicoplanin-resistant Staphylococcus aureus and fitness cost of teicoplanin resistance. Antimicrob. Agents Chemother. 50:2352–2360. http://dx.doi.org/10.1128/AAC.00073-06.

Rozen DE, McGee L, Levin BR, Klugman KP. 2007. Fitness costs of fluoroquinolone resistance in Streptococcus pneumoniae. Antimicrob. Agents Chemother. 51:412–416. http://dx.doi.org/10.1128/AAC.01161-06.

Andersson DI, Hughes D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat. Rev. Microbiol. 8:260–271. http://dx.doi.org/10.1038/nrmicro2319.

Andersson DI, Levin BR. 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. 2:489–493. http://dx.doi.org/10.1016/S1369-5274(99)00005-3.

Bjorkman J, Andersson DI. 2000. The cost of antibiotic resistance from a bacterial perspective. Drug Resist. Updat. 3:237–245. http://dx.doi.org/10.1016/S1369-5274(00)00005-3.

Sander P, Springer B, Prammanan T, Sturmfelds A, Kappler M, Pletschette M, Bottger EC. 2002. Fitness cost of chromosomal drug resistance-conferring mutations. Antimicrob. Agents Chemother. 46:1204–1211. http://dx.doi.org/10.1128/AAC.46.5.1204-1211.2002.

Valle-Silva LA, Coste AT, Ischer F, Parker JE, Kelly SL, Pinto E, Sanglard D. 2012. Azole resistance by loss of function of the sterol delta(5,(6)-desaturase gene (ERG3) in Candida albicans does not necessarily decrease virulence. Antimicrob. Agents Chemother. 56:1960–1968. http://dx.doi.org/10.1128/AAC.05720-11.

Ben-Ami R, Kontoyiannis DP. 2012. Resistance to echinocandins comes at a cost: the impact of FKS1 hotspot mutations on Candida albicans fitness and virulence. Virulence 3:95–97. http://dx.doi.org/10.4161/viru.3.1.18886.

Ben-Ami R, Garcia-Effron G, Lewis RE, Gamarra S, Leventakos K, Perlin DS, Kontoyiannis DP. 2011. Fitness and virulence costs of Candida albicans FKS1 hot spot mutations associated with echinocandin resistance. J. Infect. Dis. 204:626–635. http://dx.doi.org/10.1093/infdis/jir351.

Borgi E, Andreoni S, Cirasola D, Ricucci V, Scio R. Antifungal resistance does not necessarily affect Candida glabrata fitness. J. Chemother., in press.

Becker JM, Henry LK, Jiang W, Kolten Y. 1995. Reduced virulence of Candida albicans mutants affected in multidrug resistance. Infect. Immun. 63:4515–4518.

Schulz B, Weber K, Schmidt A, Borg-von Zepelin M, Ruhmkne M. 2011. Difference in virulence between fluconazole-susceptible and fluconazole-resistant Candida albicans in a mouse model. Mycoses 54:e522–e530. http://dx.doi.org/10.1111/j.1439-0507.2010.01970.x.

Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gyge SE, Morschhauser J, Rogers PD. 2012. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation inazole-resistant clinical isolates of Candida albicans. Eukaryot. Cell 11:1289–1299. http://dx.doi.org/10.1128/EC.00015-12.

Gillam AM, Tsay EY, Kirsch DR. 1984. Isolation of the Candida albicans gene for orotidine-5’-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol. Gen. Genet. 198:179–182. http://dx.doi.org/10.1007/BF00328721.