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Mutations in the multidrug resistance regulator *MRR1*, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *Candida albicans* strains

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Summary

Overexpression of the *MDR1* gene, encoding a multidrug efflux pump of the major facilitator superfamily, is a major cause of resistance to the widely used antifungal agent fluconazole and other toxic substances in the fungal pathogen *Candida albicans*. We found that all tested clinical and *in vitro* generated *C. albicans* strains that had become fluconazole-resistant by constitutive *MDR1* upregulation contained mutations in the *MRR1* gene, which encodes a transcription factor that controls *MDR1* expression. Introduction of the mutated alleles into a drug-susceptible *C. albicans* strain resulted in activation of the *MDR1* promoter and multidrug resistance, confirming that the amino acid substitutions in Mrr1p were gain-of-function mutations that rendered the transcription factor constitutively active. The majority of the *MDR1* overexpressing strains had become homozygous for the mutated *MRR1* alleles, demonstrating that the increased resistance level conferred by two gain-of-function alleles provides sufficient advantage to select for the loss of heterozygosity in the presence of fluconazole both *in vitro* and within the human host during therapy. Loss of heterozygosity usually occurred by mitotic recombination between the two chromosome 3 homologues on which *MRR1* is located, but evidence for complete loss of one chromosome and duplication of the chromosome containing the mutated *MRR1* allele was also obtained in two *in vitro* generated fluconazole-resistant strains. These results demonstrate that gain-of-function mutations in *MRR1* are the major, if not the sole, mechanism of *MDR1* overexpression in fluconazole-resistant strains and that this transcription factor plays a central role in the development of drug resistance in *C. albicans*.

Keywords

Candida albicans; efflux pump; fluconazole; gain-of-function mutation; multidrug resistance; transcription factor

Introduction

Infections by the opportunistic pathogenic yeast *Candida albicans* are frequently treated with the antimycotic agent fluconazole, which inhibits the biosynthesis of ergosterol, the major sterol in the fungal cell membrane. *C. albicans* can develop resistance to fluconazole,

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especially during long-term treatment of oropharyngeal candidiasis, a condition that affects many individuals infected with the human immunodeficiency virus (HIV) and AIDS patients (White *et al.*, 1998). Fluconazole resistance can be caused by different mechanisms, including alterations in the sterol biosynthesis pathway, increased expression of the *ERG11* gene encoding the drug target enzyme, sterol 14α-demethylase (Erg11p), mutations in Erg11p that result in reduced affinity to fluconazole, and overexpression of multidrug efflux pumps that actively transport fluconazole and many other, structurally unrelated toxic compounds out of the cell. The analysis of serial isolates from individual patients has shown that several of these mechanisms are often combined to result in a stepwise development of clinically relevant fluconazole resistance (Morschhäuser, 2002; White *et al.*, 1998).

C. albicans possesses two types of efflux pumps whose overexpression is responsible for fluconazole resistance in many clinical isolates, the highly homologous ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p and the major facilitator Mdr1p (Sanglard *et al.*, 1995; Sanglard *et al.*, 1996, 1997; Wirsching *et al.*, 2000). The molecular basis for the constitutive upregulation of the genes encoding these efflux pumps has recently been elucidated. Expression of *CDR1* and *CDR2* is controlled by the zinc cluster transcription factor Tac1p (transcriptional activator of *CDR* genes), which binds to a drug response element that is present in the promoter region of both of these genes and induces their expression in response to steroids and certain toxic chemicals (Coste *et al.*, 2004; de Micheli *et al.*, 2002). Mutations in Tac1p result in constitutive activity of this transcription factor and, consequently, *CDR1* and *CDR2* overexpression (Coste *et al.*, 2006; Coste *et al.*, 2007; Znaidi *et al.*, 2007). Although other transcription factors have also been reported to regulate *CDR1* expression (Chen *et al.*, 2004; Gaur *et al.*, 2004), so far only mutations in Tac1p have been found as the cause of drug resistance mediated by this efflux pump in clinical *C. albicans* isolates.

MDR1 expression is also controlled by a transcription factor of the zinc cluster family, termed Mrr1p (multidrug resistance regulator) (Morschhäuser *et al.*, 2007). *MRR1* was identified as one of the genes that were commonly upregulated in three different *MDR1* overexpressing, fluconazole-resistant *C. albicans* isolates as compared with genetically related, fluconazole-susceptible isolates from the same patients. *MRR1* inactivation in two fluconazole-resistant isolates resulted in the loss of *MDR1* expression and increased susceptibility to fluconazole and other metabolic inhibitors that are supposed to be substrates of the Mdr1p efflux pump, like cerulenin and brefeldin A. Deletion of *MRR1* from the drug-susceptible *C. albicans* wild-type model strain SC5314 also abolished *MDR1* expression in the presence of inducing chemicals, like benomyl and hydrogen peroxide, demonstrating that Mrr1p mediates both inducible *MDR1* expression in drug-susceptible strains and constitutive *MDR1* overexpression in drug-resistant strains. The two *MDR1* overexpressing clinical isolates that were studied in detail had become homozygous for mutated *MRR1* alleles containing a P683S or a G997V mutation. Introduction of these mutated alleles into a drug-susceptible strain resulted in constitutive *MDR1* overexpression and multidrug resistance. These results showed that, similar to *CDR1/CDR2* upregulation by Tac1p, *MDR1* overexpression is also caused by gain-of-function mutations in its regulator, Mrr1p. However, additional transcription factors have been implicated in the control of *MDR1* expression, namely, Cap1p and Mcm1p, both of which have binding sites in the *MDR1* promoter (Harry *et al.*, 2005; Hiller *et al.*, 2006; Riggle and Kumamoto, 2006; Rognon *et al.*, 2006). Therefore, it seemed possible that *MDR1* overexpression in drug-resistant *C. albicans* isolates might also be due to mutations in regulatory factors other than *MRR1*. To investigate how frequently *MDR1* overexpression is caused by alterations in *MRR1*, we analyzed a panel of well-documented matched pairs of drug-susceptible and drug-resistant *C. albicans* isolates in which fluconazole resistance was associated with *MDR1* overexpression for the presence of mutations and genomic rearrangements involving *MRR1*.

Results

MDR1 overexpressing clinical *C. albicans* isolates contain mutations in the transcription factor *MRR1*

To investigate whether *MDR1* overexpression in other fluconazole-resistant, clinical *C. albicans* isolates is also caused by mutations in the transcription factor Mrr1p, we cloned and sequenced the *MRR1* alleles from seven additional resistant isolates and genetically related susceptible isolates from the same patients which had been described in the literature. One of these matched pairs of isolates (B3/B4) came from our own group (Franz *et al.*, 1999), three pairs (1442/2271, 1490/1587 and 5044/5052) were provided by Thomas Patterson (Lopez-Ribot *et al.*, 1998; Perea *et al.*, 2001), two pairs (DSY291/DSY292 and DSY2285/DSY2286) by Dominique Sanglard (Calabrese *et al.*, 2000; Sanglard *et al.*, 1995), and one isolate pair (5833/6692) was obtained from Martine Raymond (Saidane *et al.*, 2006) (see Table S1). The nucleotide sequences of all *MRR1* alleles found in these isolates have been deposited in GenBank (see Experimental procedures for accession numbers) and amino acid differences in the encoded proteins are listed in Table 1.

Two polymorphic *MRR1* alleles were found in the susceptible isolate B3. Allele 1 was identical to the *MRR1* allele of *C. albicans* strain SC5314 (orf19.7372), which was used for genome sequencing. Allele 2 differed from it at 41 nucleotide positions, five of which resulted in amino acid exchanges. In addition, allele 2 encoded five tandem repeats of the amino acids NPQS (positions 165–168 in Mrr1p). Only one *MRR1* allele was obtained from the resistant isolate B4, which was identical to allele 2 of the matched isolate B3 except for a G2633A substitution that resulted in a G878E exchange in the encoded protein.

The susceptible isolate 1442 also contained two polymorphic *MRR1* alleles. Allele 1 corresponded to *MRR1* of strain SC5314 but contained ten silent nucleotide exchanges. Allele 2 differed from allele 1 by 31 nucleotide substitutions, five of which resulted in amino acid exchanges, and also contained five tandem repeats of the NPQS sequence. The matched resistant isolate 2271 contained only one *MRR1* allele, which was identical to allele 1 of isolate 1442 except for a single A1049T substitution that resulted in a Q350L exhange in Mrr1p.

Two polymorphic *MRR1* alleles were found in the susceptible isolate 1490, which differed from one another by 27 silent nucleotide exchanges. The matched resistant isolate 1587 contained only allele 2 with an additional A2407G exchange that caused an N803D mutation in the encoded protein.

The *MRR1* alleles obtained from the susceptible isolate 5044 were identical and differed from *MRR1* of strain SC5314 by 26 nucleotide exchanges, three of which resulted in amino acid exchanges. The matched resistant isolate 5052 contained the same allele, but with a C2048A mutation in both copies that resulted in a P683H exchange in Mrr1p.

The susceptible isolate DSY291 contained two polymorphic *MRR1* alleles, one of which was identical to *MRR1* from SC5314. Allele 2 differed from allele 1 at 28 positions, which resulted in four amino acid exchanges, and encoded two tandem repeats of the NPQS sequence. The matched resistant isolate DSY292 contained the same two alleles, however, allele 1 exhibited an additional C2048A exchange that resulted in a P683H mutation in the encoded protein. An identical mutation had been found in isolate 5052, but in an otherwise different *MRR1* allele (see above).

The *MRR1* alleles in the susceptible isolate DSY2285 were identical and differed from *MRR1* of strain SC5314 only by a G3058C exchange that resulted in an E1020Q

substitution. In the matched resistant isolate DSY2286 both alleles contained an additional C2687T mutation that caused a T896I exchange in Mrr1p.

The suceptible isolate 5883 contained two polymorphic *MRR1* alleles that differed from one another at 14 positions, with two of the polymorphisms resulting in amino acid differences. Both alleles encoded five tandem repeats of the NPQS sequence. These two alleles were also found in the matched resistant isolate 6692, however, each of them exhibited a single nucleotide substitution. Allele 1 contained a C1079T exchange that caused a T360I mutation and allele 2 had an A1005C substitution that resulted in a K335N mutation in Mrr1p.

In summary, all analyzed *MDR1* overexpressing clinical *C. albicans* isolates contained a mutation in the transcription factor *MRR1*. Like the two previously described isolates F5 and G5 (Morschhäuser *et al.*, 2007), five of the seven resistant isolates analyzed in the present study (B4, 2271, 1587, 5052, DSY2286) had become homozygous for the mutated *MRR1* allele. One isolate (6692) had aquired independent mutations in both *MRR1* alleles and one resistant isolate (DSY292) had retained a wild-type allele in addition to the mutated allele.

In vitro *generated,* MDR1 overexpressing *C. albicans* strains have aquired *MRR1* mutations

Riggle and Kumamoto recently isolated five fluconazole-resistant derivatives of *C. albicans* laboratory strains, which themselves were generated from the wild-type strain SC5314, that overexpressed *MDR1* after passage in medium containing fluconazole (Riggle and Kumamoto, 2006). We investigated whether *MDR1* upregulation in these *in vitro* generated strains was also caused by mutations in *MRR1*. Sequencing of the *MRR1* alleles of these strains showed that all of them indeed had aquired single point mutations in this transcription factor, resulting in the amino acid substitutions T381I in strain CAPR510, A880E in CAPR513, W893R in CAPR514, R873T in CAPR515, and L998F in CAPR517. Strains CAPR510, CAPR514, CAPR515, and CAPR517 had become homozygous for the mutated alleles while strain CAPR513 was found to be heterozygous and also retained one original *MRR1* allele of the parental strain SC5314.

Table 1 summarizes all polymorphisms that were found in the *MRR1* alleles of the investigated strains and which resulted in amino acid differences in Mrr1p. The fact that all *MDR1* overexpressing, fluconazole-resistant strains contained *MRR1* alleles that differed from the corresponding alleles in the matched susceptible isolates (for clinical isolates) or the susceptible parental strain (for *in vitro* generated strains) by a single nucleotide exchange strongly suggested that the resulting amino acid substitutions in Mrr1p rendered the transcription factor constitutively active and were responsible for *MDR1* overexpression in these strains.

MRR1 *gain-of-function mutations* cause constitutive *MDR1* overexpression and multidrug resistance

To verify that the *MRR1* mutations found in the fluconazole-resistant strains were indeed gain-of-function mutations causing *MDR1* overexpression, we introduced each of the mutated alleles into a derivative of the drug-susceptible *C. albicans* strain SC5314 from which the endogenous *MRR1* alleles had been deleted and which contained a P_{MDR1}-*GFP* reporter gene fusion that could be used to monitor *MDR1* promoter activity (see Fig. 1). The amino acid sequences of Mrr1p encoded by the mutated *MRR1* alleles of strains CAPR510 (*MRR1*^{T381I}), CAPR513 (*MRR1*^{A880E}), CAPR514 (*MRR1*^{W893R}), CAPR515 (*MRR1*^{R873T}), CAPR517 (*MRR1*^{L998F}), 2271 (*MRR1*^{Q350L}), 1587 (*MRR1*^{N803D}), and DSY292 (*MRR1*^{P683H}) were identical to that of Mrr1p of strain SC5314, except for the putative gain-of-function mutations, and the corresponding transformants could therefore directly be compared with control strains into which the wild-type allele had been reintroduced in the

same way. As the mutated *MRR1* alleles from isolates B4, DSY2286, and 6692 contained additional amino acid differences not found in Mrr1p of strain SC5314, we incorparated only the relevant part of these *MRR1* alleles into the expression cassette to generate the *MRR1*$^{G878E,\ 1020Q}$, *MRR1*$^{T896I,\ E1020Q}$, *MRR1*$^{S171P,\ L248V,\ V341E,\ T360I}$, and *MRR1*$^{S171P,\ L248V,\ K335N,\ V341E}$ alleles (see Experimental procedures). Expression cassettes containing the corresponding parts of the nonmutated alleles (*MRR1*^{E1020Q} and *MRR1*$^{S171P,\ L248V,\ V341E}$) served as controls in these cases. The mutated *MRR1* allele from isolate 5052 was not tested because the identical P683H mutation was present in the *MRR1*^{P683H} allele from isolate DSY292. Two independent transformants were kept in each case and used to quantify *MDR1* promoter activity by FACS analysis. As can be seen in Fig. 2, expression of all mutated *MRR1* alleles resulted in constitutive activation of the *MDR1* promoter, while no *MDR1* promoter activity was observed in the control transformants carrying the nonmutated *MRR1* alleles. These results demonstrated that the *MRR1* mutations that had occurred in the fluconazole-resistant strains resulted in constitutive activity of the transcription factor and caused *MDR1* overexpression.

In a complementary approach to investigate the effect of the *MRR1* mutations on drug resistance, the same alleles described above were also introduced into *mrr1*Δ mutants of the wild type strain SC5314 and the susceptibility of the transformants to fluconazole and other metabolic inhibitors to which *MDR1* overexpression confers resistance was tested (see Fig. 3). As previously reported (Morschhäuser *et al.*, 2007), reintroduction of the original wild-type allele (*MRR1*) did not confer increased resistance to cerulenin, brefeldin A, or fluconazole, and the same result was obtained with the two control alleles *MRR1*^{E1020Q} and *MRR1*$^{S171P,\ L248V,\ V341E}$ which contained amino acid exchanges that were commonly found in susceptible isolates. In contrast, all gain-of-function alleles conferred increased resistance to cerulenin and brefeldin A, two compounds that are good substrates of the Mdr1p efflux pump (Hiller *et al.*, 2006). Expression of a single copy of most of the alleles was also sufficient to increase fluconazole resistance of the strains, except for the *MRR1*^{W893R} and *MRR1*^{R873} alleles, which also had the weakest effect on *MDR1* promoter activity (see Fig. 2) and probably have to be present in two copies to result in significantly increased fluconazole resistance, as in strains CAPR514 and CAPR515 in which they were found. These results confirmed that all the mutated *MRR1* alleles confer multidrug resistance.

Contribution of mutated MRR1 *alleles* to drug resistance in heterozygous and homozygous C. albicans strains

As described above, in addition to the aquisition of point mutations in *MRR1*, most of the fluconazole-resistant *C. albicans* strains had also undergone genomic changes and become homozygous for the mutated *MRR1* allele. To assess the contribution of the *MRR1* alleles to drug resistance in clinical isolates, we deleted one or both *MRR1* alleles in the two heterozygous resistant isolates DSY292 and 6692 as well as in three additional isolates that had become homozygous for a mutated *MRR1* allele (isolates B4, 2271, DSY2286). Two independent series of heterozygous and homozygous *mrr1*Δ mutants were generated from each parental strain using the *SAT1*-flipping strategy (Reuβ *et al.*, 2004). In all cases, two rounds of gene deletion were sufficient to obtain homozygous *mrr1*Δ mutants, demonstrating that *MRR1* gene amplification was not involved in resistance development in these strains. The susceptibilities of the mutants to fluconazole, cerulenin, and brefeldin A were then compared with those of the respective resistant parental isolates and matched susceptible isolates. As can be seen in Fig. 4, the increased resistance to these inhibitors observed in the parental resistant isolates was completely or almost completely lost in all homozygous *mrr1*Δ mutants (*i.e.*, the MIC decreased to the level of the matched susceptible isolate or was only one dilution step higher), except for the fluconazole resistance of isolates DSY292 and 6692 (see below and discussion). Deletion of one *MRR1* copy in the strains

that had become homozygous for a mutated *MRR1* allele (B4, 2271, DSY2286) resulted in an intermediate phenotype, demonstrating that both alleles contributed to the resistance of the clinical isolates. The same result was seen in the heterozygous mutants generated from the clinical isolate 6692, which contained two different mutated *MRR1* alleles. Sequencing showed that both randomly selected heterozygous deletion mutants had retained the *MRR1* allele with the K335N mutation. The partial loss of drug resistance in the heterozygous mutants demonstrated that both mutated *MRR1* alleles contributed to the resistant phenotype in this clinical isolate. In contrast, the two heterozygous deletion mutants of isolate DSY292 behaved differently. One of the heterozygous *MRR1*/*mrr1*Δ mutants (strain A) displayed the same resistance as the parental isolate (resistance to cerulenin was even slightly increased) and sequencing showed that in this strain the nonmutated *MRR1* allele was deleted and the allele with the P683H mutation was retained. The other heterozygous *MRR1*/*mrr1*Δ mutant (strain B) exhibited the same sensitivity to cerulenin and brefeldin A as the homozygous *mrr1*Δ mutants and sequencing demonstrated that in this strain the *MRR1*^{P683H} allele was deleted and the nonmutated allele was retained. This result showed that only the *MRR1* allele with the P683H gain-of-function mutation contributed to the increased drug resistance of isolate DSY292 as compared with the matched susceptible isolate DSY291. Interestingly, while the resistance to cerulenin and brefeldin A was strongly reduced in the *mrr1*Δ mutants of DSY292, fluconazole resistance of this strain was not or only slightly affected by the *MRR1* deletion. This is probably due to the fact that DSY292 contains mutations in *ERG11* that confer fluconazole resistance (see discussion). In some cases, we observed minor differences in the drug susceptibilities of the two independently generated *mrr1*Δ mutants of a particular strain, indicating that unspecific alterations which slightly affected drug resistance had also occurred during the construction of the mutants. However, in all cases the importance of *MRR1* in drug resistance of the clinical isolates was clearly evident.

A comparison of the effects of *MRR1* deletion in the resistant clinical isolates and expression of the corresponding *MRR1* allele in the highly susceptible strain SC5314 showed that the contribution of a specific *MRR1* gain-of-function allele to drug resistance was largely comparable in the different strain backgrounds. For example, expression of a single copy of the *MRR1*^{G878E} allele from isolate B4 in strain SC5314 resulted in 16-fold increased resistance to cerulenin, 8-fold increased resistance to brefeldin A, and 4-fold increased resistance to fluconazole (Fig. 3), while deletion of the remaining copy of the same allele from the heterozygous mutants of isolate B4 decreased resistance to these drugs in the homozygous mutants by 16- to 32-fold, 16-fold, and 4-fold, respectively (Fig. 4, top panels). Similarly, expression of the *MRR1*^{K335N} allele from isolate 6692 in strain SC5314 increased the resistance of this strain to cerulenin, brefeldin A, and fluconazole by 16-fold, 16-fold, and 4-fold, respectively (Fig. 3), while deletion of the same allele from the heterozygous mutants of isolate 6692 decreased resistance to these drugs by 16-fold, 8-fold, and 4-fold, respectively (Fig. 4, bottom panels). In the few cases where major differences were observed, these are presumably related to the different genetic backgrounds. For example, the contribution of the *MRR1*^{P683H} allele to fluconazole resistance in isolate DSY292 is probably masked by the *ERG11* mutations in this isolate (see above and discussion), and the strong effect of deletion of the *MRR1*^{T896I} allele on fluconazole resistance in isolate DSY2286 could be explained by strain-specific differences in other Mrr1p target genes that contribute to fluconazole resistance (Morschhäuser *et al.*, 2007).

Loss of heterozygosity in *C. albicans* strains containing *MRR1* gain-of-function mutations is caused by mitotic recombination and chromosome loss

Loss of *MRR1* heterozygosity in the resistant strains could have occurred by mitotic recombination between the two homologues of chromosome 3, on which *MRR1* is located, or by loss of one chromosome 3 homologue and duplication of the other copy containing the

mutated *MRR1* allele. Both types of events have been found as a mechanism of loss of heterozygosity for genes located on chromosome 5 (Coste *et al.*, 2006; Coste *et al.*, 2007; Wu *et al.*, 2005; Wu *et al.*, 2007). Loss of *MRR1* heterozygosity by mitotic recombination must involve at least one cross over event between the centromere and the *MRR1* locus on the right arm of chromosome 3. In the absence of additional recombination events this would result in homozygosity of the region between the cross over site and the telomere, but polymorphisms on the other chromosome arm would be retained. In contrast, loss of one chromosome 3 homologue would result in the loss of all polymorphisms on chromosome 3. Several allelic polymorphisms in genes located on chromosome 3 have been reported for strain SC5314 (Jones *et al.*, 2004). To distinguish between chromosome loss and mitotic recombination, we first analyzed the four *in vitro* generated derivatives of strain SC5314 that had become homozygous for a mutated *MRR1* allele for the presence of polymorphisms in *CAP1*, a gene that is located on the left arm of chromosome 3 (see Fig. 5). The two *CAP1* alleles in strain SC5314 can easily be distinguished by restriction site polymorphisms, one allele containing an *Eco*RI site and the other allele an *Sph*I site. As illustrated in Fig. 5A, strains CAPR515 and CAPR517 retained these polymorphisms, thus excluding chromosome loss as the cause of *MRR1* homozygosity in these strains. In contrast, strains CAPR510 and CAPR514 contained only the *CAP1* allele with the *Sph*I site but had lost the allele with the *Eco*RI site, pointing to chromosome loss or multiple recombination events on both arms of chromosome 3 in these strains. We, therefore, looked for the presence of additional polymorphic markers on chromosome 3. Strains CAPR515 and CAPR517 contained the known polymorphisms of strain SC5314 in *MEP3* and *SAP9* on the left arm and *AAP1* on the right arm of chromosome 3, demonstrating that the recombination event that resulted in homozygosity for the mutated *MRR1* allele occurred between *AAP1* and *MRR1*. Strains CAPR510 and CAPR514 had lost heterozygosity for all these markers. As *SAP9* and *AAP1* are located on both sides of and very close to the centromere on chromosome 3, we consider it unlikely that homozygosity was caused by two independent recombination events in the small regions between the centromere and *SAP9* on the left arm and between the centromere and *AAP1* on the right arm. These two strains most probably became homozygous for the mutated *MRR1* allele by loss of the chromosome containing the wild-type allele.

To investigate by which mechanism the fluconazole-resistant, clinical *C. albicans* isolates F5 and G5 described in a previous study (Morschhäuser *et al.*, 2007) and isolates B4, 2271, 1587, DSY2286, and 5052 analyzed in the present study had become homozygous for their mutated *MRR1* alleles, we first tested whether the matched susceptible isolates contained any of the heterozygous markers on chromosome 3 described above. Isolate 5044 was homozygous for all tested markers, therefore, the mechanism of loss of *MRR1* heterozygosity in the matched resistant isolate 5052 could not be investigated. The other susceptible isolates contained one or more polymorphic alleles on chromosome 3, as illustrated in Fig. 5B. In all these cases, the matched resistant isolates retained the polymorphisms, which shows that the loss of *MRR1* heterozygosity was caused by mitotic recombination and not by chromosome loss. Altogether, these results demonstrate that mitotic recombination is the major cause of loss of heterozygosity for *MRR1* on chromosome 3, but chromosome loss can also occur, at least during the *in vitro* selection of *MDR1* overexpressing, fluconazole-resistant strains.

Identification of a clinical isolate that represents the missing link from drug susceptibility to homozygosity for a mutated *MRR1* allele and drug resistance

Most of the *MDR1* overexpressing, drug-resistant *C. albicans* isolates had become homozygous for mutated *MRR1* alleles, while the matched susceptible isolates from the same patients contained two nonmutated alleles. One must assume that a gain-of-function mutation first appeared in one allele and that the advantage gained by becoming

homozygous for the mutated allele is sufficient to rapidly select for such an event in the presence of antimycotic therapy. Isolates F2 and F5 are from a series in which the first two described isolates (F1 and F2) did not detectably express *MDR1* whereas the last two described isolates (F4 and F5) displayed high *MDR1* transcript levels. This series also included an isolate, F3, that exhibited intermediate *MDR1* expression levels and intermediate fluconazole resistance (Franz *et al.*, 1998). We speculated that this isolate might represent the missing link between the susceptible isolate F2 with two polymorphic wild-type *MRR1* alleles and the resistant isolate F5 that had become homozygous for the *MRR1*^{P683S} allele and strongly expressed *MDR1*. We therefore amplified the *MRR1* alleles from isolate F3 and sequenced the relevant part of the PCR product. We found that F3 indeed still possessed both *MRR1* alleles found in the suscpetible isolate F2, but contained the *MRR1*^{P683S} mutation in allele 1, while allele 2 remained unchanged. This finding in a clinical isolate confirmed that a gain-of-function mutation in one *MRR1* allele results only in intermediate resistance levels and explains why most *MDR1* overexpressing, fluconazole-resistant isolates obtained from patients have become homozygous for mutated *MRR1* alleles.

Discussion

The results of this study demonstrate that constitutive *MDR1* upregulation in fluconazole-resistant *C. albicans* strains is tightly linked to mutations in *MRR1*, a transcription factor that controls *MDR1* expression. All nine clinical *C. albicans* isolates that have been investigated to date contain gain-of-function mutations in *MRR1* that render the transcription factor constitutively active and cause upregulation of the *MDR1* efflux pump and multidrug resistance. In addition, all five *in vitro* generated, *MDR1* overexpressing strains that were available also contain mutated *MRR1* alleles. Therefore, our results strongly suggest that mutations in *MRR1* are the main, if not the sole cause of *MDR1*-mediated multidrug resistance in *C. albicans*.

It is presently unknown how Mrr1p is activated to induce expression of its target genes in response to environmental signals and how the gain-of-function mutations affect the activity of the transcription factor. Fig. 6 shows the location of all activating mutations that have been identified so far in Mrr1p. There are four major mutational hot spots which are located in different regions of the protein. Four mutations (K335N, Q350L, T360I, T381I) lie between amino acid positions 335 and 381, five mutations (R873T, G878E, A880E, W893R, T896I) cluster in a small stretch between amino acids 873 and 896, and two mutations are located at positions 997 and 998 (G997V and L998F). In addition, the proline at position 683 is mutated in three different *MDR1* overexpressing isolates (two P683H substitutions and one P683S exchange). As suggested previously (Morschhäuser *et al.*, 2007), it is likely that these mutations relieve the transcription factor from inhibition by an autoregulatory domain or another repressing factor and render it constitutively active. Apart from the predicted DNA binding domain in the N-terminal part of the protein, which is typical for the zinc cluster transcription factor family, other functional domains of Mrr1p remain to be experimentally defined. It seems reasonable to assume that such a large protein of more than 1100 amino acid residues is subject to multiple regulatory inputs that may affect its interaction with other proteins, binding to the promoters of its target genes, and/or transcriptional activation. The knowledge gained from our present study about activating mutations that have occurred in different regions of Mrr1p in drug-resistant *C. albicans* strains should help to understand how the activity of the transcription factor is regulated once its functional domains and interacting proteins as well as *in vivo* DNA binding sites in the *C. albicans* genome have been identified, which are major aims for future investigations.

We have previously demonstrated that *MRR1* alleles containing gain-of-function mutations can activate *MDR1* expression and confer drug resistance in the presence of a wild-type *MRR1* allele, although a slight negative effect of a nonmutated allele on the activity of gain-of-function alleles was also observed (Morschhäuser *et al.*, 2007). Similarly, deletion of the wild-type *MRR1* allele in strain DSY292 also resulted in a detectable additional increase in cerulenin resistance of the heterozygous mutant that retained only the mutated *MRR1*^{P683H} allele (Fig. 4). The ability of *MRR1* gain-of-function alleles to activate *MDR1* expression in the presence of a wild-type *MRR1* allele is in contrast to the activation of *CDR1* and *CDR2* by the transcription factor Tac1p, where *TAC1* gain-of-function alleles conferred hyperactive phenotypes only when homozygous (Coste *et al.*, 2006). Nevertheless, the presence of two instead of only one mutated *MRR1* allele also results in increased drug resistance. In two clinical *C. albicans* isolates that were examined in a previous study (Morschhäuser *et al.*, 2007) as well as in four additional isolates investigated in our present work, the deletion of one of the two mutated *MRR1* copies resulted in a partial loss of drug resistance. These findings explain why the majority of *MDR1* overexpressing, fluconazole-resistant strains had become homozygous for a mutated *MRR1* allele. Apparently, the increased fluconazole resistance provides sufficient advantage in the presence of the drug both *in vitro* and *in vivo* during therapy to select for the loss of heterozygosity once a gain-of-function mutation has occurred in one of the *MRR1* alleles. Further support for this scenario came from the analysis of the serial isolates from patient F, where we identified an intermediate isolate (F3) that contained the P683S mutation in only one of the two *MRR1* alleles and displayed intermediate *MDR1* expression and fluconazole resistance. Isolate F5, which was obtained from a later infection episode, had become homozygous for the mutated *MRR1* allele and exhibited higher *MDR1* transcript levels and further increased fluconazole resistance (Franz *et al.*, 1998, and this study).

Only one of the clinical isolates, DSY292, had retained a wild-type *MRR1* allele that did not contribute to the drug resistance of this strain. Deletion of the mutated *MRR1*^{P683H} allele largely abolished the increased resistance of DSY292 to cerulenin and brefeldin A, which in other drug-resistant strains has been shown to be mediated mostly or exclusively by *MDR1* overexpression (Wirsching *et al.*, 2001). In contrast, fluconazole resistance of strain DSY292 was not or only slightly affected by deletion of *MRR1*. It was previously shown that DSY292 has aquired a Y132H mutation in one *ERG11* allele, in addition to the G464S and R467K mutations that were already present in both *ERG11* alleles of the matched isolate DSY291 (Sanglard *et al.*, 1998). The presence of the Y132H mutation resulted in a more than 8-fold increased fluconazole resistance of cells expressing this *ERG11* allele (Sanglard *et al.*, 1998), explaining why DSY292 retained a high level of resistance to fluconazole after deletion of *MRR1*. We speculate that the P683H mutation may have occurred in one *MRR1* allele and been selected before the appearance of the Y132H mutation in *ERG11*. The high fluconazole resistance conferred by the *ERG11* mutations then probably diminished the contribution of the *MRR1* mutation and prevented selection for *MRR1* homozygosity in DSY292. Mutated *MRR1* alleles also contribute only partially to the overall fluconazole resistance of other strains that exhibit additional resistance mechanisms, like isolate G5, which contains a G464S mutation in both *ERG11* alleles (Franz *et al.*, 1998; Morschhäuser *et al.*, 2007), or isolate 6692 (see Fig. 4), which exhibits increased resistance to other azoles that are not Mdr1p substrates, although the basis of this resistance has not been identified (Saidane *et al.*, 2006).

Interestingly, we also found one *MDR1* overexpressing clinical isolate that had aquired independent gain-of-function mutations in either of its *MRR1* alleles, both of which contributed to the drug resistance of this isolate. A similar finding has been reported by Coste *et al*., who identified two independent hyperactive *TAC1* alleles in a fluconazole-resistant isolate that overexpressed *CDR1* and *CDR2* (Coste *et al.*, 2007). However, most of

the *MDR1* overexpressing strains had become homozygous for a mutated *MRR1* allele (11 out of 14 strains analysed so far), presumably because genomic alterations that result in homozygosity occur more frequently than the aquisition of an independent mutation in the second allele. A more detailed analysis of these strains showed that loss of heterozygosity was usually caused by mitotic recombination, although duplication of the chromosome containing the mutated *MRR1* allele and loss of the other copy of chromosome 3 seems to have occurred in two of the *in vitro* selected fluconazole-resistant strains. These results are in line with recent findings by Wu *et al.*, who reported that loss of one copy of chromosome 5, followed by duplication of the remaining homolog, is the most common mechanism generating homozygosity for the mating type locus (*MTL*) in strains cultured in the laboratory, whereas *MTL* homozygosity in natural *C. albicans* strains usually occurs by mitotic recombination, which allows the retention of many polymorphic genes and results in a smaller decrease in competitiveness than the complete loss of heterozygosity on that chromosome (Wu *et al.*, 2005; Wu *et al.*, 2007). While we can not exclude the possibility that loss of heterozygosity may have occurred during storage and propagation of the strains in the laboratory, the fact that most susceptible isolates were heterozygous, while most resistant isolates were homozygous for mutated *MRR1* alleles indicates that loss of heterozygosity has in most, if not all cases been selected for during fluconazole therapy of the patients.

Genomic alterations involving chromosome 5, which result in aneuploidies that are sometimes combined with an increased copy number of mutated *TAC1* and *ERG11* alleles have been found in fluconazole-resistant *C. albicans* strains that overexpress *CDR1* and *CDR2* (Coste *et al.*, 2007). Trisomy for chromosome 3, on which *MRR1* is located, has also been reported in *in vitro* generated, fluconazole-resistant *C. albicans* strains, however, no *MDR1* overexpression was seen in these strains (Perepnikhatka *et al.*, 1999). Our analyses indicate that *MDR1* overexpression is not usually associated with an increase in the copy number of *MRR1*. All seven *MDR1* overexpressing clinical *C. albicans* isolates in which the *MRR1* alleles were inactivated by targeted gene deletion in this and a previous study (Morschhäuser *et al.*, 2007) contained only two copies of *MRR1*. Additional strains, which had become homozygous for mutated *MRR1* alleles but retained heterozygosity for other genes on chromosome 3 (strains 1587, CAPR515, and CAPR517), also had not amplified one of the chromosome 3 homologues, as direct sequencing of the PCR products showed equal signal intensities at heterozygous positions (data not shown). Although we have shown that the presence of two instead of only one mutated *MRR1* allele results in increased drug resistance, it seems that the selection pressure was not strong enough to result in a further stable increase in the *MRR1* copy number in these strains.

In summary, this study demonstrates that gain-of-function mutations in the transcription factor Mrr1p, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *C. albicans* strains. This underscores the important role of Mrr1p in the development of multidrug resistance in *C. albicans* and illustrates again how the genomic flexibility of this fungal pathogen contributes to its ability to adapt to the selection pressure caused by the presence of fluconazole both *in vitro* and in the human host during antimycotic therapy.

Experimental procedures

Strains and growth conditions

C. albicans strains used in this study are listed in Table S1. All strains were stored as frozen stocks with 15% glycerol at −80°C and subcultured on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar per liter) at 30°C. For routine growth of the strains YPD liquid medium was used. Selection of nourseothricin-resistant (Nou^R) transformants

was performed on YPD agar plates containing 200 μg ml^{-1} nourseothricin (Werner Bioagents, Jena, Germany). To obtain nourseothricin-sensitive (Nou^S) derivatives in which the *SAT1* flipper was excised by FLP-mediated recombination, transformants were grown overnight in YCB-BSA-YE medium (23.4 g yeast carbon base, 4 g bovine serum albumin, 2 g yeast extract per litre, pH 4.0) without selective pressure to induce the *SAP2* promoter controlling *caFLP* expression. One hundred to 200 cells were then spread on YPD plates containing 20 μg ml^{-1} of nourseothricin and grown for 2 days at 30°C. Nou^S clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 100 μg ml^{-1} of nourseothricin as described previously (Reuβ *et al.*, 2004).

Plasmid constructions

The coding region and flanking sequences of the *MRR1* alleles of the different *C. albicans* strains was amplified by polymerase chain reaction (PCR) with the primers ZCF36-3 and ZCF36-6, which bind in the *MRR1* upstream and downstream regions, respectively (primer sequences are given in Table S2). The PCR products were digested at the introduced *Sac*I and *Apa*I restriction sites and cloned into the vector pBluescript to generate plasmids pZCF36-B3-1, pZCF36-B3-2, pZCF36-B4, pZCF36-1442-1, pZCF36-1442-2, pZCF36-2271, pZCF36-1490-1, pZCF36-1490-2, pZCF36-1587, pZCF36-5044, pZCF36-5052, pZCF36-DSY291-1, pZCF36-DSY291-2, pZCF36-DSY292-1, pZCF36-DSY2285-1, pZCF36-DSY2285-2, pZCF36-DSY2286, pZCF36-5833-1, pZCF36-5833-2, pZCF36-6692-1, pZCF36-6692-2, pZCF36-CAPR510, pZCF36-CAPR513, pZCF36-CAPR514, pZCF36-CAPR515 and pZCF36-CAPR517. The *Sac*I-*Pst*I fragments from pZCF36-CAPR510, pZCF36-CAPR513, pZCF36-CAPR514, pZCF36-CAPR515, pZCF36-CAPR517, pZCF36-DSY292-1, pZCF36-2271, pZCF36-1587 and pZCF36-DSY2286 were then substituted for the corresponding fragment in the previously described plasmid pZCF36K2 (Morschhäuser *et al.*, 2007) to produce pZCF36K6, pZCF36K7, pZCF36K8, pZCF36K9, pZCF36K10, pZCF36K13, pZCF36K14, pZCF36K15 and pZCF36K19, respectively (see Fig. 1 for illustration). Similarly, the *Eco*RI-*Pst*I fragments from pZCF36-B3-2 and pZCF36-B4 were substituted for the corresponding fragment in pZCF36K2 to yield pZCF36K11 and pZCF36K12, respectively. For the generation of pZCF36K16 and pZCF36K17, the *Sac*I-*Eco*RI fragments from pZCF36-5833-1 and pZCF36-6692-1, respectively, were substituted for the corresponding fragment in pZCF36K2. pZCF36K18 was obtained by ligating the *Sac*I-*Nde*I fragment from pZCF36-6692-1 and the *Nde*I-*Eco*RI fragment from pZCF36-6692-2 into the *Sac*I/*Eco*RI-digested pZCF36K2.

C. albicans transformation

C. albicans strains were transformed by electroporation (Köhler *et al.*, 1997) with the following gel-purified linear DNA fragments: The *Sac*I-*Apa*I fragments from plasmids pZCF36K6-K19 were used to insert mutated *MRR1* alleles and the corresponding nonmutated alleles into one the disrupted *mrr1*Δ loci of strains SCMRR1M4A and -B and the reporter strain CAG48MRR1M4B. The *Sac*I-*Apa*I fragment from plasmid pZCF36M2 (Morschhäuser *et al.*, 2007) was used for deletion of *MRR1* in the drug-resistant clinical *C. albicans* isolates. Selection of nourseothricin-resistant transformants was performed as described previously (Reuβ *et al.*, 2004). The correct integration of each construct was confirmed by Southern hybridization of *Nsi*I-digested genomic DNA with *MRR1*-specific probes.

Isolation of genomic DNA and Southern hybridization

Genomic DNA from *C. albicans* was isolated as described previously (Millon *et al.*, 1994). 10 μg of DNA was digested with *Nsi*I, separated on a 1% agarose gel and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was

performed with the Amersham ECL™ Direct Nucleic Acid Labelling and Detection System (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

DNA sequencing and analysis of allelic polymorphisms

The sequences of the cloned *MRR1* alleles were determined using universal forward and reverse primers and the primers listed in Table S2. The loss of heterozygosity in resistant isolates and the absence of the gain-of-function mutations in susceptible isolates was confirmed by direct sequencing of PCR products amplified from genomic DNA of the strains with primers covering the relevant regions. The presence or absence of allelic polymorphisms in the *CAP1*, *MEP1*, *SAP9*, and *AAP1* genes in the SC5314 derivatives and in the clinical isolates was determined by amplifying parts of these genes and direct sequencing of the *MEP1*, *SAP9*, and *AAP1* PCR products with the primers listed in Table S2. Heterozygosity for the *CAP1* alleles was tested by digesting the respective PCR products with *Eco*RI and (separately) *Sph*I and analysing the fragments by agarose gel electrophoresis. For isolates F2 and F5, in which the *CAP1* alleles did not exhibit these restriction site polymorphisms, the PCR products were also sequenced. The results of these analyses are described in detail in Table S3.

FACS analysis

Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm band-pass filter. Twenty thousand cells were analyzed per sample and were counted at low flow rate. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software.

Drug susceptibility tests

Stock solutions of the drugs were prepared as follows. Fluconazole (1 mg ml^{-1}) was dissolved in water and cerulenin (5 mg ml^{-1}) and brefeldin A (5 mg ml^{-1}) were dissolved in dimethylsulfoxide (DMSO). In the assays, serial twofold dilutions in the assay medium were prepared from the following initial concentrations: cerulenin, 100 μg ml^{-1}; brefeldin A, 200 μg ml^{-1}; fluconazole, 100 μg ml^{-1}. Susceptibility tests were carried out in high resolution medium (14.67 g HR-Medium [Oxoid GmbH, Wesel, Germany], 1 g $NaHCO_3$, 0.2 M phosphate buffer [pH 7.2]), using a previously described microdilution method (Ruhnke *et al.*, 1994).

Nucleotide sequence accession numbers

The coding sequences of the *MRR1* alleles described in the present work and in a previous study (Morschhäuser *et al.*, 2007) have been deposited in GenBank with the following accession numbers: EU139261 (*MRR1*$^{F2\text{-}1}$), EU139262 (*MRR1*$^{F2\text{-}2}$), EU139263 (*MRR1*^{F5}), EU139264 (*MRR1*$^{G2\text{-}1}$), EU139265 (*MRR1*$^{G2\text{-}2}$), EU139266 (*MRR1*^{G5}), EU497768 (*MRR1*$^{B3\text{-}1}$), EU497744 (*MRR1*$^{B3\text{-}2}$), EU497745 (*MRR1*^{B4}), EU497746 (*MRR1*$^{1442\text{-}1}$), EU497747 (*MRR1*$^{1442\text{-}2}$), EU497748 (*MRR1*^{2271}), EU497749 (*MRR1*$^{1490\text{-}1}$), EU497750 (*MRR1*$^{1490\text{-}2}$), EU497751 (*MRR1*^{1587}), EU497752 (*MRR1*^{5044}), EU497753 (*MRR1*^{5052}), EU497754 (*MRR1*$^{DSY291\text{-}1}$), EU497755 (*MRR1*$^{DSY291\text{-}2}$), EU497756 (*MRR1*$^{DSY292\text{-}1}$), EU497757 (*MRR1*^{DSY2285}), EU497758 (*MRR1*^{DSY2286}), EU497759 (*MRR1*$^{5833\text{-}1}$), EU497760 (*MRR1*$^{5833\text{-}2}$), EU497761 (*MRR1*$^{6692\text{-}1}$), EU497762 (*MRR1*$^{6692\text{-}2}$), EU497763 (*MRR1*^{CAPR510}), EU497764 (*MRR1*$^{CAPR513\text{-}2}$), EU497765 (*MRR1*^{CAPR514}), EU497766 (*MRR1*^{CAPR515}), and EU497767 (*MRR1*^{CAPR517}).

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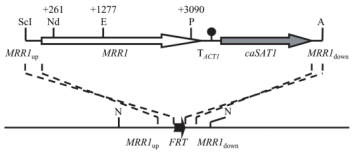

Fig. 1.

Structure of the DNA cassettes (top) used for integration of different *MRR1* alleles into *mrr1*Δ mutants (bottom) in which the endogenous *MRR1* copies had been deleted and replaced by the target sequence of the Flp recombinase (*FRT*, black arrow). The *MRR1* coding region is represented by the white arrow and the upstream ($MRR1_{up}$) and downstream regions ($MRR1_{down}$) by the solid lines. The transcription termination sequence of the *ACT1* gene (T_{ACT1}) is indicated by the filled circle and the *caSAT1* selection marker by the grey arrow. Only relevant restriction sites are shown: A, *Apa*I; E, *Eco*RI; N, *Nsi*I; Nd, *Nde*I; P, *Pst*I; ScI, *Sac*I. The nucleotide positions of the *Nde*I, *Eco*RI, and *Pst*I sites, which were used to replace specific fragments within the *MRR1* coding region, are given. The *MRR1* upstream region is identical in all plasmids and corresponds to that of strain SC5314.

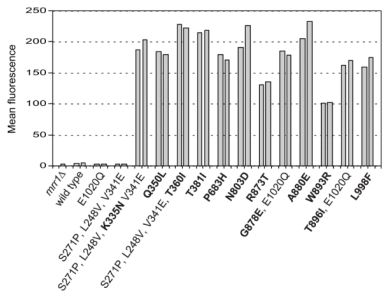

Fig. 2.

MDR1 promoter activity in an *mrr1*Δ mutant carrying a P_{MDR1}-*GFP* reporter fusion (*mrr1*Δ) and in transformants into which *MRR1* alleles with the gain-of-function mutations (highlighted in bold) from strains CAPR510 (T381I), CAPR513 (A880E), CAPR514 (W893R), CAPR515 (R873T), CAPR517 (L998F), 2271 (Q350L), 1587 (N803D), 5052 and DSY292 (P683H), B4 (G878E, E1020Q), DSY2286 (T896I, E1020Q), and 6692 (S171P, L248V, V341E, T360I and S171P, L248V, K335N, V341E) or the corresponding control alleles without the mutations (wild type, E1020Q, and S171P, L248V, V341E) were reintroduced, as outlined in Fig. 1. The strains were grown to log phase in YPD medium and the mean fluorescence of the cells was determined by flow cytometry. Results from two independent transformants are shown in each case.

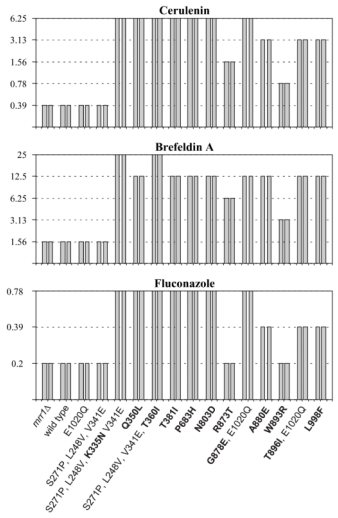

Fig. 3.

MICs (in μg ml^{-1}) of cerulenin, brefeldin A, and fluconazole for two independently constructed homozygous *mrr1*Δ mutants of the wild-type parental strain SC5314 and transformants carrying the indicated *MRR1* alleles (see legend to Fig. 2).

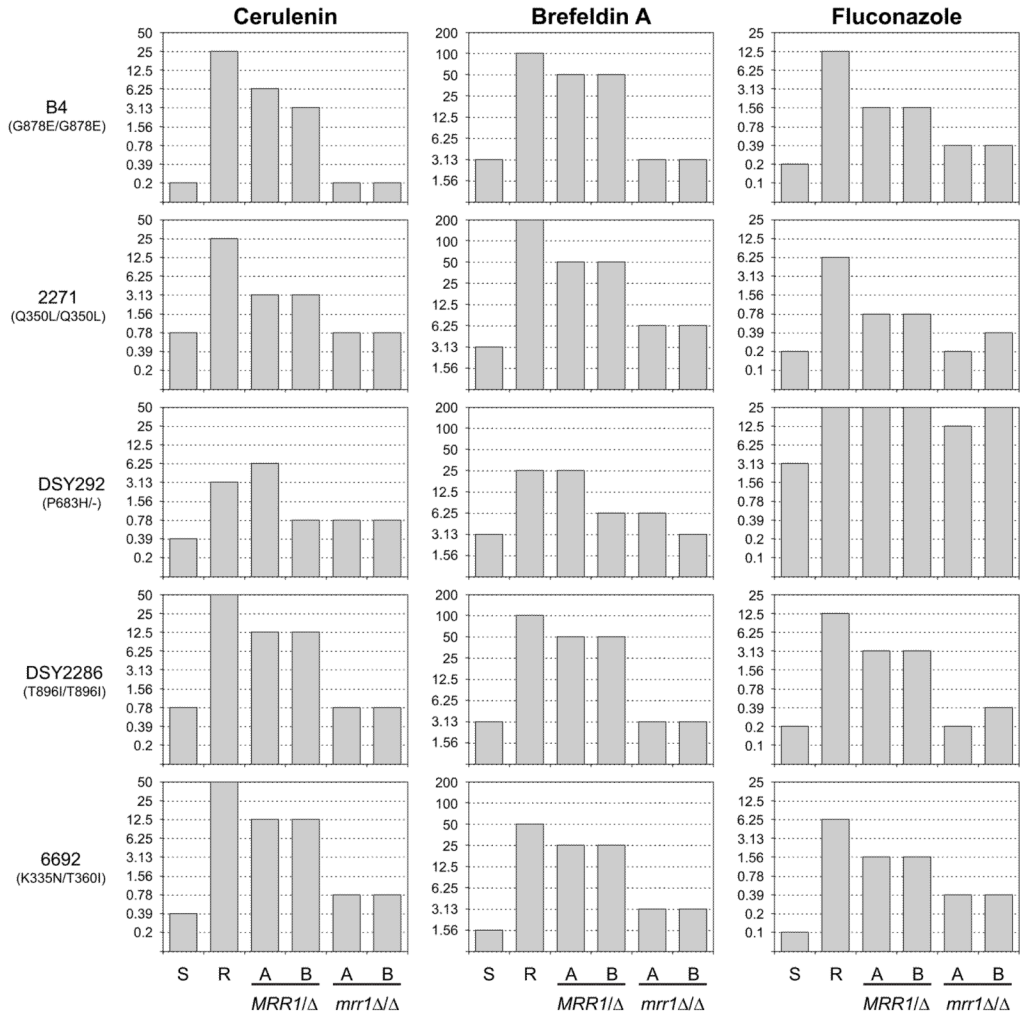

Fig. 4.

Susceptibilities of the matched fluconazole-susceptible (S) and resistant (R) clinical *C. albicans* isolate pairs and the two independent series (A and B) of heterozygous (*MRR1*/Δ) and homozygous (*mrr1*Δ/Δ) *mrr1* deletion mutants generated from the resistant isolates to cerulenin, brefeldin A, and fluconazole. The names of the resistant parental isolates and the Mrr1p gain-of-function mutations found in these isolates are given to the left of the respective panels. MICs of each compound are given in μg ml^{-1}.

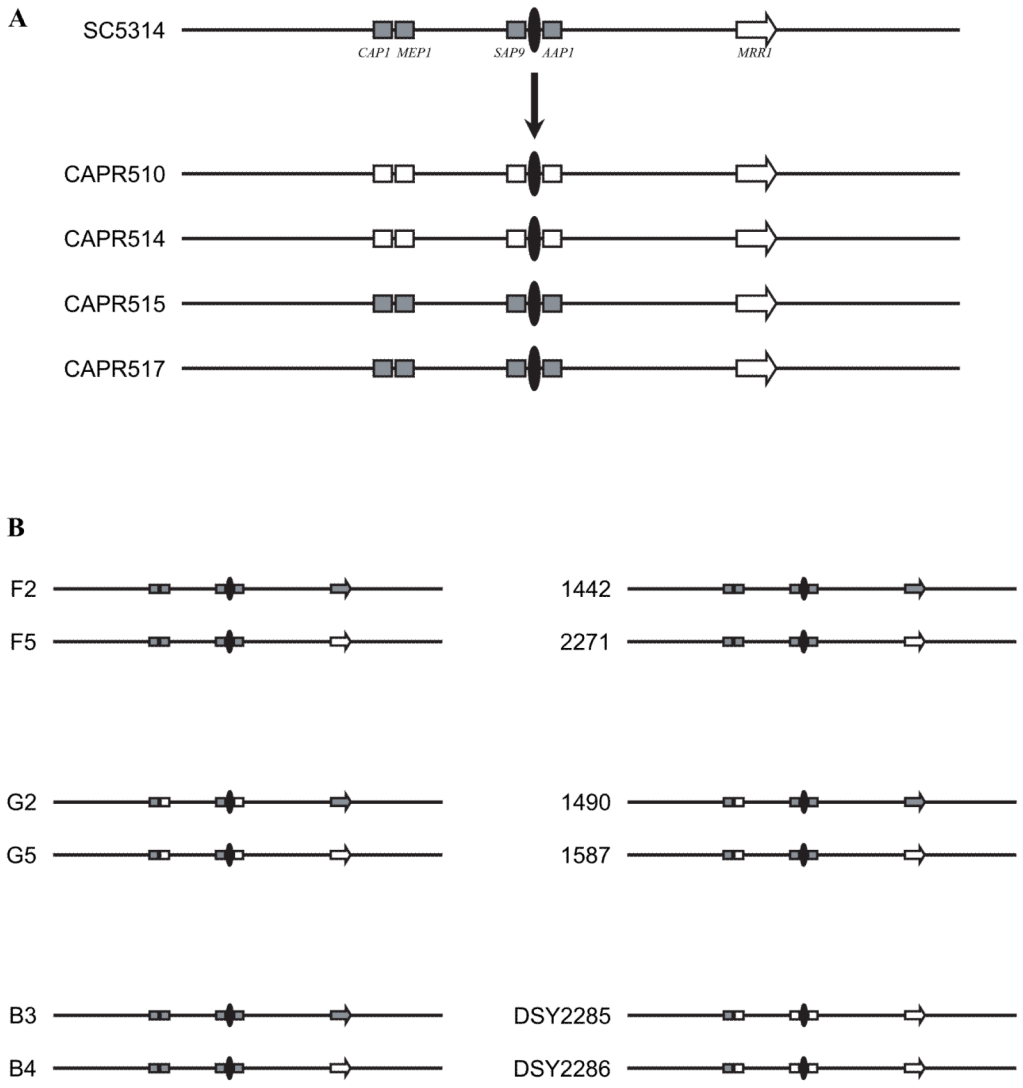

Fig. 5.

Mechanism of loss of *MRR1* heterozygosity in *MDR1* overexpressing, fluconazole-resistant *C. albicans* strains. (A) shows the position of known polymorphic markers (gray boxes) on chromosome 3, on which *MRR1* (white arrow) is located, in strain SC5314. The *in vitro* generated, fluconazole-resistant derivatives CAPR515 and CAPR517 have retained these polymorphisms whereas CAPR510 and CAPR514 have become homozygous for all tested polymorphic alleles, as indicated by the white boxes. The location of the centromere is indicated by the black oval. (B) shows the results of a similar analysis of six clinical isolate pairs in which the resistant isolate had become homozygous for a mutated *MRR1* allele. Gray shading: allelic polymorphisms detected, white: no allelic polymorphisms found.

Fig. 6.

Location of all known gain-of-function mutations in Mrr1p. The Mrr1p protein is represented as a linear bar and the DNA binding domain at the N-terminus is indicated by black shading.

TABLE 1

Allelic polymorphisms and mutations resulting in amino acid differences in Mrr1p

Strain	Allele	Amino acid polymorphisms
SC5314		-
CAPR510		**T381I**
CAPR513	1	-
	2	**A880E**
CAPR514		**W893R**
CAPR515		**R873T**
CAPR517		**L998F**
F2	1	-
	2	S171P, V341E, E1020Q
F5		**P683S**
G2	1	E1020Q
	2	S171P, V341E, E1020Q
G5		S171P, V341E, **G997V**, E1020Q
B3	1	-
	2	V27I, S171P, L248V, V341E, E1020Q, 5x NPQS
B4		V27I, S171P, L248V, V341E, **G878E**, E1020Q, 5x NPQS
1442	1	-
	2	V27I, S171P, L248V, V341E, E1020Q, 5x NPQS
2271		**Q350L**
1490	1	-
	2	-
1587		**N803D**
5044		S171P, V341E, E1020Q
5052		S171P, V341E, **P683H**, E1020Q
DSY291	1	-
	2	S16I, T73K, S171P, E1020Q, 2x NPQS
DSY292	1	**P683H**
	2	S16I, T73K, S171P, E1020Q, 2x NPQS
DSY2285		E1020Q
DSY2286		**T896I**, E1020Q
5833	1	S171P, L248V, V341E, 5x NPQS
	2	V27I, S171P, L248V, V341E, E1020Q, 5x NPQS
6692	1	S171P, L248V, V341E, **T360I**, 5x NPQS
	2	V27I, S171P, L248V, **K335N**, V341E, E1020Q, 5x NPQS

Only amino acids that differ from those of Mrr1p of strain SC5314 are listed, gain-of-function mutations in resistant strains are highlighted in bold. Matched isolates are grouped together in gray blocks, as are the *in vitro* generated strains CAPR510-CAPR517 and their parent SC5314. The previously analyzed isolate pairs F2/F5 and G2/G5 (Morschhäuser *et al.*, 2007) are included for completeness. The number of tandem repeats of the amino acid sequence NPQS (amino acids 165–168 in Mrr1p of strain SC5314) is indicated for alleles that contain more than one copy of this sequence.