A Mutation in Tac1p, a Transcription Factor Regulating *CDR1* and *CDR2*, Is Coupled With Loss of Heterozygosity at Chromosome 5 to Mediate Antifungal Resistance in *Candida albicans*

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ABSTRACT

TAC1, a *Candida albicans* transcription factor situated near the mating-type locus on chromosome 5, is necessary for the upregulation of the ABC-transporter genes *CDR1* and *CDR2*, which mediate azole resistance. We showed previously the existence of both wild-type and hyperactive *TAC1* alleles. Wild-type alleles mediate upregulation of *CDR1* and *CDR2* upon exposure to inducers such as fluphenazine, while hyperactive alleles result in constitutive high expression of *CDR1* and *CDR2*. Here we recovered *TAC1* alleles from two pairs of matched azole-susceptible (DSY294; FH1: heterozygous at mating-type locus) and azole-resistant isolates (DSY296; FH3: homozygous at mating-type locus). Two different *TAC1* wild-type alleles were recovered from DSY294 (*TAC1-3* and *TAC1-4*) while a single hyperactive allele (*TAC1-5*) was isolated from DSY296. A single amino acid (aa) difference between *TAC1-4* and *TAC1-5* (Asn977 to Asp or N977D) was observed in a region corresponding to the predicted activation domain of Tac1p. Two *TAC1* alleles were recovered from FH1 (*TAC1-6* and *TAC1-7*) and a single hyperactive allele (*TAC1-7*) was recovered from FH3. The N977D change was seen in *TAC1-7* in addition to several other aa differences. The importance of N977D in conferring hyperactivity to *TAC1* was confirmed by site-directed mutagenesis. Both hyperactive alleles *TAC1-5* and *TAC1-7* were codominant with wild-type alleles and conferred hyperactive phenotypes only when homozygous. The mechanisms by which hyperactive alleles become homozygous was addressed by comparative genome hybridization and single nucleotide polymorphism arrays and indicated that loss of *TAC1* heterozygosity can occur by recombination between portions of chromosome 5 or by chromosome 5 duplication.

CANDIDA *albicans* is an opportunistic pathogen that causes oral and systemic infections in immuno-compromised patients as well as vaginal infections in immunocompetent women. To prevent and treat Candida infections, immunocompromised patients are often treated for a long time with antifungal agents among which is the class of azoles. As azoles are fungistatic, rather than fungicidal, *C. albicans* cells repetitively exposed to these antifungals can adapt to the drug pressure and eventually become resistant to azoles. The most important mechanism of resistance to azoles is the overexpression of multidrug transporters, encoded by either the major facilitator efflux pump *CaMDR1* (*m*ulti*d*rug *r*esistance 1) or the ABC transporters *CDR1* (*c*andida *d*rug *r*esistance) and *CDR2*. Upregulation of *CaMDR1* confers resistance to fluconazole, while upregulation of *CDR1* and *CDR2* confers resistance to mul-

tiple azoles (itraconazole, fluconazole, voriconazole). Understanding the transcriptional control of these genes, by both *cis*- and *trans*-acting effectors, is therefore important for determining how azole resistance and transport mechanisms are regulated in *C. albicans*.

CaMDR1 expression is controlled by at least two regulatory promoter *cis*-acting regions as reported recently by HARRY *et al.* (2005). Several elements of *CDR* genes are important for the regulation of *CDR1* and *CDR2*. A *b*asal *r*esponse *e*lement (BRE) is located between nt −860 and −810 in the *CDR1* promoter, and a *d*rug *r*esponse *e*lement (DRE) is present in the promoters of both *CDR1* and *CDR2* (DE MICHELI *et al.* 2002). The BRE regulates basal expression of *CDR1* (DE MICHELI *et al.* 2002), while the DRE sequence (5′-CGGAA/TATCGGATA-3′) is crucial for the upregulation of these genes in azole-resistant strains as well as for the transient upregulation of both genes in the presence of different drugs such as oestradiol, progesterone, or fluphenazine in azole-susceptible strains. In addition, another BRE (located between −243 and −234) and a

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negative regulatory element (NRE) located within the −289 region have been reported in *CDR1* (Puri *et al.* 1999; Gaur *et al.* 2004). Finally, in the same gene, Karnani *et al.* (2004) identified SRE1 and SRE2 (steroid response elements) between −696 and −521.

Trans-acting factors regulating *CDR1* and *CDR2* were reported recently. C. G. Chen *et al.* (2004) described a potential activator of *CDR1* identified by screening of a *C. albicans* genomic library expressed in a *Saccharomyces cerevisiae* strain, which contained a *CDR1* promoter/*lacZ* fusion. This factor, *CaNDT80*, is a homolog to a meiosis-specific transcription factor in *S. cerevisiae* (C. G. Chen *et al.* 2004). Deletion of *CaNDT80* in *C. albicans* conferred hypersensitivity to azoles and decreased the inducible expression of *CDR1*. Recently, our laboratory discovered Tac1p (**t**ranscriptional **a**ctivator of **C**DR), a transcription factor belonging to the family of zinc-finger proteins with a Zn_2Cys_6 motif (Coste *et al.* 2004). Tac1p binds to the DRE, which contains two CGG triplets typical of the DNA-binding sites of Zn_2Cys_6 transcription factors. Tac1p is responsible for transient upregulation of both *CDR* genes in azole-susceptible strains in the presence of inducers. Interestingly, *TAC1* is located close to (within ∼14 kb) the mating-type-like (*MTL*) locus. Previous studies reported a strong correlation between homozygosity at the mating-type locus and azole resistance in a number of clinical isolates (Rustad *et al.* 2002). In our previous study, we showed that a clinical azole-resistant strain (DSY296) that is homozygous at the mating-type locus contains a *TAC1* allele that is sufficient to confer fluconazole resistance to a laboratory strain lacking *TAC1* (Coste *et al.* 2004). This type of allele was defined as "hyperactive" because it caused constitutive high expression of *CDR1* and *CDR2* in a *tac1*Δ/Δ mutant. In contrast, *TAC1* alleles of the matched azole-susceptible clinical strain (DSY294) or of a laboratory strain (CAF2-1), which are strains heterozygous at the mating-type locus, were not able to confer azole resistance to a *tac1*Δ/Δ mutant. These alleles were defined as "wild-type" alleles. Using *C. albicans* microarrays, we also showed that Tac1p regulates the expression of at least three other genes: *RTA3*, *IFU5*, and *HSP12* (Coste *et al.* 2004; Karababa *et al.* 2004). Northern blot analysis showed that Tac1p also regulates *PDR16* (D. Sanglard, unpublished data), a gene shown to be overexpressed in azole-resistant strains upregulating *CDR1* and *CDR2* (De Deken and Raymond 2004). Interestingly, all four of these Tac1p-regulated genes contain a putative DRE in their promoters.

In *S. cerevisiae*, the functional homolog of *CDR1* and *CDR2*, *PDR5*, is known to be regulated by at least two Zn_2Cys_6 transcription factors (*PDR1* and *PDR3*). These transcription factors bind as homo- and heterodimers to a *cis*-acting pleiotropic drug responsive element containing two CGG triplets (Katzmann *et al.* 1994; Carvajal *et al.* 1997). Point mutations in *PDR1* and *PDR3* lead to increased *PDR5* expression and drug resistance (Carvajal *et al.* 1997; Nourani *et al.* 1997; Anderson *et al.* 2003). Carvajal *et al.* (1997) found that a F815S mutation (F815S) in the putative activation domain of Pdr1p is responsible for strong constitutive *PDR5* expression. Point mutations in other regions of *PDR1* also affect the regulation of *PDR5* expression, but have a more moderate effect than the F815S mutation.

In this study, we analyzed *TAC1* alleles of matched azole-susceptible and azole-resistant clinical *C. albicans* isolates. We identified a single point mutation (N977D) in both hyperactive *TAC1* alleles, which is sufficient to confer hyperactivity as measured by upregulation of *CDR1* and *CDR2* and levels of drug resistance. We also show that hyperactive alleles carrying this mutation are codominant with other wild-type alleles such that only strains homozygous for hyperactive alleles show high expression levels of *CDR1* and *CDR2*. We also show that homozygosity at *MTL* accompanies the acquisition of *TAC1* homozygosity via at least two mechanisms, but that *MTL* homozygosity does not contribute to the azole resistance phenotype.

MATERIALS AND METHODS

Strains and media: The *C. albicans* strains used in this study are listed in Table 1. These strains were grown either in complete medium YEPD (1% Bacto peptone, Difco Laboratories, Basel, Switzerland), 0.5% yeast extract (Difco), and 2% glucose (Fluka, Buchs, Switzerland) or in minimal medium yeast nitrogen base (Difco) and 2% glucose (Fluka). When grown on solid media, 2% agar (Difco) was added to either of the media. *Escherichia coli* DH5α was used as a host for plasmid constructions and propagation. DH5α was grown in Luria–Bertani broth (LB) or on LB plates, supplemented with ampicillin (0.1 mg/ml) when required.

Yeast transformation: *C. albicans* cells from 0.2 ml stationary-phase culture were resuspended in 0.1 ml of a solution containing 200 mm lithium acetate (pH 7.5), 40% (w/v) PEG 8000, 15 mg/ml DTT, and 250 µg/ml denatured salmon sperm DNA. Transforming DNA (1–5 µg) was added to the yeast suspension, which was incubated for 60 min at 43.5°. Transformation mixtures were plated directly onto selective plates. For transformation involving the dominant marker *SAT1*, the transformation mixture was incubated at room temperature overnight in 1 ml YEPD and plated the day after on YEPD agar plates containing 200 µg/ml of nourseothricin (Werner Bio-agent, Jena, Germany).

Drug susceptibility testing: Drug susceptibility testing was performed by spotting cells onto solid agar plates containing the tested drugs. Yeast cultures were grown overnight in YEPD and diluted to a density of 1.5×10^7 cells/ml and serial 10-fold dilutions were performed to a final dilution step containing 1.5×10^3 cells/ml. Four microliters of each dilution were spotted onto YEPD plates with or without drugs. Plates were incubated for 48 hr at 35°.

Drug susceptibility testing was also performed in microtiter plates with twofold serial dilutions of fluconazole (range is from 128 to 0.06 µg/ml) or terbinafine (range is from 32 to 0.015 µg/ml). Yeast cultures were grown overnight in YEPD and inoculated at a density of 10^4 cells/ml in a total volume of 200 µl containing the serial dilution of fluconazole or terbinafine. Microtiter plates were incubated at 35° during 48 hr and optical densities read with a microtiter plate reader at a

TABLE 1

Strains used in this study

Strain	Parental strain	Genotype	Reference
CAF2-1	SC5314	$ura3\Delta::imm434/URA3$	Fonzi and Irwin (1993)
CAF4-2	CAF2-1	$ura3\Delta::imm434/ura3\Delta::imm434$	Fonzi and Irwin (1993)
DSY2875	CAF4-2	$tac1\text{-}1\Delta::hisG/TAC1\text{-}2$	Coste *et al.* (2004)
DSY2903	DSY2875	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG\text{-}URA\text{-}hisG$	Coste *et al.* (2004)
DSY2906	DSY2903	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG$	Coste *et al.* (2004)
DSY2937-35	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1$	Coste *et al.* (2004)
DSY2925-47	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}3$	Coste *et al.* (2004)
DSY2925-18	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}4$	Coste *et al.* (2004)
DSY2984	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}5$	This study
VTY9	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}5^{D977N}$	This study
VTY21	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}1^{N977D}$	This study
VTY28	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}4^{N977D}$	This study
DSY3010-80	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}6$	This study
DSY3010-113	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}7\text{-}FH1$	This study
DSY3013	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}7\text{-}FH3$	This study
ACY11	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}6^{N977D}$	This study
ACY12	DSY2906	$tac1\text{-}1\Delta::hisG/tac1\text{-}2\Delta::hisG, LEU2::TAC1\text{-}7^{D977N}$	This study
DSY294		Azole-susceptible clinical strain ($MTLa/MTL\alpha$)	Coste *et al.* (2004)
DSY3040	DSY294	$ura3\Delta::FRT/ura3\Delta::FRT$	This study
DSY3058	DSY3040	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG\text{-}URA3\text{-}hisG$	This study
DSY3075	DSY3058	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG$	This study
DSY3082	DSY3075	$tac1\text{-}3\Delta::hisG/tac1\text{-}4\Delta::hisG\text{-}URA3\text{-}hisG$	This study
DSY3089	DSY3082	$tac1\text{-}3\Delta::hisG/tac1\text{-}4\Delta::hisG$	This study
DSY3102-2	DSY3089	$tac1\text{-}3\Delta::hisG/tac1\text{-}4\Delta::hisG, LEU2::TAC1\text{-}5$	This study
DSY3287-1	DSY3089	$tac1\text{-}3\Delta::hisG/tac1\text{-}4\Delta::hisG, LEU2::TAC1\text{-}3$	This study
DSY 3288-3	DSY3089	$tac1\text{-}3\Delta::hisG/tac1\text{-}4\Delta::hisG, LEU2::TAC1\text{-}4$	This study
DSY3053-1	DSY3040	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG\text{-}URA3\text{-}hisG$	This study
DSY3168-1	DSY3053-1	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG$	This study
DSY3219-2	DSY3168	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG, tac1::TAC1\text{-}5$	This study
DSY3220-1	DSY3168	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG, tac1::TAC1\text{-}3$	This study
DSY3221-3	DSY3168	$TAC1\text{-}4/tac1\text{-}3\Delta::hisG, tac1::TAC1\text{-}4$	This study
DSY3053-2	DSY3040	$TAC1\text{-}3/tac1\text{-}4\Delta::hisG\text{-}URA3\text{-}hisG$	This study
DSY3168-2	DSY3053-2	$TAC1\text{-}3/tac1\text{-}4\Delta::hisG$	This study
DSY3222-2	DSY3168-2	$TAC1\text{-}3/tac1\text{-}4\Delta::hisG, tac1::TAC1\text{-}5$	This study
DSY3223-1	DSY3168-2	$TAC1\text{-}3/tac1\text{-}4\Delta::hisG, tac1::TAC1\text{-}3$	This study
DSY3224-1	DSY3168-2	$TAC1\text{-}3/tac1\text{-}4\Delta::hisG, tac1::TAC1\text{-}4$	This study
DSY296	DSY294	Azole-resistant clinical strain ($MTL\alpha/MTL\alpha$)	Coste *et al.* (2004)
DSY3041	DSY296	$ura3\Delta::FRT/ura3\Delta::FRT$	This study
DSY3059	DSY3041	$TAC1\text{-}5/tac1\text{-}5\Delta::hisG\text{-}URA3\text{-}hisG$	This study
DSY3076	DSY3059	$TAC1\text{-}5/tac1\text{-}5\Delta::hisG$	This study
DSY3210-1	DSY3076	$TAC1\text{-}5/tac1\text{-}5\Delta::hisG, tac1::TAC1\text{-}5$	This study
DSY3211-4	DSY3076	$TAC1\text{-}5/tac1\text{-}5\Delta::hisG, tac1::TAC1\text{-}3$	This study
DSY3215-1	DSY3076	$TAC1\text{-}5/tac1\text{-}5\Delta::hisG, tac1::TAC1\text{-}4$	This study
DSY3083	DSY3076	$tac1\text{-}5\Delta::hisG/tac1\text{-}5\Delta::hisG\text{-}URA3\text{-}hisG$	This study
DSY3090-8	DSY3083	$tac1\text{-}5\Delta::hisG/tac1\text{-}5\Delta::hisG$	This study
DSY3284-1	DSY3090-8	$tac1\text{-}5\Delta::hisG/tac1\text{-}5\Delta::hisG, LEU2::TAC1\text{-}5$	This study
DSY3285-1	DSY3090-8	$tac1\text{-}5\Delta::hisG/tac1\text{-}5\Delta::hisG, LEU2::TAC1\text{-}3$	This study
DSY3286-2	DSY3090-8	$tac1\text{-}5\Delta::hisG/tac1\text{-}5\Delta::hisG, LEU2::TAC1\text{-}4$	This study
FH1		Azole-susceptible clinical strain ($MTLa/MTL\alpha$)	Marr *et al.* (1997)
FH3	FH1	Azole-resistant clinical strain ($MTL\alpha/MTL\alpha$)	Marr *et al.* (1997)
DSY3132-14	FH1	$TAC1\text{-}7/tac1\text{-}6\Delta::FRT$	This study
DSY3132-11	FH1	$TAC1\text{-}6/tac1\text{-}7\Delta::FRT$	This study
DSY3133-15	FH3	$TAC1\text{-}7/tac1\text{-}7\Delta::FRT$	This study
DSY3157-2	FH1	$TAC1\text{-}6/TAC1\text{-}7/TAC1\text{-}7$	This study
DSY3301-4	DSY3157-2	$TAC1\text{-}7/TAC1\text{-}7/TAC1\text{-}7$	This study

wavelength of 540 nm. The minimal inhibitory concentration (MIC) was determined as the drug concentration required to decrease the optical density of the drug-free culture by at least 50%.

Efflux of rhodamine 6G: To measure the drug efflux capacity of *C. albicans* strains with specific *TAC1* alleles and *CDR1/CDR2* expression, rhodamine 6G (R6G) efflux was measured by fluorescence assays with whole cells. *C. albicans* cultures grown overnight in YEPD were diluted in 5 ml YEPD and allowed to grow at 30° under constant agitation until a density of 2×10^7 cells/ml was obtained. Cells were centrifuged, washed with 5 ml PBS (pH 7), and resuspended in 2 ml PBS. The cells were incubated for 1 hr at 30° under constant agitation in PBS to energy deprive cells. R6G was next added at a concentration of 10 µg/ml and the incubation was continued for 1 hr, thus facilitating R6G accumulation. After this incubation time, cells were sedimented by centrifugation, washed with PBS at 4°, and resuspended in a final volume of 200 µl PBS. Fifty microliters of individual strains were diluted in 150 µl PBS and aliquoted in a 96-well microtiter plate, which was placed in a SpectraMax Gemini fluorimeter with temperature control set at 30°. Baseline emission of fluorescence (excitation wavelength: 344 nm; emission wavelength: 555 nm) was recorded as relative fluorescence units (RFU) for 5 min and glucose (1% final concentration) was next added to each strain to initiate R6G efflux. As a negative control, no glucose was added to separate aliquots of each strain. Data points were recorded in duplicate for 60 min at 1-min intervals.

Immunoblots: *C. albicans* cell extracts for immunoblotting were prepared by an alkaline extraction procedure from cells grown to midlog phase. Briefly, cells (5 OD$_{540nm}$) were resuspended in an Eppendorf tube with 500 µl water and 150 µl of a solution containing 1.85 M NaOH and 7.5% β-mercaptoethanol. This mixture was incubated on ice for 10 min. Proteins were next precipitated with 150 µl of a 50% trichloroacidic acid solution and the suspension was left on ice for another 10 min. Precipitated proteins were sedimented by a centrifugation step at maximal speed in a microfuge for 15 min. The sediment was resuspended in 50 µl of loading buffer (40 mM Tris–HCl pH 6.8, 8 M urea, 5% SDS, 0.1 M EDTA, 1% β-mercaptoethanol, and 0.1 mg/ml bromophenol blue) and incubated at 37° for 10 min. Nonsolubilized material was cleared by a centrifugation step for 10 min. Ten microliters of solubilized yeast proteins were separated by 10% SDS–PAGE and transferred by Western blot on a nitrocellulose membrane. The membrane was stained by Ponceau reagent (0.25% Ponceau S in 40% methanol and 15% acetic acid) for 5 min to verify that protein extracts were evenly transferred. Immunodetection of Cdr1p and Cdr2p was performed with rabbit polyclonal anti-Cdr1p and anti-Cdr2p antibodies as described previously (DE MICHELI *et al.* 2002) by chemoluminescence with an ECL kit according to the recommendations of the manufacturer (Amersham Biosciences, Otelfingen, Switzerland).

Construction of gene disruption cassettes: Four different *TAC1* disruption cassettes were designed in this study. Three cassettes—C333, C357, and C343 in plasmids pDS1052, pDS1142, and pDS1102—were designed using the "Ura"-blaster system. C333 and C357 bear the deletion of a small portion of 271 bp between nt +1153 and +1424 with respect to the first ATG codon of *TAC1*. C343 was designed to delete a larger region of 1931 bp between nt +502 and +2433. C358 carried by the plasmid pDS1196 integrates the *SAT1*-flipper system (REUSS *et al.* 2004), in which a region of 1924 bp was deleted between nt +501 and +2425.

To construct these different deletion cassettes, the entire *TAC1* ORF was first amplified from genomic DNA using the cloning primers CaZNC2–*Bam*HI and CaZNC2–Xho (see supplemental Table S3 at http://www.genetics.org/supplemental/).

For the construction of cassettes C333 and C343, *TAC1* was amplified from the genomic DNA of CAF2-1 but for the construction of the cassette C357, *TAC1* was amplified with genomic DNA from DSY2875 to specifically amplify the *TAC1-2* allele. PCR fragments were cloned into pBluescript KS+ to yield pDS1048 and pDS1138 (supplemental Table S2 at http://www.genetics.org/supplemental/). For disruption with cassettes C333 and C357, pDS1048 and pDS1138 were digested with *Pst*I and *Bgl*II and the 3.7-kb *Pst*I–*Bgl*II fragment containing the "Ura"-blaster cassette from pMB7 was cloned into compatible sites to yield pDS1052 and pDS1142, respectively. For disruption with cassette C343, a deletion was created from pDS1048 using primers Znc2–BG2 and Znc2–PST (supplemental Table S3 at http://www.genetics.org/supplemental/). The obtained PCR fragment was digested with *Pst*I and *Bgl*II and the 3.7-kb *Pst*I–*Bgl*II fragment from pMB7 was inserted to obtain pDS1102 (supplemental Table S2 at http://www.genetics.org/supplemental/). For transformation in *C. albicans*, linear fragments were obtained by digestion of the plasmids with *Nsi*I and *Xmn*I, thus liberating cassettes C333 and C357, and by *Apa*I and *Sac*I, liberating C343. For the cassette C358, *TAC1* was amplified from CAF2-1 genomic DNA. The obtained PCR fragment was cloned into the pMTL21 (CHAMBERS *et al.* 1998) to yield pDS1141. A deletion was created using pDS1141 as template with the primers Znc2–*Apa* and Znc2–*Sac*II. Next, the *Apa*I–*Sac*II fragment of pSFS2 comprising the *SAT1*-flipper cassette was cloned into the previously *Apa*I- and *Sac*II-digested PCR fragment to obtain pDS1196. For the transformation in *C. albicans*, a linear fragment was obtained by digestion of the plasmid with *Sac*I and *Sph*I, thus liberating the C358 cassette. *C. albicans* strains DSY3053-1 and DSY3053-2 were obtained using the C357 disruption cassette. DSY3058 and DSY3059 were obtained using the C343 cassette. DSY3082, DSY3083, DSY2875, and DSY2903 were obtained using the C333 cassette. DSY3132-11, DSY3132-14, and DSY3133-15 were obtained using the C358 cassette.

To obtain *ura3* mutants of the clinical isolates DSY294 and DSY296, the two *URA3* alleles were deleted using the *SAT1*-flipping strategy (REUSS *et al.* 2004). For this purpose, the *SAT1*-flipper cassette was substituted for the *MPA*^{R}-flipper cassette in the previously described plasmid pSFIU4 (STRAUSS *et al.* 2001) to result in pSFSU1, in which the *SAT1* flipper is flanked by *URA3* upstream and downstream sequences. The insert from this plasmid was then used to inactivate *URA3* in the clinical *C. albicans* isolates by two rounds of targeted integration and subsequent FLP-mediated excision of the *SAT1*-flipper cassette, generating strains DSY3040 and DSY3041. Transformations were performed by electroporation and selection of nourseothricin-resistant transformants was performed as described (REUSS *et al.* 2004).

Construction of revertant strains: The revertant strains from each homozygous mutant generated in this study were obtained by transformation of *C. albicans* *ura3* derivatives with the pRC2312-derived plasmid pDS178 containing the *URA3* and *LEU2* markers as described previously (DE MICHELI *et al.* 2002). To generate revertants from *tac1*Δ/Δ mutant strains, *TAC1* ORFs flanked by 500 bp were amplified from genomic DNA of strains SC5314, DSY294, DSY296, and FH1 and FH3 with primers Znc2-5–*Bam*B and Znc2-3–*Xho* (supplemental Table S3 at http://www.genetics.org/supplemental) and inserted into pDS178 previously digested by *Bam*HI and *Xho*I to yield pDS1097 (containing the *TAC1-1* allele), pDS1098-1 and pDS1098-9 (containing the *TAC1-4* and *TAC1-3* alleles), pDS1099 (containing the *TAC1-5* allele), pDS1045 (containing the *TAC1-6* allele), and pDS1048 (containing the *TAC1-7* allele), respectively. For each amplified allele, *TAC1* was sequenced from several plasmids to rule out PCR artifacts. These plasmids either were linearized by *Sal*I and transformed

into *C. albicans* DSY2906, DSY3089, and DSY3090, allowing integration into the genomic *LEU2* locus, or were linearized by *Bst*BI, allowing integration in the *TAC1* locus of strains DSY3168-1, DSY3168-2, and DSY3076. For each reintegration of *TAC1* alleles, several independent transformants (approximately four to five) were tested for phenotypes (susceptibility assays, immunodetection of Cdr1p and Cdr2p) and correct integration. The transformants containing identical *TAC1* alleles generally had the same phenotypes, but only a single revertant for individual alleles was selected and presented in this study. Integration at the *TAC1* locus for strains DSY2906, DSY3089, and DSY3090 was also performed and yielded phenotypes comparable to those obtained by integration at the *LEU2* locus (data not shown).

Southern blots: Southern blots were performed as described previously (Sanglard *et al.* 1995). Radioactive signals were revealed by exposure to Kodak BioMax MR films (Amersham Biosciences, Otelfingen, Switzerland). Signals obtained in blotted membranes were quantified by counting of radioactivity with the help of an Instant Imager (Perkin-Elmer, Rotkreuz, Switzerland).

Site-directed mutagenesis: For site-directed mutagenesis of *TAC1-1*, *TAC1-4*, and *TAC1-5*, the previously cloned alleles were amplified from pDS1097, pDS1098-1, and pDS1099, respectively, using the forward primer Zn2-5BAMB (Table S3) and with the reverse primers TAC1-Asn-Asp-977 (primer for pDS1097 and pDS1098-1) and TAC1-Asp-Asn-977 (primer for pDS1099). These primers introduce a modification of codon 977 from Asn to Asp in *TAC1-1* and *TAC1-4* and from Asp to Asn in *TAC1-5*. Amplified products were cloned in the pDS178 backbone yielding pVT21, pVT28, and pVT9 containing the mutated *TAC1-1^{N977D}*, *TAC1-4^{N977D}*, and *TAC1-5^{D977N}* alleles, respectively. These plasmids were linearized by *Sal*I and transformed into *C. albicans* DSY2906 as described above.

Single nucleotide polymorphism and comparative genomic hybridization: Single nucleotide polymorphism (SNP) microarray hybridization and comparative genomic hybridization (CGH) were performed as described previously (Forche *et al.* 2005; Selmecki *et al.* 2005). Additional SNP markers were designed from specific regions of chromosome 5 using *C. albicans* genome data deposited at http://candida.bri.nrc.ca/candida/alignments/index.cfm?chr=5. The search for SNP markers not present on the microarrays was performed by amplification of specific regions of chromosome 5 from genomic DNA using different V5 and V3 primer pairs (see supplemental Table S3 at http://www.genetics.org/supplemental/) followed by sequencing the PCR products using a AB Prism, 3130 genetic analyzer (AB Applied Biosystems). Sequences were analyzed for polymorphisms using the Contig Express software (InforMax).

RESULTS

Analysis of *TAC1* alleles isolated from azole-susceptible and azole-resistant *C. albicans* strains: To analyze the different *TAC1* alleles present in either azole-susceptible or azole-resistant strains, two sets of clinical strains were first chosen. A first set of matched strains reported previously (Sanglard *et al.* 1995, 1998) and consisting of the azole-susceptible strain DSY294 (also known as C43) and the azole-resistant strain DSY296 (also known as C56) originated from an HIV-positive patient with oropharyngeal candidiasis who was treated with fluconazole. A second set of strains described by

Marr *et al.* (1998, 2001) consisted of azole-susceptible strains FH1 and FH2 and of strains FH3–FH8, which developed azole resistance. These strains were isolated from a bone marrow transplant patient suffering from invasive candidiasis and treated with fluconazole. FH1 and FH2 are heterozygous at the mating-type locus (Rustad *et al.* 2002). FH3–FH8 are homozygous at the mating-type locus (Rustad *et al.* 2002). *TAC1* alleles of strains DSY294, DSY296, and FH1–FH8 were cloned and introduced in a *tac1*Δ/Δ mutant (strain DSY2906) derived from SC5314. The azole resistance phenotypes of the transformants were analyzed by drug susceptibility assays and immunoblotting detection of Cdr1p and Cdr2p.

Sequencing of individual *TAC1* alleles from DSY294 revealed two distinct alleles, *TAC1-3* and *TAC1-4* (Table 2). Both of these alleles had the properties of wild-type alleles when reintroduced in a *tac1*Δ/Δ mutant. First, they did not confer resistance to terbinafine or fluconazole (Figure 1A). Second, they did not result in constitutive high levels of Cdr1p or Cdr2p expression under normal growth conditions; rather, Cdr1p was detected at basal levels and Cdr2p could not be detected (Figure 1B, lanes 3 and 4). The Cdr1p and Cdr2p levels were comparable to those found in CAF2-1 and in the *tac1*Δ/Δ mutant (Figure 1B, lanes 1–4). However, Cdrp1 and Cdr2p were still inducible as in CAF2-1, since exposure of *TAC1-3* or *TAC1-4* revertant strains to fluphenazine led to high Cdr1p and Cdr2p levels (Figure 1B, lanes 1, 3, and 4). The identification of two distinct *TAC1* alleles in DSY294 is consistent with the heterozygosity of DSY294 at the mating-type locus, given that *TAC1* is located at a distance of ∼14 kb from this locus. In contrast, only a single *TAC1* allele (*TAC1-5*, Table 2) was recovered from the matched azole-resistant strain DSY296. The isolation of a single *TAC1* allele from DSY296 is consistent with homozygosity of this strain at the mating-type locus and suggests that a region >14 kb underwent loss of heterozygosity (LOH) during the acquisition of drug resistance.

The *TAC1-5* allele was considered to be a hyperactive allele. First, when reintroduced into a *tac1*Δ/Δ mutant, it conferred higher terbinafine resistance than that observed in strains containing the wild-type *TAC1-3* and *TAC1-4* alleles (Figure 1A). Second, it conferred constitutively high levels of Cdr1p and Cdr2p (Figure 1B, lane 5), which are comparable to levels seen in strains carrying wild-type alleles and exposed to fluphenazine (Figure 1B, lanes 1, 3, and 4).

Similar analyses of *TAC1* alleles were performed in FH1–FH8 strains. Two different *TAC1* alleles were isolated from FH1 and FH2 (Table 2). One allele, *TAC1-6*, was wild type, and the other, *TAC1-7*, was hyperactive as defined above for *TAC1* alleles of DSY294 and DSY296. *TAC1-6* does not confer terbinafine resistance (Figure 1A) and does not express high levels of Cdr1p and Cdr2p under normal growth conditions (Figure 1B,

TABLE 2

TAC1 alleles present in the strains used in this study

Strain	Name of allele	Type of allele	Polymorphism of *TAC1* alleles (position of the nonsynonymous codons)																					
			47	104	131	170	189	199	206	207	377	396	558	772	776	829	869	904	935	937	941	944	977
CAF2-1	*TAC1-1* orf19.3188 (contig19-10170)	Wild type	TTA (L)	TTT (F)	CTA (L)	ATG (M)	TTT (F)	AGT (S)	CGT (R)	GTT (V)	GCT (A)	AAC (N)	ATT (I)	AAT (N)	GAC (D)	GAA (E)	CGA (R)	GAG (E)	TCA (S)	TCG (S)	CTG (S)	AAT (N)	AAT (N)
	TAC1-2ᵃ orf19.10700 (contig19-20170)	Wild type	AAA (K)	GTC (V)	CTA (L)	ATG (M)	TTT (F)	AAT (N)	CAC (H)	GCT (A)	GCT (A)	AGC (S)	ATT (I)	AAA (K)	AAC (N)	CAA (Q)	CGA (R)	GAG (E)	TTA (L)	TCG (S)	CCG (P)	AAT (N)	AAT (N)
DSY294	*TAC1-3*	Wild type	AAA (K)	TTT (F)	ATA (I)	ATG (M)	TCT (S)	AAT (N)	CAC (H)	GCT (A)	GCT (A)	AGC (S)	GTT (V)	AAA (K)	AAC (N)	GAA (E)	CGA (R)	GAG (E)	TCA (S)	TCG (S)	CTG (S)	AAT (N)	AAT (N)
	TAC1-4	Wild type	AAA (K)	TTT (F)	CTA (L)	GTG (V)	TTT (F)	AAT (N)	CAC (H)	GCT (A)	GTT (V)	AGC (S)	GTT (V)	AAA (K)	AAC (N)	CAA (Q)	CAA (Q)	GAG (E)	TCA (S)	TCG (S)	CTG (S)	AAT (N)	AAT (N)
DSY296	*TAC1-5*	Hyperactive	AAA (K)	TTT (F)	CTA (L)	GTG (V)	TTT (F)	AAT (N)	CAC (H)	GCT (A)	GTT (V)	AGC (S)	GTT (V)	AAA (K)	AAC (N)	CAA (Q)	CAA (Q)	GAG (E)	TCA (S)	TCG (S)	CTG (S)	AAT (N)	GAT (D)
FH1 and FH2	*TAC1-6*	Wild type	AAA (K)	TTT (F)	ATA (I)	ATG (M)	TCT (S)	AAT (N)	CAC (H)	GCT (A)	GCT (A)	AGC (S)	GTT (V)	AAA (K)	AAC (N)	GAA (E)	CGA (R)	GGG (G)	TCA (S)	TTG (L)	CTG (S)	TAT (Y)	AAT (N)
	TAC1-7	Hyperactive	AAA (K)	TTT (F)	CTA (L)	ATG (M)	TTT (F)	AAT (N)	CAC (H)	GCT (A)	GCT (A)	AGC (S)	ATT (I)	AAT (N)	AAC (N)	GAA (E)	CGA (R)	GAG (E)	TCA (S)	TCG (S)	CCG (P)	AAT (N)	GAT (D)
FH3	*TAC1-7*	Hyperactive	AAA (K)	TTT (F)	CTA (L)	ATG (M)	TTT (F)	AAT (N)	CAC (H)	GCT (A)	GCT (A)	AGC (S)	ATT (I)	AAT (N)	AAC (N)	GAA (E)	CGA (R)	GAG (E)	TCA (S)	TCG (S)	CCG (P)	AAT (N)	GAT (D)

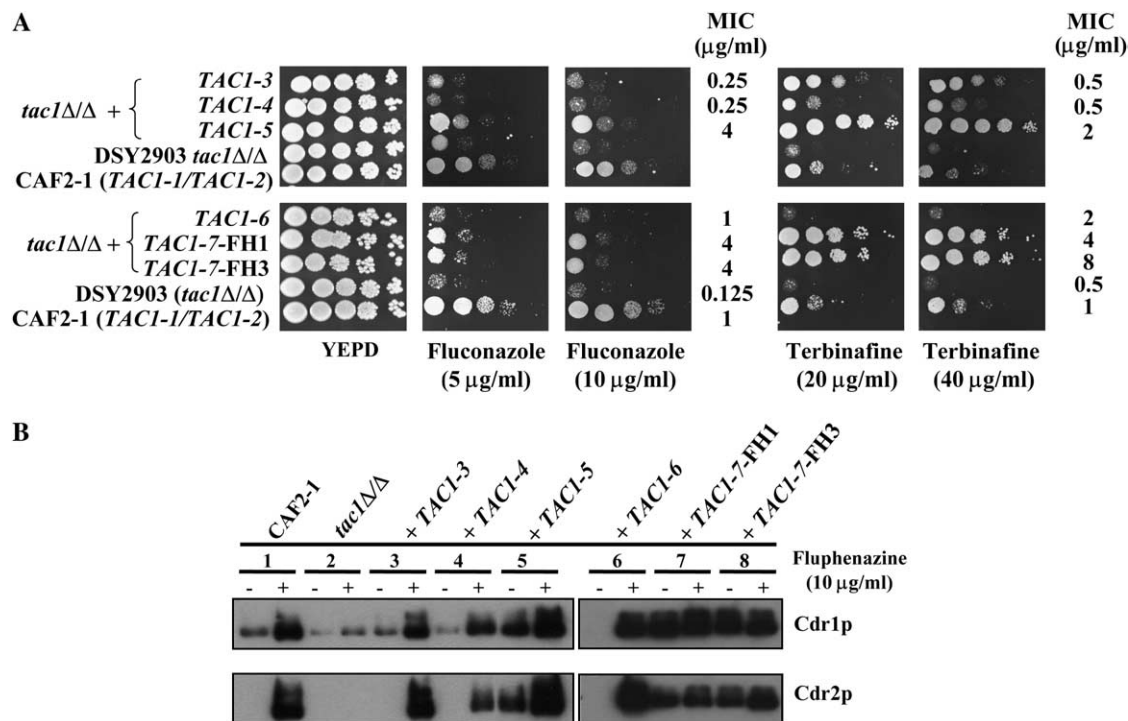ᵃ This allele is not corrected in CGD and contains a stop codon in the ORF.

FIGURE 1.—Analysis of drug resistance properties dependent on *TAC1* alleles. (A) Drug susceptibility testing of *C. albicans tac1Δ/Δ* mutant and *TAC1* revertant strains with different *TAC1* alleles. Drug susceptibility assays were carried out by plating serial dilutions of overnight cultures onto YEPD agar plates containing different drugs as indicated. Plates were incubated for 48 hr at 35°. MIC assays were performed as described in MATERIALS AND METHODS. (B) Immunodetection of Cdr1p and Cdr2p in *tac1Δ/Δ* mutant and *TAC1* revertant strains with different *TAC1* alleles. Protein extracts of each strain were separated on SDS-10% polyacrylamide gels and immunoblotted with rabbit polyclonal anti-Cdr1p and anti-Cdr2p as described previously (DE MICHELI *et al.* 2002). *C. albicans* strains were grown in liquid YEPD to midlog phase and exposed (+) or not (−) to fluphenazine (10 μg/ml) for 20 min. The following Ura$^+$ strains correspond to the following genotypes: CAF2-1, *TAC1-1/TAC1-2*; DSY2903, *tac1-1Δ/tac1-2Δ*; DSY2925-47, *tac1Δ/Δ + TAC1-3*; DSY2925-18, *tac1Δ/Δ + TAC1-4*; DSY2984, *tac1Δ/Δ + TAC1-5*; DSY3010-80, *tac1Δ/Δ + TAC1-6*; DSY3010-113, *tac1Δ/Δ + TAC1-7*-FH1; DSY3013, *tac1Δ/Δ + TAC1-7*-FH3. For phenotypes and genotypes of the different strains of this study refer to supplemental Table S1 at http://www.genetics.org/supplemental/.

lane 6). In contrast, *TAC1-7* conferred terbinafine resistance (Figure 1A) and expressed constitutive high levels of Cdr1p and Cdr2p (Figure 1B, lane 7). When strains FH3–FH8 were screened for *TAC1* alleles, all attempts to recover alleles different from *TAC1-7* were unsuccessful. These results indicate that strains FH3–FH8 are homozygous for the *TAC1-7* allele. Consistent with this, these strains were also homozygous at the *MTL*, suggesting that they underwent an LOH event encompassing >14 kb of chromosome 5.

Taken together, the analysis of *TAC1* alleles from clinical strains identified five new alleles in addition to the *TAC1-1* and *TAC1-2* previously characterized from strain CAF2-1. Nucleotide polymorphisms in all these alleles are presented in Table 2. Because of the high diversity of *TAC1* alleles, their nomenclature was modified (Table 2) as compared to results of our previous study (COSTE *et al.* 2004). Among the five new alleles, three were defined as wild type and two as hyperactive. Furthermore, the two hyperactive alleles were the only alleles that were homozygous in two independent clinical, azole-resistant strains, a feature consistent with the linked homozygosity at the mating-type locus.

TAC1 alleles can be hyperactive through a mutation in the C-terminal domain: *TAC1* sequences were aligned to identify differences between wild-type and hyperactive alleles. Wild-type *TAC1-4* and hyperactive *TAC1-5* alleles differed by only one base in codon 977 (Table 2). This nonsynonymous point mutation changes asparagine (N) in *TAC1-4* to aspartic acid (D) in *TAC1-5*. Furthermore, none of the wild-type alleles contain this point mutation. Importantly, the *TAC1-7* hyperactive allele contains the same nucleotide change that yields a N977D mutation in Tac1p. These results strongly suggest a relationship between the presence of the N977D mutation and the hyperactivity of *TAC1-5* and *TAC1-7*. Nineteen other codons with nonsynonymous polymorphisms were detected among the seven allele sequences compared in this study (Table 2). Since these were present in both wild-type and hyperactive alleles, they were not associated with azole resistance.

To test the role of the N977D mutation in *TAC1* hyperactivity, Asp^{977} was introduced by site-directed mutagenesis into wild-type alleles *TAC1-1*, *TAC1-4*, and *TAC1-6*. The complementary experiment, replacing Asp^{977} with Asn^{977} in the hyperactive alleles *TAC1-5* and *TAC1-7*, was
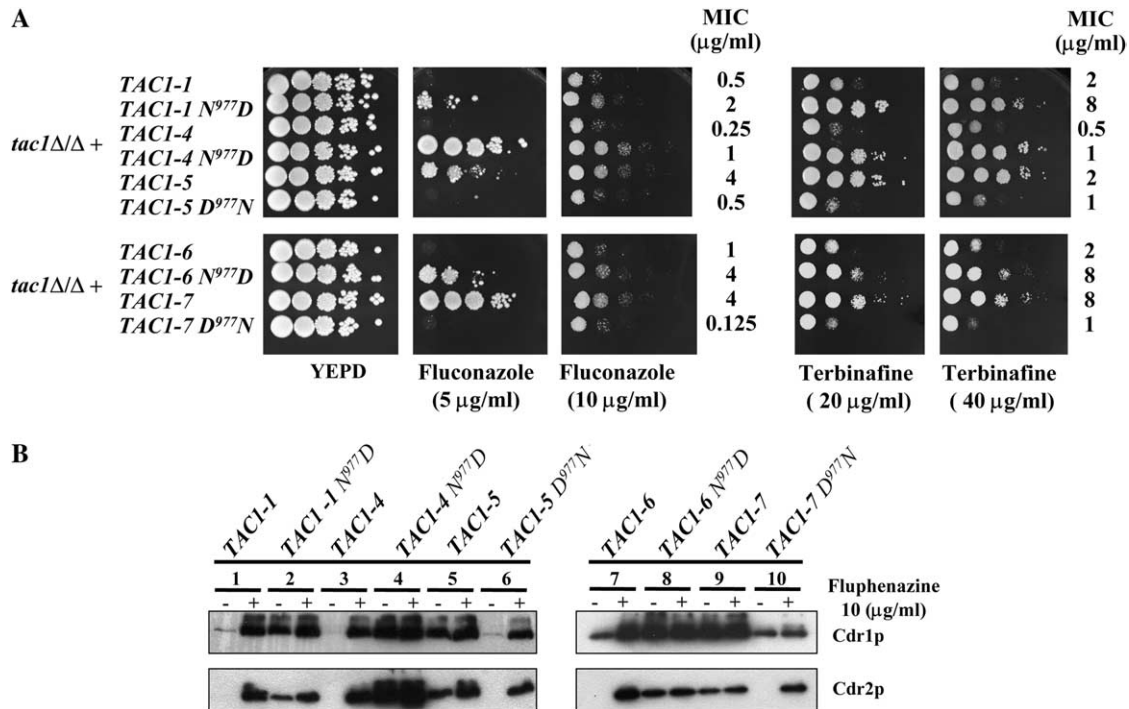
FIGURE 2.—Effect of the mutation N977D on the properties of *TAC1*. (A) Drug susceptibility testing of *C. albicans tac1*Δ/Δ mutant and *TAC1* revertant strains with different *TAC1* alleles containing the N977D substitution. Drug susceptibility assays were carried out as described in Figure 1. MIC assays were performed as described in MATERIALS AND METHODS. (B) Immunodetection of Cdr1p and Cdr2p in *C. albicans tac1*Δ/Δ mutant and *TAC1* revertant strains containing different *TAC1* alleles with the N977D substitution. See legend of Figure 1B for other details. The following strains correspond to the following genotypes: DSY2937-35, *tac1*Δ/Δ + *TAC1-1*; VTY21, *tac1*Δ/Δ + *TAC1-1^{N977D}*; VTY28, *tac1*Δ/Δ + *TAC1-4^{N977D}*; ACY11, *tac1*Δ/Δ + *TAC1-6^{N977D}*; ACY12, *tac1*Δ/Δ + *TAC1-7^{D977N}*; VTY9, *tac1*Δ/Δ + *TAC1-5^{D977N}*. See legend of Figure 1 for other strains and genotype designations.

also performed. The new alleles *TAC1-1^{N977D}*, *TAC1-4^{N977D}*, *TAC1-6^{N977D}*, *TAC1-5^{D977N}*, and *TAC1-7^{D977N}* were then introduced into a *tac1*Δ/Δ mutant strain and the drug resistance phenotypes, as well as the Cdr1p and Cdr2p levels, were measured in cells with or without exposure to fluphenazine. The presence of Asp^{977} in the modified alleles *TAC1-1^{N977D}*, *TAC1-4^{N977D}*, and *TAC1-6^{N977D}* increased resistance to fluconazole and terbinafine relative to the corresponding wild-type alleles (Figure 2A). Moreover, the presence of Asp^{977} in the modified alleles resulted in constitutive high levels of Cdr1p and Cdr2p (Figure 2B, lanes 2, 4, and 8). In contrast, expression of the corresponding wild-type alleles resulted in basal levels of Cdr1p. High levels of Cdr1p and Cdr2p expression were seen in strains carrying the wild-type alleles only upon fluphenazine exposure (Figure 2B, lanes 1, 3, and 7). When Asp^{977} was replaced by Asn^{977} in the hyperactive alleles (modified alleles *TAC1-5^{D977N}* and *TAC1-7^{D977N}*), the ability to grow on plates containing antifungal agents and the constitutive high levels of Cdr1p and Cdr2p were not maintained (Figure 2). Modified alleles *TAC1-5^{D977N}* and *TAC1-7^{D977N}* (Figure 2B, lanes 6 and 10) did not mediate constitutive high levels of Cdr1p and Cdr2p as compared to the nonmodified alleles *TAC1-5* and *TAC1-7* (Figure 2B, lanes 5 and 9). The phenotypes obtained by the strains carrying these modified hyperac-

tive alleles were similar to those obtained with strains containing the wild-type alleles *TAC1-1*, *TAC1-4*, and *TAC1-6* (Figure 2B, lanes 1, 3, and 7). Taken together, these analyses demonstrate that a single point mutation, the replacement of Asn by Asp at position 977 of Tac1p, is sufficient to modify the activity of the transcription factor into a hyperactive state.

Role of *TAC1* in drug susceptibility and Cdr1p/Cdr2p levels in clinical strains: As mentioned above, strains DSY294 and DSY296 are matched azole-susceptible and azole-resistant isolates. To demonstrate that azole resistance was coupled with the presence of hyperactive alleles, *TAC1* was inactivated in the background of these clinical strains. First, the *ura3* auxotrophic marker was introduced into these strains using the dominant marker *caSAT1* (REUSS *et al.* 2004). The deletion of *TAC1* in DSY294 had a moderate effect on fluconazole MIC in both the heterozygous and the homozygous mutants (Figure 3A, top). The fluconazole MIC values decreased from 1 μg/ml for DSY294 to 0.25 and 0.5 μg/ml in both mutant strains. Furthermore, in the *tac1*Δ/Δ mutant, fluphenazine exposure did not increase Cdr1p and Cdr2p levels (Figure 3B, lane 3). This result is similar to observations of CAF2-1 and its *tac1*Δ/Δ derivative strain (COSTE *et al.* 2004). These results highlight the crucial role of *TAC1* in both basal and induced
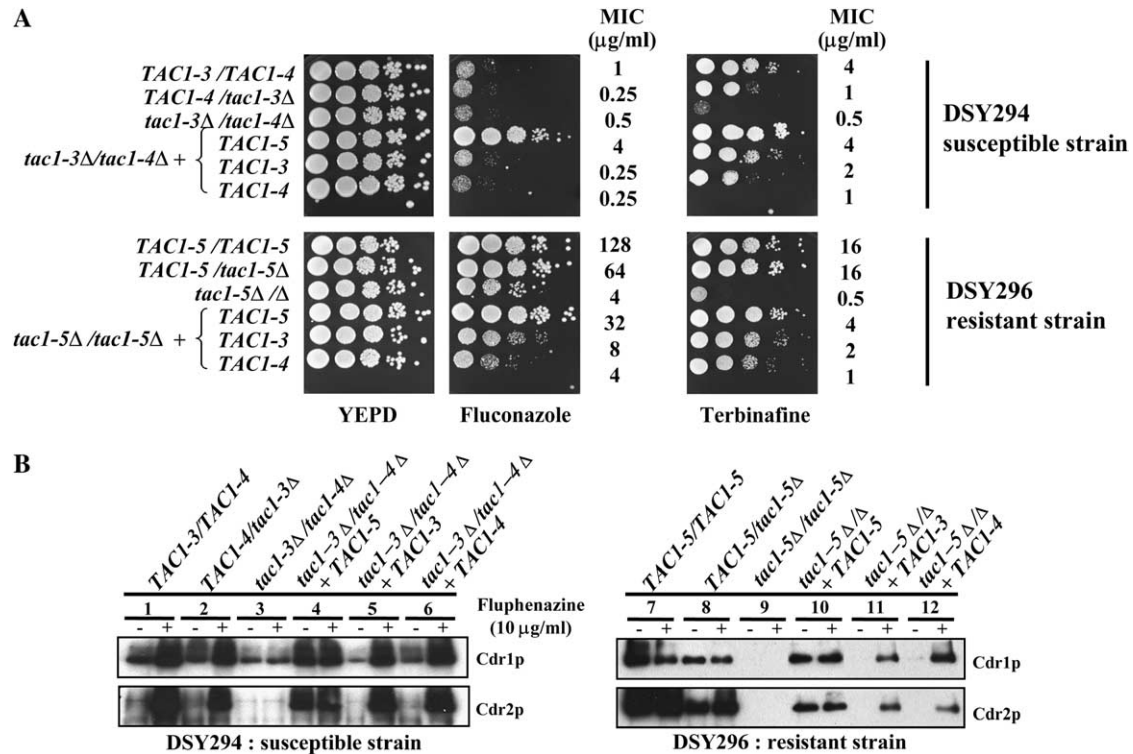
FIGURE 3.—Analysis of *TAC1* alleles from the clinical strains DSY294 and DSY296. (A) Drug susceptibility testing of *C. albicans* *tac1Δ/Δ* mutant and *TAC1* revertant clinical strains containing specific *TAC1* alleles. Drug susceptibility assays were carried out onto YEPD medium containing 2.5 μg/ml of fluconazole and 1 μg/ml cyclosporin A for the DSY294-derived strains and 5 μg/ml of fluconazole and 1 μg/ml cyclosporin A for the DSY296-derived strains. Cyclosporin A alone had no effect on the growth of these strains. All strains were spotted onto agar medium containing 20 μg/ml of terbinafine. Plates were incubated for 48 hr at 35°. MIC assays were performed as described in MATERIALS AND METHODS. (B) Immunodetection of Cdr1p and Cdr2p in *C. albicans* *tac1Δ/Δ* mutant and *TAC1* revertant clinical strains. See legend of Figure 1 for other details. The following strains correspond to the following genotypes: DSY294, *TAC1-3/TAC1-4*; DSY296, *TAC1-5/TAC1-5*; DSY3058, *TAC1-4/tac1-3Δ*; DSY3082; *tac1-3Δ/tac1-4Δ*; DSY3287-1, *tac1-3Δ/tac1-4Δ* + *TAC1-3*; DSY3288-3, *tac1-3Δ/tac1-4Δ* + *TAC1-4*; DSY3102-2, *tac1-3Δ/tac1-4Δ* + *TAC1-5*; DSY3059, *TAC1-5/tac1-5Δ*; DSY3083, *tac1-5Δ/Δ*; DSY3285-1, *tac1-5Δ/Δ* + *TAC1-3*; DSY3286-2, *tac1-5Δ/Δ* + *TAC1-4*; DSY33284-1, *tac1-5Δ/Δ* + *TAC1-5*.

expression of *CDR1* and *CDR2* in this clinical strain. The deletion of one copy of *TAC1-5* from DSY296 resulted in a slight decrease of fluconazole resistance (MIC varying from 128 to 64 μg/ml), while deletion of both *TAC* copies in DSY296 resulted in a significant reduction in fluconazole resistance (MIC varying from 128 to 4 μg/ml) (Figure 3A, bottom). A slight decrease in Cdr1p and Cdr2p levels was observed in *TAC1-5/tac1-5Δ* as compared to the wild-type strain (Figure 3B, right, lane 8), thus indicating that the *TAC1-5* hyperactive allele copy number has an impact on expression levels of these proteins. In the *tac1-5Δ/Δ* homozygous mutant, Cdr1p/Cdr2p levels were almost undetectable both with and without fluphenazine exposure, demonstrating a direct relationship between the presence of *TAC1* hyperactive alleles and azole resistance in these clinical strains (Figure 3B, lane 9). Interestingly, the MIC of fluconazole, but not of terbinafine, was higher in the *tac1Δ/Δ* DSY296-derived strain (4 μg/ml) than in the *tac1Δ/Δ* DSY294-derived strain (0.5 μg/ml). This discrepancy may be explained by the presence of a mutation in both *ERG11* alleles in DSY296 (G464S) that alters binding of

azoles to Erg11p and therefore contributes to fluconazole resistance (SANGLARD *et al.* 1998).

To characterize the properties of *TAC1* alleles from DSY294 and DSY296, the activity of *TAC1-3*, *TAC1-4*, and *TAC1-5* was first assessed in the DSY294 and DSY296 backgrounds, which are mating type heterozygous and homozygous, respectively. Each type of *TAC1* allele was reintroduced into the *tac1Δ/Δ* mutant strains derived from DSY294 and DSY296. *TAC1* activity profiles for these alleles were similar to those observed in the CAF2-1 background. *TAC1-3* and *TAC1-4* did not confer resistance to fluconazole or terbinafine (Figure 3A) in either strain backgrounds. Consistently, they exhibited basal levels of Cdr1p and no Cdr2p and did mediate high Cdr1p/Cdr2p levels in the presence of fluphenazine (Figure 3B, lanes 5, 6, 11, and 12). *TAC1-5* restored resistance to fluconazole and terbinafine in either strain backgrounds (Figure 3A) and constitutive high Cdr1p and Cdr2p levels (Figure 3B, lanes 4 and 10).

To correlate Cdr1p and Cdr2p levels with the capacity to efflux an ABC-transporter substrate, rhodamine 6G efflux was monitored in clinical strains DSY294 and
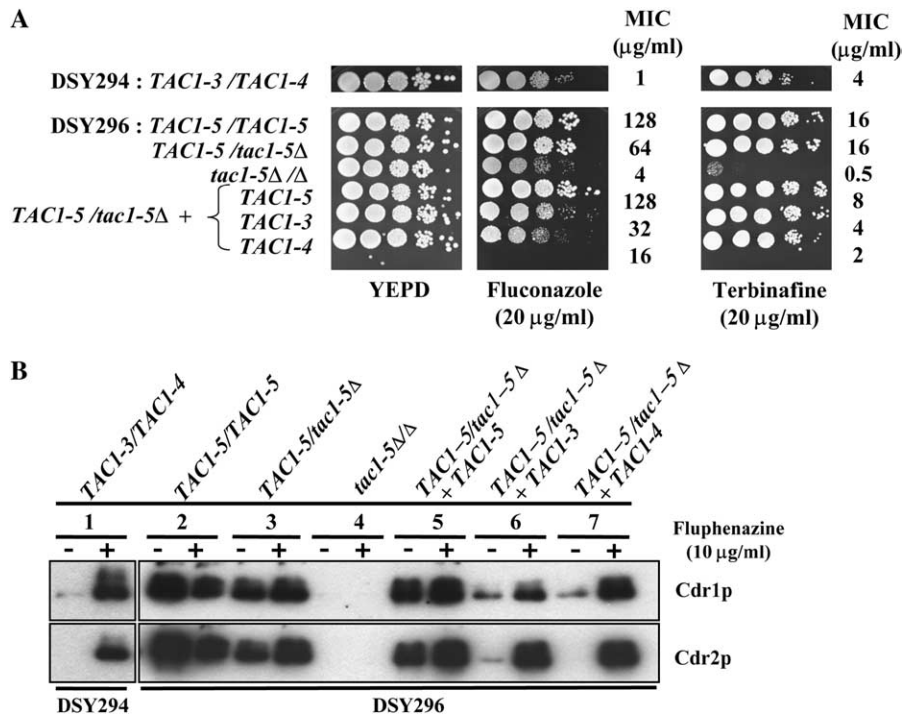
A

B

FIGURE 4.—Codominance of the *TAC1-5* hyperactive allele. (A) Drug susceptibility testing of the *C. albicans* clinical isolates DSY294 and DSY296, heterozygous (*TAC1-5/tac1-5Δ*) and homozygous (*tac1-5Δ/Δ*) mutants derived from DSY296, and transformants of the heterozygous mutant in which a *TAC1-3*, *TAC1-4*, or *TAC1-5* allele was reintroduced. See legend of Figure 1 for other details. (B) Immunodetection of Cdr1p and Cdr2p in the strains listed above. See legend of Figure 1 for other details. The following strains correspond to the following genotypes: DSY3211-4, *TAC1-5/tac1-5Δ + TAC1-3*; DSY3215-1, *TAC1-5/tac1-5Δ + TAC1-4*; DSY3210-1, *TAC1-5/tac1-5Δ + TAC1-5*. See the legend of Figure 3 for other strain and genotype designations.

DSY296 as well as in the *tac1Δ/Δ* derivatives (supplemental Figure S1, A and B, at http://www.genetics.org/supplemental/). The DSY296 strain exhibited higher rhodamine 6G efflux rates (average V_{max}: 5.7 RFU/sec) than the DSY294 strain (average V_{max}: 1.3 RFU/sec), which is consistent with the differences in Cdr1p and Cdr2p levels between the two strains. In the *tac1Δ/Δ* mutant strains from both isolates, rhodamine 6G efflux rates were similar to the rates observed in DSY294. The presence of *TAC1-5* in the background of DSY294 *tac1Δ/Δ* mutants resulted in higher rhodamine 6G efflux rates (V_{max} values between 3.8 and 4.1 RFU/sec) than those observed in DSY294, in *tac1Δ/Δ* mutants, and in *TAC1-3* or *TAC1-4* revertant strains (supplemental Figure S1A at http://www.genetics.org/supplemental/). Thus, the *TAC1-5* hyperactive allele is necessary for increased Cdr1p/Cdr2p levels, which is also correlated to enhanced efflux rates and is independent of strain background. Importantly, since the *MTL* locus in DSY294 is heterozygous, these studies also show that *MTL* homozygosity does not have a detectable effect on azole resistance.

Codominance of *TAC1* alleles with the N977D substitution: Homozygosity at the mating-type locus was reported to correlate with the occurrence of azole resistance (RUSTAD *et al.* 2002). Hence, azole-resistant strains used in this study are homozygous at the mating-type locus and consequently contain a single *TAC1* allele. It is possible that the development of the drug resistance phenotype necessitates hyperactive *TAC1* alleles in a homozygous state, suggesting that these alleles are recessive to wild-type alleles. This rationale is supported

by the profiles of *TAC1* alleles in strain FH1: although this strain contains both a wild-type and a hyperactive *TAC1* allele, this strain remains fluconazole susceptible.

***TAC1* status in strains DSY294 and DSY296:** The above-described analyses were performed in *tac1Δ/Δ* homozygous mutant strains and therefore the dominance/recessivity relationships of *TAC1* alleles could not be determined. To address this question, *TAC1* alleles (*TAC1-3–TAC1-5*) were reintroduced in DSY294 and DSY296 *TAC1/tac1Δ* heterozygotes, thus generating heterozygous strains, each carrying two different *TAC1* alleles.

When the *TAC1-5* allele was introduced at the genomic *TAC1* locus in the heterozygous mutant strain *TAC1-5/tac1-5Δ*, the obtained revertant (*TAC1-5/tac1-5Δ + TAC1-5*) exhibited phenotypes similar to those observed in DSY296. The fluconazole and terbinafine MICs were similar in both strains: while the fluconazole MICs were 128 μg/ml for both strains, the terbinafine MICs were 16 and 8 μg/ml for DSY296 and the revertant, respectively (Figure 4A). Constitutive high levels of Cdr1p and Cdr2p were also similar between these strains (Figure 4B, lanes 2 and 5). When wild-type allele *TAC1-3* or *TAC1-4* was introduced into the *TAC1-5/tac1-5Δ* strain, the transformants had decreased resistance phenotypes compared to DSY296. Fluconazole MIC decreased from 128 μg/ml to 32 and 16 μg/ml for the *TAC1-3* and *TAC1-4* alleles, respectively; terbinafine MICs decreased from 16 to 4 and 2 μg/ml, respectively (Figure 4A). Moreover, constitutive high Cdr1p and Cdr2p levels were reduced in these heterozygotes compared to DSY296 (Figure 4B, lanes 2, 6,
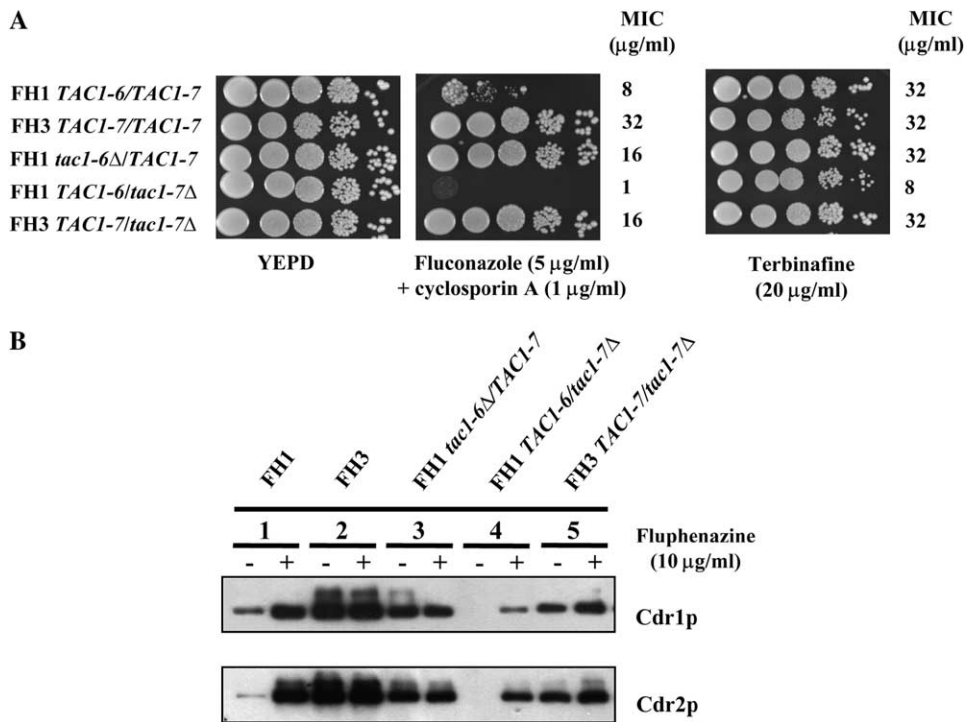
A

B

FIGURE 5.—Analysis of the *TAC1* alleles from clinical strains FH1 and FH3. (A) Drug susceptibility testing of *C. albicans* FH1 and FH3 derivatives lacking one specific *TAC1* allele. See legend of Figure 1 for other details. (B) Immunodetection of Cdr1p and Cdr2p in *C. albicans* FH1 and FH3 derivatives lacking one specific *TAC1* allele. See legend of Figure1 for other details. The following strains correspond to the following genotypes: DSY3132-11, *TAC1-6/tac1-7*Δ; DSY3132-14, *TAC1-7/tac1-6*Δ; DSY3133-15, *TAC1-7/tac1-7*Δ; FH1, *TAC1-6/TAC1-7*; FH3, *TAC1-7/TAC1-7*.

and 7). Cdr1p levels in these *TAC1-5/tac1-5*Δ + *TAC1-3* and *TAC1-5/tac1-5*Δ + *TAC1-4* strains were slightly higher than those observed in the azole-susceptible strain DSY294 (Figure 4B, lanes 1, 6, and 7). Cdr2p levels, although barely detectable in the heterozygote with *TAC1-3*, were not detectable in the heterozygote with *TAC1-4* (Figure 4B, lanes 6 and 7) as in DSY294 (Figure 4B, lane 1). Induction of Cdr1p and Cdr2p expression by fluphenazine did occur in these heterozygotes. Thus these two heterozygous strains have phenotypes intermediate between that of the azole-susceptible strain DSY294 and the azole-resistant strain DSY296. For example, the heterozygotes with *TAC1-5* and *TAC1-3* alleles or *TAC1-5* and *TAC1-4* alleles have fluconazole MICs of 32 and 16 μg/ml, respectively, *vs.* 1 μg/ml for DSY294 (*TAC1-3/TAC1-4*) and 128 μg/ml for DSY296 (*TAC1-5/TAC1-5*). Similarly, DSY294-derived heterozygous strains *TAC1-3/tac1-4*Δ and *tac1-3*Δ/*TAC1-4* transformed with the *TAC1-5* allele had an intermediate phenotype in terms of drug resistance and Cdr1p and Cdr2p levels (data not shown). Taken together, our results suggest that the hyperactivity of the *TAC1-5* allele is decreased in the presence of a wild-type allele. This implies that the hyperactive *TAC1-5* allele is codominant with wild-type alleles *TAC1-3* and *TAC1-4*. Moreover, these results confirm the codominance hypothesis of the azole resistance phenotype that was proposed from fusion experiments between DSY296 and an azole-susceptible strain with opposite mating type (Coste *et al.* 2004).

TAC1 status in strains FH1 and FH3: The development of drug resistance in strain FH1 is of interest because this strain contains both a wild-type allele (*TAC1-6*) and a hyperactive allele (*TAC1-7*). Consistent with this, strain FH1 exhibited intermediate levels of drug resistance and Cdr1p/Cdr2p levels. FH1 has MICs of 8 and 32 μg/ml for fluconazole and terbinafine, respectively (Figure 5A). Cdr1p and Cdr2p levels in normal growth conditions are higher in FH1 than those in CAF2-1 and DSY294 and are still inducible after fluphenazine exposure (Figure 5B, lane 1).

To analyze the phenotypes of individual alleles, each *TAC1* allele in strain FH1 was deleted. Whereas the FH1 strain possesses fluconazole and terbinafine MICs of 8 and 32 μg/ml, respectively (Figure 5A), the drug resistance of the *TAC1-6/tac1-7*Δ mutant decreased (fluconazole and terbinafine MICs of 1 and 8 μg/ml; Figure 5A). Both Cdr1p and Cdr2p levels decreased as well (Figure 5B, lanes 1 and 4). In contrast, when compared to FH1, the fluconazole resistance of the *tac1-6*Δ/*TAC1-7* mutant increased from 8 to 16 μg/ml and Cdr1p and Cdr2p levels increased as well (Figure 5B, lanes 1 and 3). These phenotypes resemble those of FH3 and are almost identical to those observed in the FH3-derived *TAC1-7/tac1-7*Δ strain (Figure 5, A and B, lane 2).

In summary, the hyperactive *TAC1-7* allele possesses codominant properties similar to those described for *TAC1-5*. The presence of *TAC1-7* together with a wild-type allele results in intermediate drug resistance properties. Strains carrying two hyperactive alleles (*i.e.*, DSY296 and FH3) exhibit the strongest phenotypes in terms of drug susceptibility and Cdr1p/Cdr2p levels. Therefore, development of high resistance to azoles in a *TAC1* heterozygous strain carrying one hyperactive

allele is due to LOH involving removal of the *TAC1* wild-type copy and maintenance of the hyperactive allele. Since *TAC1* is only 14 kb from the *MTL,* it appears that the mechanisms that result in LOH at *TAC1* are often accompanied by LOH at *MTL.*

Stages in the development of azole resistance: The mechanisms by which hyperactive alleles can become homozygous in azole-resistant strains remain unknown. One possibility is that, during azole exposure of a strain heterozygous at the *TAC1* locus, the chromosome carrying a *TAC1* hyperactive allele can be duplicated. This would then be followed by loss of one chromosome 5 copy with a wild-type *TAC1* allele, thus resulting in homozygosity of the remaining chromosome 5 copies. Alternatively, the chromosome 5 copy with the wild-type *TAC1* allele could be lost first and then the remaining copy could be duplicated. A third possibility is that the region containing *TAC1* (and *MTL*) undergoes a local recombination event that results in gene conversion of a long region of the chromosome.

Development of in vitro fluconazole resistance: To ask how LOH and fluconazole resistance occurs, we followed the *in vitro* acquisition of fluconazole resistance in strain FH1 and analyzed the newly resistant strains for their chromosome 5 and *TAC1* status. FH1 was spotted onto a YEPD plate supplemented with 10 μg/ml of fluconazole. The plate was incubated at 30° until resistant colonies appeared. The medium contained cyclosporine A to suppress residual growth of *C. albicans* in the presence of fluconazole as described (MARCHETTI *et al.* 2000). One resistant colony (DSY3157-2) was plated onto YEPD with 10 μg/ml fluconazole and fast-growing colonies were obtained. One of these, DSY3301-4, as well as strains FH1, FH3, and DSY3157-2, were analyzed for azole susceptibility and Cdr1p/Cdr2p protein levels. Strains FH1, DSY3157-2, and DSY3301-4 showed increasing fluconazole resistance (MIC of 8, 16, and 32 μg/ml, respectively, Figure 6A). These strains also exhibited increasing Cdr1p and Cdr2p levels as compared to FH1 under normal growth conditions (Figure 6B, lanes 1, 3 and 4). Furthermore, DSY3301-4 and FH3 had identical fluconazole MICs (32 μg/ml) and exhibited similar Cdr1p and Cdr2p levels in both the absence and the presence of fluphenazine (Figure 6B, lanes 2 and 4).

Analysis of TAC1 and chromosome 5 status: The stepwise increase of fluconazole resistance from FH1 to DSY3157-2 and DSY3301-4 could have occurred if *TAC1-7* was duplicated in DSY3157-2 while *TAC1-6* was maintained. In DSY3301-4, *TAC1-6* could have been lost, leaving two *TAC1-7* copies. To test these step hypotheses, we determined the status of the *MTL* loci. Both FH1 and DSY3157-2 were heterozygous (*MTLa/MTLα*) while both FH3 and DSY3301-4 were mating type homozygous (*MTLα/MTLα*) (Figure 6C). Southern blot analysis of the *TAC1* loci of strains FH1, FH3, DSY3157-2, and DSY3301-4 confirmed that FH1 and DSY3157-2 con-

tained two distinct alleles (1.8 and 3.2 kb), while FH3 contained only the 3.2-kb signal corresponding to *TAC1-7.* Interestingly, the signal ratio for *TAC1-7:TAC1-6* was 0.98:1 in FH1 and 2.1:1 in DSY3157-2. This analysis is consistent with the idea that, in strain DSY3157-2, the genotype is *TAC1-7/TAC1-7/TAC1-6.* Comparative genome hybridization array analysis (Figure 7) supports the first part of this step hypothesis: strains FH1 and FH3 are disomic for all genes on chromosome 5, while chromosome 5 is trisomic in strain DSY3157-2. In addition, chromosome 5 is trisomic in DSY3301-4, suggesting that this strain carries three copies of *TAC1-7, MTLα,* and all of the other genes on chromosome 5. Thus, either the copy of chromosome 5 carrying *MTLa* and *TAC1-6* was lost and another copy of a remaining chromosome 5 was duplicated or, alternatively, a region of chromosome 5 containing *MTLa* and *TAC1-6* underwent gene conversion.

Analysis of chromosome 5 alterations: To distinguish between the above mechanisms of LOH in strain DSY3301-4, we conducted SNP microarray hybridization for 13 SNP loci evenly distributed across chromosome 5 (FORCHE *et al.* 2005). This analysis showed that 12 of these loci were homozygous in all four strains. One SNP locus (marker 104), located within the *SNF1* ORF (orf19.1936), was heterozygous in strains FH1, FH3, and DSY3157-2. This locus became homozygous in strain DSY3301-4 (Figure 8, top).

To obtain additional information, 10 more genes, not present on the SNP arrays (*CRH12,* orf19.4251, orf19.1926, orf19.4288, *TRX1, TRR1, ZNC3,* orf19.4225, orf19.2646, and orf19.6680, solid triangles in Figure 8), were sequenced to determine the allelic status in each of the four strains. Of these 10 genes, 6 were uninformative because they were homozygous for all four strains. The remaining 4 genes (*CRH12,* orf19.4251, orf19.1926, orf19.4288; see Table 3) were heterozygous in strains FH1, FH3, and DSY3157-2 and homozygous in strain DSY3301-4 (markers B, E, F, and J of Figure 8, top). Since the SNP loci that became homozygous in DSY3301-4 are distributed all along chromosome 5, this result is consistent with the idea that all of chromosome 5 became homozygous in DSY3301-4. It is likely that in DSY3301-4 a homolog carrying the *TAC1-7* allele replaced the chromosome carrying *TAC1-6.*

Interestingly, the mechanism by which FH3 became homozygous for *TAC1-7* appears to be different from that of DSY3301-4; this strain shows LOH only between the *MTL* and *TAC1* loci. Rather, it appears that a mitotic recombination event occurred between the two chromosome 5 copies of strain FH3, thus leading to gene conversion of the *TAC1-6* and *MTLa* to *TAC1-7* and *MTLα* (see Figure 9, top, for schematic).

TAC1 and chromosome 5 status in clinical strains DSY294 and DSY296: To ask about the mechanism by which strain DSY296 became azole resistant, we analyzed the organization of chromosome 5 homologs in this strain
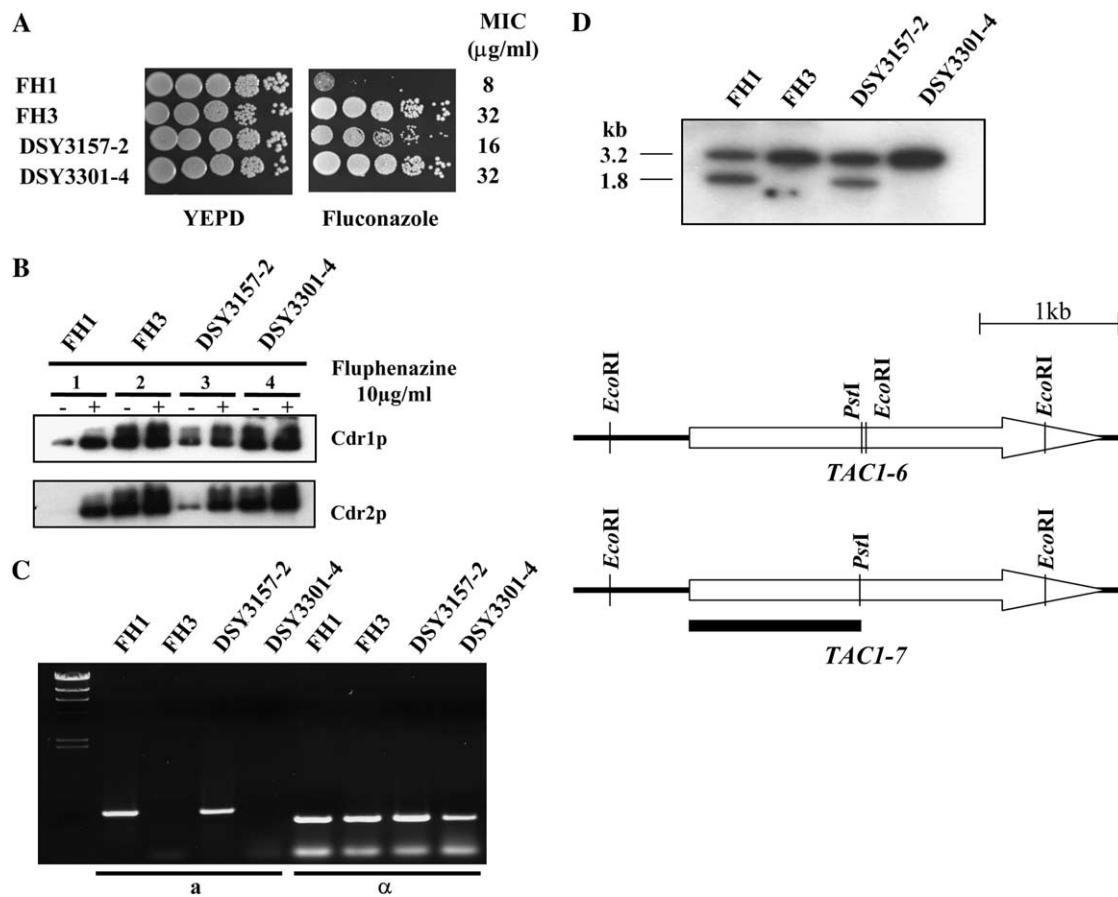
FIGURE 6.—Analysis of FH1-derived strains after *in vitro* fluconazole exposure. (A) Drug susceptibility testing of *C. albicans* FH3-, FH1-, and FH1-derived strains selected for their fluconazole resistance. DSY3157-2 was derived from a single colony of FH1 that arose after spotting onto YEPD medium with 10 μg/ml fluconazole. DSY3301-4 was derived from DSY3157-2 as a fast-growing colony onto medium with 10 μg/ml fluconazole. Drug susceptibility assays were carried out as described in Figure 1 onto YEPD medium containing 10 μg/ml fluconazole and 1 μg/ml cyclosporin A. Cyclosporin A alone had no effect on the growth of these strains. Plates were incubated for 48 hr at 35°. MIC assays were performed as described in MATERIALS AND METHODS. (B) Immunodetection of Cdr1p and Cdr2p in *C. albicans* strains FH1, FH3, DSY3157-2, and DSY3301-4. See legend of Figure 1 for other details. (C) PCR analysis of the mating-type locus. PCR was performed as described (RUSTAD *et al.* 2002). "a" and "α" denote analysis performed to detect *MTLa* and *MTLα* loci, respectively. (D) Southern blot analysis of the *TAC1* alleles in *C. albicans* FH-derivative strains. Genomic DNA of each strain was digested by *Eco*RI and Southern blot was performed as described in MATERIALS AND METHODS. Radioactivity of signals was quantified as discussed in MATERIALS AND METHODS. Restriction maps of both *TAC1-6* and *TAC1-7* alleles indicate restriction site polymorphism for *Eco*RI. The solid bar indicates the position of the labeled probe, which corresponded to the region located between the first *TAC1* initiation codon and the *Pst*I restriction site. Digestion of genomic DNA with *Eco*RI is expected to yield positive signals at 1.8 and 3.2 kb for *TAC1-6* and *TAC1-7*, respectively.

as well. Comparative genome hybridization array analysis indicated that both strains DSY294 and DSY296 carried two copies of chromosome 5 (data not shown). SNP microarray hybridization revealed that 10 of the 13 SNP markers on the array were homozygous in both strains. Markers 110 and 111 were homozygous only in DSY296 and marker 109 was heterozygous in both strains (Figure 8, bottom). Seven other genes (*CRH12*, orf19.4251, orf19.1926, *TRX1*, *TRR1*, orf19.4225 and orf19.6680) were amplified and sequenced along chromosome 5 as described above. DSY294 and DSY296 were homozygous for *TRX1*, orf19.1926, and orf19.6680 and heterozygous for orf19.4225, orf19.4251, *TRR1*, and *CRH12* (Figure 8, bottom). Since strain DSY296 is heterozygous for five polymorphisms tested here (marker

109, orf19.4225, orf19.4251, *TRR1* and *CRH12*), we conclude that LOH at *TAC1* and *MTL* was not caused by duplication of all of chromosome 5. Rather, it appears that mitotic recombination between two copies of chromosome 5 likely resulted in LOH at these loci (see Figure 9, bottom, for schematic). We propose that an intermediate strain, carrying one *TAC1-3* and one *TAC1-5* allele (derived from *TAC1-4*), underwent mitotic recombination and gene conversion leading to homozygosis of the *TAC1-5* alleles in strain DSY296.

Taken together, our results show that increased antifungal drug resistance due to constitutively high expression of the ABC transporters Cdr1p and Cdr2p can be achieved when *TAC1* alleles carry the N977D mutation and become homozygous. Homozygosis can occur
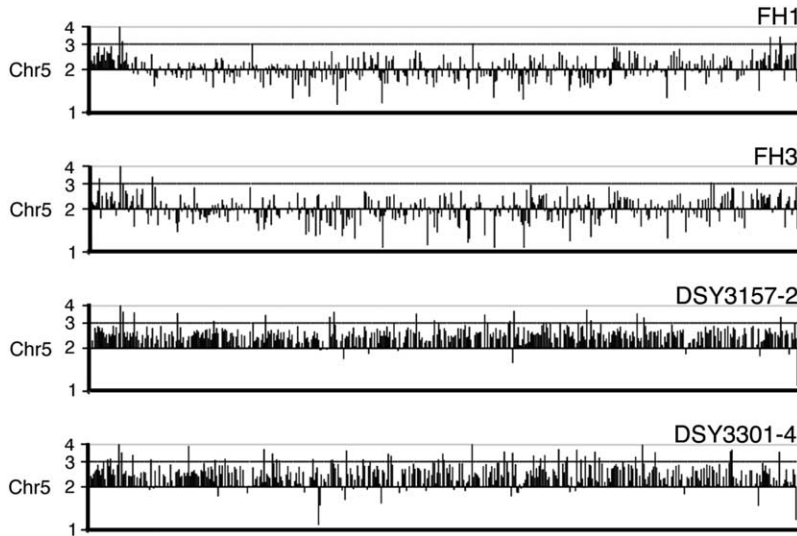
FIGURE 7.—CGH of FH-derived strains. The genomes of the tested strains were hybridized against the SC5314 genome according to the protocol published by SELMECKI *et al.* (2005). Each gene on chromosome 5 is represented by its relative intensity as compared to signals obtained in SC5314.

either by mitotic recombination between chromosome 5 copies that results in gene conversion or by the presence of extra copies of chromosome 5 carrying the hyperactive *TAC1* allele accompanied by loss of chromosome 5 carrying the wild-type *TAC1* allele.

DISCUSSION

A mutation in *TAC1* is involved in azole resistance: In this work, we established for the first time a link between a point mutation in a transcription factor and the constitutive high expression of the multidrug trans-

porters Cdr1p and Cdr2p responsible for antifungal drug resistance. It was previously shown that azole resistance can be due to point mutations in *ERG11*, which encodes the enzyme target of azoles. It is believed that these mutations can alter the affinity of azoles for their target and therefore can participate in the development of resistance (SANGLARD *et al.* 1998; PEREA *et al.* 2001). Among azole-resistant strains with altered *ERG11* alleles, MARICHAL *et al.* (1999) observed three "hot spots" localized in the region 105–165, 266–287, and 405–488 of Erg11p. Resistance to other antifungal drugs such as 5-fluorocytosine (5-FC) and caspofungin was also shown to be due to point mutations in specific
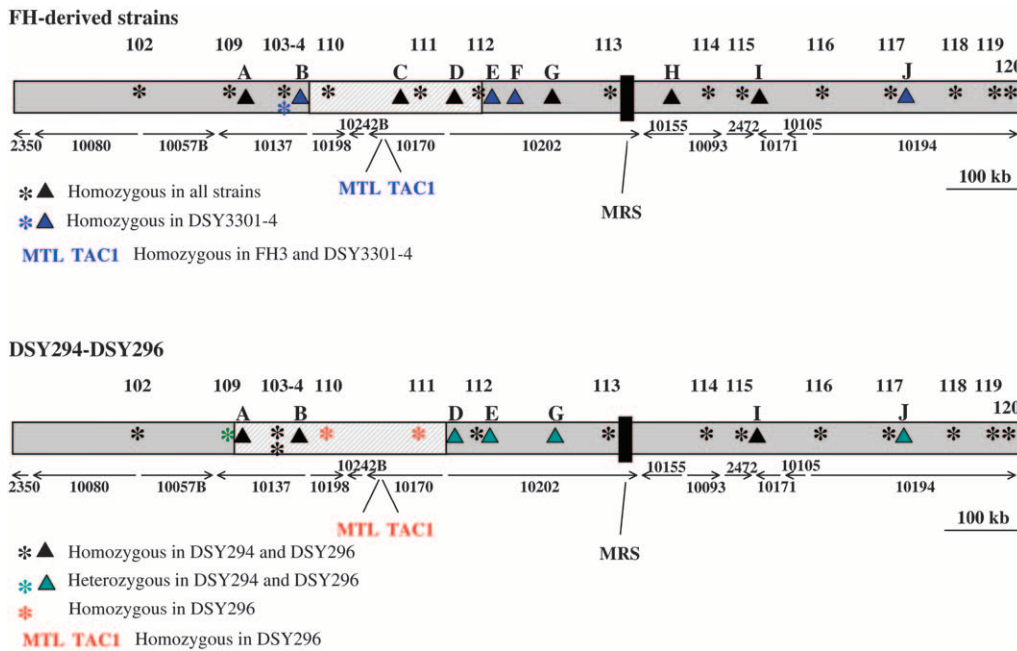

FIGURE 8.—SNP analysis of chromosome 5. The map of chromosome 5 was obtained by assembly of the different contig sequences shown by arrows. An asterisk (*) indicates SNPs of chromosome 5 as measured on the SNP microarray: 102, 1855/2172; 103, *HST3*; 104, *SNF1*; 109, 1899/2008; 110, 1445/2395; 111, 1922/2344; 112, PDE1; 113, 1969/2162; 114, *DPH5*; 115, *HEX1*; 116, 2093/2390; 117, 1817/2082; 118, 1341/2493; 119, F16n1; 120, 2340/2493. "▲" indicates additional markers of chromosome 5: A, orf19.1976 (*TRX1*); B, orf19.1926; C, *ZNC3*; D, orf19.4225; E, orf19.4251; F, orf19.4288; G, orf19.1942 (*TRR1*); H, orf19.2646; I, orf19.6680; J, CRH12. Color codes indicate modifications in SNPs for individual strains. For the strains DSY294 and DSY296, sequences of the C, F, and H markers were not available. The hatched regions on chromosome 5 delimitate the maximal region of a recombination. MRS, major repeat sequence.

TABLE 3

Nucleotide polymorphisms in chromosome 5 in FH1–FH3 and derivative strains

	Genes and position of SNPs with respect to ATG											
	orf19.4251			orf19.1926						orf19.4288:	CRHI12		
Strains	576	899	945	684	708	720	741	870	876	893	135	404	510
FH1, FH3, DSY3157-2	C or T	A or G	C or T	A or G	A or G	A or G	C or T	A or G	C or T	C or G	C or T	C or T	C or T
DSY3301-4	T	A	T	A	G	G	T	A	T	C	C	T	C

genes (Dodgson *et al.* 2004; Park *et al.* 2005). Resistance to 5-FC is restricted to clade I and due to a single point mutation, C301T, in the *FUR1* gene, encoding a phosphoribosyltransferase. This nonsynonymous mutation changes arginine to cysteine at position 101 of Fur1p (Dodgson *et al.* 2004). Recently, Park *et al.* (2005) showed that the modification of the serine 645 of CaFks1p, a subunit of the 1,3-β-D-glucan synthase, is sufficient to confer reduced susceptibility to echinocandins in *C. albicans.*

The single nucleotide mutation in codon 977 (A to G at nt 2929) in *TAC1* corresponds to a nonsynonymous modification from Asn to Asp in Tac1p. This mutation is located within a putative C-terminal activation domain of the transcription factor. The importance of this region for transcriptional activity was confirmed by preliminary experiments that deleted the C-terminal region from aa 801 to the C-terminal end of Tac1p. This deletion resulted in a truncated Tac1p unable to activate the expression of at least *CDR2* in presence of fluphenazine (D. Sanglard, unpublished results). Further experiments will be needed to elucidate how the N977D point mutation transforms Tac1p into a hyperactive state. Several mutations in the C-terminal activation domain region of the *S. cerevisiae* transcription factors Pdr1p or Pdr3p are also responsible for antifungal drug resistance and for upregulation of *PDR5*. These include *pdr1-3* (F815S; Carvajal *et al.* 1997), *pdr1-8* (L1036W; Carvajal *et al.* 1997), *pdr1-12* (L1044Q; Wendler *et al.* 1997), *PDR1-101* (T879M; Reid *et al.* 1997), *PDR1* (R821H; Tuttle *et al.* 2003), *pdr3-17* (G834D), *pdr3-18* (G834S), *pdr3-19* (L837S), and *pdr3-20* (G957N; Carvajal *et al.* 1997). Gao *et al.* (2004) showed that a strain carrying the hyperactive *pdr1-3* allele expressed *PDR5* in a drug-independent manner. This can be associated with enhanced promoter occupancy of coactivator complexes, including SAGA, Mediator, chromatin-remodeling SWI/SNF complex, and TATA-binding protein. Using chromatin immunoprecipitation, loss of contacts between histones and DNA was demonstrated at *PDR5* promoter and coding sequences (Gao *et al.* 2004). Other mechanisms to activate zinc-finger regulators have also been described, including nuclear-cytoplasmic shuffling (Gorner *et al.* 1998; Santos and de Larrinoa 2005), dimerization (Rottensteiner *et al.* 1997), DNA binding, phosphorylation (Sadowski *et al.* 1996; Kren *et al.* 2003), and unmasking of the activation domain (Sadowski *et al.* 1996). Auto-induction also can be envisaged as in the case of *PDR3* (Delahodde *et al.* 1995). Tac1p is present in the nucleus of cells in normal growth conditions (Coste *et al.* 2004). Thus, it is likely that Tac1p hyperactivity involves constitutive binding to the DRE in the promoter of *CDR1* and *CDR2*, constitutive phosphorylation of the protein, and/or a change in the conformation of the protein, which can lead to the constant unmasking of the activation domain.
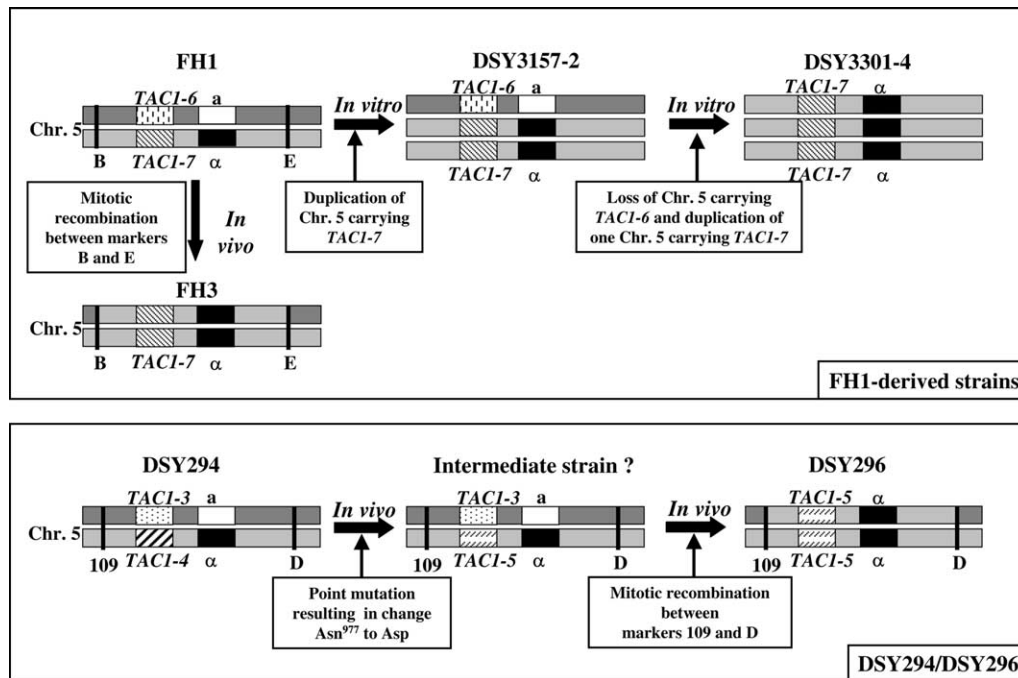
FIGURE 9.—Schematic of chromosome 5 alterations in matched azole-susceptible and azole-resistant strains obtained from patients or developed *in vitro*. (Top) Chromosome 5 alterations in FH1-derived strains both *in vitro* and *in vivo*. Chromosome 5 containing *TAC1-7* and *MTLα* was duplicated after *in vitro* fluconazole exposure in DSY3157-2; a chromosome 5 copy with *TAC1-6* and *MTLa* was lost in DSY3301-4 and replaced by another chromosome 5 copy containing *TAC1-7* and *MTLα* after a second fluconazole exposure; in FH3, a portion of chromosome 5 underwent mitotic recombination between markers B and E, resulting in *TAC1-7* and *MTLα* homozygosity. (Bottom) Chromosome 5 alterations in DSY294 and DSY296 *in vivo*. In DSY296, a portion of chromosome 5 underwent mitotic recombination between markers 109 and D, resulting in *TAC1-5* and *MTLα* homozygosity. An intermediate strain between DSY294 and DSY296 in which a single nucleotide change in *TAC1-4* yielded *TAC1-5* resulting in Asn^{977} to Asp could have existed. *TAC1-3*, *-4*, and *-6* are defined as wild-type alleles; *TAC1-5* and *-7* are defined as hyperactive alleles. "a" and "α" designate MTL types. Position of markers 109, B, D, and E are indicated in Figure 8.

TAC1 hyperactive alleles are codominant with wild-type alleles: Much like *PDR1* F815S and other *PDR1* gain-of-function alleles, hyperactive *TAC1* N977D alleles are codominant with wild-type alleles. This suggests that high levels of antifungal drug resistance cannot be achieved in the presence of wild-type alleles. Rather, homozygosis of the hyperactive alleles is necessary for the development of high levels of azole resistance. Codominance of these alleles probably reflects the fact that Tac1p is a transcription factor belonging to the Zn_2–Cys_6 family, which often dimerize to bind a *cis*-acting element (MAMNUN *et al.* 2002). When two different *TAC1* alleles are expressed in the same cell, heterodimer formation is likely. A corollary to this hypothesis, which remains to be tested, is that heterodimers containing one hyperactive and one wild-type Tac1p cannot direct high levels of basal Cdr1p and Cdr2p expression.

Codominance is a feature shared with other alleles of genes involved in antifungal resistance such as *ERG11* or *FUR1*. WHITE (1997) showed that the R467K mutation in *ERG11* alone is not sufficient to confer azole resistance. Loss of allelic variation in *ERG11* is required to confer strong azole resistance (WHITE 1997). This observation was also made independently for other *ERG11* alleles in our laboratory (SANGLARD *et al.* 1998). DODGSON *et al.* (2004) found a semidominant relationship between the hyperactive and wild-type alleles of *FUR1* for the development of 5-FC resistance.

Duplication of and recombination between chromosome 5 homologs as mechanisms resulting in *TAC1* homozygosity: To observe strong drug resistance, the

codominance of *TAC1* N977D alleles requires of loss of allelic variation. This property of *TAC1* may explain the link between *MTL* homozygosity and antifungal drug resistance. In this study, we reconstituted the loss of allelic variation at the *TAC1* locus *in vitro*. Drug resistance increased in two steps: the first step corresponded to a duplication of the *TAC1-7* hyperactive allele along with all other chromosome 5-linked genes; the second step involved the replacement of the remaining wild-type *TAC1-6* allele by the hyperactive *TAC1-7* allele while the strain remained trisomic for chromosome 5. SNP analysis suggests that the mechanism by which this occurred was loss of the *MTLa* chromosome 5 homolog and gain of a third copy of the *MTLα* homolog. The order of these events is not clear, but appears to have involved all of chromosome 5.

We also observed other mechanisms of LOH: duplication and exchanges of parts of chromosome 5. Clinical strain FH3 did not undergo LOH for all investigated SNP markers and for 10 other genes situated on chromosome 5 (see Figure 8). In FH3, the LOH that led to homozygosity of *TAC1* and of *MTL* includes flanking regions that compose a 250-kb region delimitated by heterozygous loci orf19.1926 and orf19.4251 (Figure 9, hatched region of chromosome 5). Similarly, SNP analysis of strains DSY294 and DSY296 revealed that a mitotic recombination event in DSY296 comprising an ~300-kb fragment bordered by heterozygous loci 1899/2008 and orf19.4225 resulted in LOH at *TAC1* and *MTL*. This homozygosity was not restricted to the *MTLa/α* genes but extended into the *PAP* genes within *MTL*

as well (data not shown). This observation is not in agreement with the findings of GOLDMAN *et al.* (2004), which indicated that recombination occurring within the *MTL* locus of azole-resistant clinical isolates leads to homozygosity for the a/α genes but not to other genes of the *MTL* locus.

The experiments undertaken with strains DSY3157-2 and DSY3301-4 also revealed that chromosome 5 can become trisomic prior to the loss of one chromosome 5 copy. These results are partially consistent with those presented by WU *et al.* (2005), who showed that *MTL* homozygosity can occur by loss of one copy of chromosome 5 followed by duplication of the remaining copy. Our work suggests that several mechanisms contribute to LOH, which is consistent with the recognized elasticity of the *C. albicans* genome (IWAGUCHI *et al.* 2001; X. CHEN *et al.* 2004; IWAGUCHI *et al.* 2004). Although the number of isolates investigated here was limited, LOH at chromosome 5 was obtained by mitotic recombination in the investigated clinical isolates, whereas it was obtained by chromosome 5 loss and duplications in laboratory conditions. This difference might reflect a fitness cost for such events under the conditions encountered in the host. Therefore chromosome 5 mitotic recombinations rather might be selected *in vivo*. Additional azole-resistant clinical isolates should be investigated to address this question.

Association among azole resistance, *TAC1*, and the mating-type locus: The short distance between *TAC1* and *MTL* has interesting consequences. Under selective drug pressure, codominance of *TAC1* favors LOH at this locus and indirectly contributes to the appearance of *MTL* homozygous yeast strains. Importantly, our work with different *TAC1* alleles in different strain backgrounds that were heterozygous or homozygous for *MTL* did not detect any contribution of *MTL* in drug resistance. Nonetheless, selective pressure for LOH at the *TAC1* locus appears to facilitate the exchange of genetic material within a yeast population. For example, mating-competent strains that emerge from LOH in this region have the capacity to mate with cells of the opposite mating type. Even though mating in a *C. albicans* population under *in vivo* conditions has been reported, evidence for genetic rearrangement and exchange after mating is still sparse. However, the link between *TAC1* and mating-type locus enables us to test the effect of drug pressure on the ability to propagate a drug resistance genotype.

Genetic transfer of drug resistance in bacteria is a common feature; however, genetic transfer in fungi and especially in *C. albicans* has not yet been reported. Animal models will be used in future studies to test this hypothesis. Interestingly, in some clinical isolates, azole resistance mediated by upregulation of *CDR1* and *CDR2* is not linked to homozygosity at the mating-type locus (D. SANGLARD, unpublished results). The *TAC1* alleles of these isolates are probably dominant and may carry

mutation(s) different from the N977D mutation described here. A catalog of existing mutations in *TAC1* alleles in the population of azole-resistant isolates is necessary to identify and characterize other mutations responsible for Tac1p hyperactivity as well as their distribution in the population of clinical strains. First, this catalog will help to establish critical domains necessary for *TAC1* hyperactivity and, second, it will assist the design of molecular diagnostic tools for the detection of such alleles.

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