Genome-Wide Expression and Location Analyses of the 
Candida albicans Tac1p Regulon

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A major mechanism of azole resistance in Candida albicans is overexpression of the genes encoding the ATP binding cassette transporters Cdr1p and Cdr2p due to gain-of-function mutations in Tac1p, a transcription factor of the zinc cluster family. To identify the Tac1p regulon, we analyzed four matched sets of clinical isolates representing the development of CDR1- and CDR2-mediated azole resistance by using gene expression profiling. We identified 31 genes that were consistently up-regulated with CDR1 and CDR2, including TAC1 itself, and 12 consistently down-regulated genes. When a resistant strain deleted for TAC1 was examined similarly, expression of almost all of these genes returned to levels similar to those in the matched azole-susceptible isolate. Using genome-wide location (ChIP-chip) analysis (a procedure combining chromatin immunoprecipitation with hybridization to DNA intergenic microarrays), we found 37 genes whose promoters were bound by Tac1p in vivo, including CDR1 and CDR2. Sequence analysis identified nine new genes whose promoters contain the previously reported Tac1p drug-responsive element (CGGNGCGG), including TAC1. In total, there were eight genes whose expression was modulated in the four azole-resistant clinical isolates in a TAC1-dependent manner and whose promoters were bound by Tac1p, qualifying them as direct Tac1p targets: CDR1, CDR2, GPX1 (putative glutathione peroxidase), LCB4 (putative sphingosine kinase), RTA3 (putative phospholipid flipase), and orf19.1887 (putative lipase), as well as IPU5 and orf19.4898 of unknown function. Our results show that Tac1p binds under nonactivating conditions to the promoters of its targets, including its own promoter. They also suggest roles for Tac1p in regulating lipid metabolism (mobilization and trafficking) and oxidative stress response in C. albicans.

Candida albicans causes mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis, the most frequent opportunistic infection among patients with AIDS (25, 40). Azole antifungal agents have proven effective in the management of oropharyngeal candidiasis; however, with increased use of these agents, treatment failures that have been associated with the emergence of azole-resistant strains of C. albicans have occurred (47, 52, 56, 63, 82). The azole antifungals target lanosterol demethylase (Erg11p), a key enzyme in the ergosterol biosynthesis pathway (38). Several mechanisms of resistance to the azole antifungal agents have been described for C. albicans, including increased expression of genes encoding multidrug efflux pumps (27, 28, 47, 67, 69, 80, 81). These include the gene encoding a transporter of the major facilitator superfamily (MDR1) and genes encoding two ATP binding cassette (ABC) transporters (CDR1 and CDR2) (27, 28, 47, 67, 69, 80). Overexpression of these efflux pumps is presumed to prevent accumulation of effective concentrations of the azole antifungal agents within the fungal cell. Among studies examining multiple matched azole-susceptible and -resistant sets of isolates, some isolates overexpress only MDR1, whereas others overexpress only CDR1 and CDR2 (47, 56). These observations suggest that two distinct transcriptional pathways are involved in regulating these efflux pumps. Previous studies have shown that a wild-type CDR1 pro-
moter fused to a luciferase reporter construct becomes activated when placed in an azole-resistant isolate that overexpresses CDR1 and CDR2 (20). By use of this system, it was shown that a conserved DNA sequence element in the CDR1 and CDR2 promoters, named drug response element (DRE) (5'-CGGAAA/TATCGG), was necessary for CDR1 constitutive as well as drug-inducible transcriptional activation (20). These results suggested that a gain-of-function mutation in a transcription factor was the cause of the CDR1 and CDR2 constitutive overexpression in azole-resistant isolates (20). The zinc cluster transcription factor Tac1p (for transcriptional activator of CDR genes) was recently identified and shown to be responsible for CDR1 and CDR2 transcriptional activation (15, 16). An amino acid change from asparagine to aspartic acid at position 977 (N977D) in Tac1p was able to confer increased expression of CDR1 and CDR2 accompanied by decreased azole susceptibility (15). It was also shown that the DNA-binding domain of Tac1p fused to the glutathione S-transferase protein binds in vitro to the CDR1 and CDR2 DRE (16).

Interestingly, increasedazole resistance is observed when a gain-of-function mutation is present in both TAC1 alleles (15). The TAC1 locus is on chromosome 5. Homozygosity at the TAC1 locus occurs either through mitotic recombination between copies of chromosome 5 or through the presence of extra copies of chromosome 5 harboring a gain-of-function TAC1 allele and loss of chromosome 5 with the wild-type TAC1 allele (15). Selmecki et al. demonstrated that a specific segmental aneuploidy, consisting of an isochromosome composed of the two left arms of chromosome 5, is associated withazole resistance. Increases and decreases inazole resistance were found to be strongly associated with gain and loss of this isochromosome (73). Recent work by Coste et al. showed that the effect of specific mutations inazole resistance genes located on chromosome 5 (TAC1 and ERG11) can be enhanced further by loss of heterozygosity and/or addition of extra copies of chromosome 5 (14).

In addition to CDR1 and CDR2, other targets of Tac1p (RTA3, HSP12, and IFU5), all of which contain a putative DRE in their promoter region, have been identified previously (16). In a separate study, it was shown that PDR1, encoding a putative phosphatidylinositol transfer protein contributing to clinicalazole resistance, is also a target of Tac1p (66, 85). In the present study, we used functional genomic approaches, namely, genome-wide expression and location profiling, to identify the Tac1p regulon. Our results provide a more comprehensive picture of the molecular effects of Tac1p mutations inazole-resistant clinical isolates and suggest other important cellular functions for Tac1p in C. albicans.

### MATERIALS AND METHODS

#### Strains and growth media.

The C. albicans strains used in this study are listed in Table 1. The matched clinical isolate sets and strain SZY31 were grown in yeast-peptone-dextrose (YPD) broth (Sigma-Aldrich, St. Louis, MO) at 30°C. The CAI4 strain and its epitope-tagged derivatives were grown at 30°C in YPD yeast-peptone-dextrose (YPD) broth (Sigma-Aldrich, St. Louis, MO) at 30°C. In Table 1, the matched clinical isolate sets and strain SZY31 were grown in yeast-peptone-dextrose (YPD) broth (Sigma-Aldrich, St. Louis, MO) at 30°C. The CAI4 strain and its epitope-tagged derivatives were grown at 30°C in YPD broth, supplemented or not with 100 μg/ml of 5-fluorocytosine (5-FOA) (Toronto Research Chemicals, Inc., Toronto, Ontario, Canada). The Escherichia coli DH5α bacterial strain was used for DNA cloning and maintenance of the plasmid constructs.

**Construction of C. albicans expression microarrays.** The nucleotide sequences corresponding to 6,165 open reading frames (ORFs) for C. albicans were downloaded from the Galar Fungal European Consortium (assembly 6, http://www.pasteur.fr/Galar_Fungal/CandidaDB). We set out to design two nonoverlapping probe sets targeting the 3'-600-bp region of each ORF. Each probe set consisted of 13 perfect-match 25-mer probes and their corresponding mismatch control probes containing a single mismatch in the center of the oligonucleotide. For ORFs less than 600 bp in length, the sequence was divided in two equal segments for subsequent design procedures. Optimum probe sets were selected by the Affymetrix design team based on their model, which, among other things,
considers probe hybridization quality and cross-hybridization potential. Consequently, in some cases, only one probe set was selected for a given ORF. For quality control and normalization purposes, we made two to three additional probe sets spanning the entire sequence of the *C. albicans* 18S rRNA gene (GenBank accession no. M60382) and genes encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase), actin, and Mdr1p (Bmr1p) in addition to the standard *A. thaliana* BiocBCD, cre, DAP, PHE, LYS, and THR. In the end, the GeneChip array contained 10,736 probe sets, including nine controls, 6,123 unique ORFs, and duplicate probe sets for 4,604 ORFs. The duplicate probe sets are made to distinct regions of the ORF, thereby allowing two independent measurements of the mRNA level for that particular gene. The *C. albicans* custom Affymetrix NimbleExpression arrays (CANO43a500004N) were manufactured by NimbleGen Systems (1) per our specification.

**RNA preparation for microarrays.** The matched clinical isolate sets and strain SZY31 were grown in YPD broth at 30°C in a shaking incubator to mid-log phase as described previously (61). The cell pellets were frozen and stored at −80°C prior to RNA preparation. Experiments were repeated independently three times. Total RNA was isolated using a hot sodium dodecyl sulfate (SDS)-phenol method (70). Frozen cell pellets were suspended in 12 ml of 50 mM sodium acetate (pH 5.2), 10 mM EDTA at room temperature, after which one ml of 20% SDS and 12 ml of acid phenol (Fisher Scientific, Waltham, MA) was added. This mixture was incubated for 10 min at 65°C with vortexing each minute, cooled on ice for 5 min, and centrifuged for 15 min at 12,000 × g. Supernatants were transferred to new tubes containing 15 ml of chloroform, mixed, and centrifuged at 200 × g for 10 min. The aqueous layer was removed to new tubes, and RNA was precipitated with 1 volume isopropanol and 0.1 volume 2 M sodium acetate (pH 5.0) and then collected by centrifugation at 17,000 × g for 35 min at 4°C. The RNA pellet was suspended in total 5 ml by use of 2 μl of the double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA) per the manufacturer’s instructions. First- and second-strand cDNA synthesis were washed at 25°C with 6×SSPE (1×SSPE = 0.1 M NaCl, 0.01 M NaH²PO₄, and 1 mM EDTA [pH 7.7]), 0.01% Tween 20 followed by a stringent wash at 50°C with 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 0.1 M NaCl, 0.01% Tween 20. Hybridizations and washes employed an Affymetrix Fluidics station 450 with the standard EukGE-WS2v5 protocol. The arrays were then stained with phycoerythrin-conjugated streptavidin (Molecular Probes/Invitrogen) for preprocessing and data analysis. The change in fluorescence of SYBR green I dye in every cycle was monitored by the software system, and the cycle threshold (Cₚ) above the background for each reaction was calculated. The Cₚ value of the 18S rRNA gene was subtracted from that of the gene of interest to obtain a ΔCₚ value. The ΔCₚ value of an arbitrary calibrator (e.g., untreated sample) was subtracted from the ΔCₚ value of each sample to obtain a ΔΔCₚ value. The gene expression level relative to the calibrator was expressed as 2⁻ΔΔCₚ Statistical analysis was performed using R software, version 2.5.0 (www.r-project.org). For each gene, the fold change was calculated using Student’s t test. The statistical significance threshold was fixed at α = 0.05.

**Generation of a hemagglutinin (HA)-tagged Tacl-expressing strain.** A DNA fragment overlapping positions −402 to +937 of the *C. albicans URA3* ORF (URA3 marker) was PCR amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA) from plasmid pCaEXP (12) by using primers 5'-ATCACCTCTCGAGATAGTAACAGATGGTTAAGATTTTGGAACAAGGAAAACGTTTCAGGTTTTTGAGGTACCCAGTATTTGGCATTGAAA-3' (the lowercase sequence corresponds to positions 9004 to 9272 of the *C. albicans URA3* gene). This PCR product (720 bp) was digested with XbaI and SmaI (introduces an XbaI site, underlined). The resulting fragment 1,400 bp was digested with *SacI*, ligated with the above-mentioned DNA fragment, and cloned into the *SacI*-digested *pUC19* vector. The plasmid was transformed into *C. albicans* strain CAI4. Counterselection of the *URA3* marker (72), generating *URA3*ΔCₚ is expressed as 2⁻ΔΔCₚ Statistical analysis was performed using R software, version 2.5.0 (www.r-project.org). For each gene, the fold change was calculated using Student’s t test. The statistical significance threshold was fixed at α = 0.05.

**Q-PCR for expression data.** An aliquot of the RNA preparations from the samples used in the microarray experiments was saved for quantitative real-time reverse transcription (RT)-PCR follow-up studies. First-strand cDNAs were synthesized from 2 μg of total RNA in a 21-μl reaction volume by use of a SuperScript first-strand synthesis kit for RT-PCR (Invitrogen) in accordance with the manufacturer’s instructions. Quantitative real-time PCRs (Q-PCRs) were performed in triplicate using a 7000 sequence detection system (Applied Biosystems, Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and the 18S rRNA gene by use of Primer Express software (Applied Biosystems, Inc.) and an Oligo analysis and plotting tool (QIAGEN, Valencia, CA) and are shown in Table 2. The PCR conditions consisted of AmpliTaq Gold activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified by using software provided with the 7000 sequence detection system. The change in fluorescence of SYBR green I dye in every cycle was monitored by the software system, and the cycle threshold (Cₚ) above the background for each reaction was calculated. The Cₚ value of the 18S rRNA gene was subtracted from that of the gene of interest to obtain a ΔCₚ value. The ΔCₚ value of an arbitrary calibrator (e.g., untreated sample) was subtracted from the ΔCₚ value of each sample to obtain a ΔΔCₚ value. The gene expression level relative to the calibrator was expressed as 2⁻ΔΔCₚ Statistical analysis was performed using R software, version 2.5.0 (www.r-project.org). For each gene, the fold change was calculated using Student’s t test. The statistical significance threshold was fixed at α = 0.05.

**TABLE 2. Primers used for quantitative real-time PCR expression analysis**

| Gene          | Primer pair* | Amplicon size (bp) |
|---------------|--------------|--------------------|
| 18S rRNA      | 5'-CCACAGGCCGAGTTCTACAAAGA-3' | 135 |
|               | 5'-CGATGGGAATTTGAGGCACAT-3'   |       |
| CDR1         | 5'-ATTCTAAAGTGCTCCTGGCAAGATG-3' | 140 |
|               | 5'-AGTTCGCGCATAAATTTGAATTTTC-3' |       |
| PDR16        | 5'-GTCGGGGACAGGATCTACATTGAC-3' | 62  |
|               | 5'-TTGATGACCAACAGGAGTTGCTT-3' |       |
| TAC1         | 5'-TGCCAAATGTATTTAGCATTAGG-3' | 71  |
|               | 5'-TGCCATGACGGTGTAATTTTG-3'   |       |

* F, forward; R, reverse.
containing 5-FOA as described previously (8), except that uracil was replaced with uridine.

*C. albicans* transformation. *C. albicans* transformations were performed as described previously (35), with minor modifications. The cells were grown overnight in 15 ml of YPD medium, diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in 100 ml of fresh YPD, and allowed to grow to an OD₆₀₀ of 0.4. The cells were harvested, washed once with 10 ml of sterile water, and resuspended in 1 ml of 1× lithium acetate (LiAc) solution (35). Cells (100 ml) were transferred to a sterile tube containing 6 µg of gel-purified PCR fragment and 100 µg of denatured salmon sperm DNA as the carrier. A LiAc-40% polyethylene glycol solution (700 µl of 10 mM Tris-HCl, pH 7.5, 1 ml EDTA, pH 8.0, 100 mM LiAc, 40% [wt/vol] polyethylene glycol 4000) was added, and the cell suspensions were incubated overnight at 30°C with gentle rotation. The cells were heat shocked at 42°C for 15 min and plated on synthetic complete medium lacking uracil.

Genomic DNA isolation and Southern blot analysis. *C. albicans* genomic DNA was prepared as described previously for *S. cerevisiae* (62). For the Southern hybridization, genomic DNAs (500 ng) were digested to completion with HindIII and EcoRV, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane (Hybond-N; Amersham Biosciences, Piscataway, NJ). Hybridization, hybridization, and washing steps were carried out as previously described (66). The *TAC1* probe used consists of a 3′-P-terminated 927-bp PCR-amplified fragment from *SC5314* genomic DNA overlapping positions 10 to +2946 in the *TAC1* ORF. The membrane was exposed to a FUJIFILM imaging plate screen. The signal was quantified using the Multi Gauge program, version 2.3 (FUJIFILM). The membrane was subsequently exposed to Kodak XAR film at −80°C.

Total protein preparation and Western blotting. Total protein was prepared from 2 OD units of strains CAI4 and SZY63 grown overnight, as described previously for *S. cerevisiae* (62). Extracts were boiled for 5 min, and 35 µl (out of 100 µl total) was separated by electrophoresis on an SDS-8%-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane with a Trans Blot SD semidry transfer apparatus (Bio-Rad, Hercules, CA) and analyzed with a mouse anti-HA monoclonal antibody [HA probe (F-7): sc-7392; Santa Cruz Biotechnology, Inc., Santa Cruz, CA] at a dilution of 1:2,000 by use of a chemiluminescence detection system under conditions recommended by the manufacturer (Pierce Biotechnology, Inc., Rockford, IL).

Chromatin immunoprecipitation (ChIP). Three independent cultures (50 ml each) of strains CAI4 and SZY63 were grown in YPD medium to an OD₆₀₀ of 1 of 2 OD units of strains CAI4 and SZY63 grown overnight, as described previously for *S. cerevisiae* (62). The signal was quantified using the Multi Gauge program, version 2.3 (FUJIFILM). The membrane was subsequently exposed to Kodak XAR film at −80°C.

Total protein was prepared from 2 OD units of strains CAI4 and SZY63 grown overnight, as described previously for *S. cerevisiae* (62). Extracts were boiled for 5 min, and 35 µl (out of 100 µl total) was separated by electrophoresis on an SDS-8%-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane with a Trans Blot SD semidry transfer apparatus (Bio-Rad, Hercules, CA) and analyzed with a mouse anti-HA monoclonal antibody [HA probe (F-7): sc-7392; Santa Cruz Biotechnology, Inc., Santa Cruz, CA] at a dilution of 1:2,000 by use of a chemiluminescence detection system under conditions recommended by the manufacturer (Pierce Biotechnology, Inc., Rockford, IL).

Analysis of the genome-wide location data. Q-PCR was performed with three independent CAI4 and SZY63 ChIP samples prepared as described above, except that the cell cultures were carried out in the presence of dimethyl sulfoxide (0.08%). Quantification of the DNA recovered from the CAI4 and SZY63 ChIPs was performed using a Quant-iT PicoGreen double-stranded DNA assay kit (Molecular Probes/Invitrogen). A standard curve was prepared using *C. albicans* SC5314 genomic DNA quantified by fluorometry and serially diluted in TE buffer (0, 0.1, 0.01, and 0.001 ng/µl). The CAI4 and SZY63 ChIP samples (1 µl) were resuspended in 49 µl of TE buffer. The samples (50 µl) were transferred, in duplicate, in a black 96-well plate (Costar 3696; Corning, Inc., Corning, NY), and 50 µl of the PicoGreen reagent was added. The fluorescence was measured using an Eppendorf fluorescent plate reader (Perkin-Elmer, Waltham, MA) under conditions of optimal excitation and emission wavelengths of 485 and 535 nm, respectively. The DNA concentration of the ChIP samples was an average 500 pg/µl, yielding approximately 25 ng of total IP DNA per ChIP sample (50 µl).

Q-PCR assays were designed using the Universal ProbeLibrary (Roche Applied Science, Indianapolis, IN) (formerly the Exiqon ProbeLibrary) and TaqMan (Integrated DNA Technologies [IDT], Coralville, IA) methodologies. The different primer and probe combinations used for Q-PCR are listed in Table 3. The *CDR1, PDR6, TAC1, and FUR1* promoter sequences were submitted to the web-based ProbeFinder software (version 2.34; Roche Applied Science) available on the Roche Applied Science website. The software assigned optimal PCR-specific primer sequences to be combined with the corresponding Universal ProbeLibrary probe for each promoter sequence (Universal ProbeLibrary probes; Roche Diagnostics Corp., Basel, Switzerland). We also used the PrimerQuest tool from the IDT website (http:// primerquest.idtdna.com) to design a custom TaqMan probe for the *CDR1* promoter (with its corresponding forward and reverse primers) that binds closer to the DRE motif than the Universal ProbeLibrary probe. The *C. albicans* homologue of *S. cerevisiae* *SPS4* (orf19.7568) was used as the reference promoter and FUR1 was used as a control to perform statistical analyses. The *SPS4* and FUR1 genes were selected because (i) they were not modulated in our microarray expression study and (ii) their promoters were not enriched in the ChIP-chip experiments. The primer sets were designed based on the coordinates of the *SPS4* and FUR1 promoters by use of ProbeFinder software (Table 3).

Q-PCR mixtures were prepared using TaqMan universal PCR master mix (Applied Biosystems, Inc.) according to the manufacturer’s instructions. For reactions using probes from the Universal ProbeLibrary, 200 pg of ChIP DNA, 250 nM of each forward and reverse primer, 100 nM of the probe, 5 µl of TaqMan universal PCR master mix, and water were combined in a final volume of 10 µl. Mixtures for Q-PCR using the TaqMan probe were prepared using the same conditions except that the probe and the primers (forward and reverse) were added to final concentrations of 100 nM and 200 nM, respectively. Reactions were performed in a MicorAmp optical 384-well reaction plate (Applied Biosystems, Inc.) by use of an ABI 7900 HT real-time PCR instrument with 1 cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. Each biological replicate sample (three) was processed in triplicate.

Data analysis was performed using Sequence Detection System software (SDS 2.2.2; ABI). For each sample, *C₅* values were determined using the Sequence Detection System software. The sevelfold enrichments of the targets (*CDR1, PDR6, and TAC1* promoters) were calculated using relative quantification according to the 2−ΔΔC₅ method, where ΔΔC₅ = C₅ target − C₅ calibrator (SPS4 promoter) and ΔΔC₅ = C₅ test(SZY63 ChIP sample) − C₅ calibrator(CAI4 ChIP sample) (46). The FUR1 promoter was used as a negative control to confirm the sevelfold enrichment obtained using the 2−ΔΔC₅ method. Statistical analysis was performed using R software (version 2.5.1; www.r-project.org), and the ΔΔC₅ values were compared using Welch’s t test. The statistical significance threshold was fixed at α = 0.001. 

DRE motif analysis. The promoter sequences (1.5 kb upstream of the starting ATG) were retrieved from the Saccharomyces Genome Database (CGD) (http://www.yeastgenome.org) and analyzed for the presence of potential DRE motifs by use of fuzznuc software from EMBOB (http://emboss.bioinformatics.nl/).
we were initially considered to be differentially expressed if (i) C. albicans to custom-designed Affymetrix microarrays and J. Berman, personal communication). chromosomal rearrangements or aneuploidies, as determined mutations in Tac1p. The eight strains did not show major CDR1 and CDR2 is isolate 3 to identify the genes coregulated with and an L962 N969 deletion in the second allele of 5674 (85), and an A736C amino acid substitution in one allele DSY296) (15), an N972D amino acid substitution in isolate 3 exhibits increased expression of CDR1 and CDR2 overexpression (Table 1). Three of these azole-resistant isolates possess previously defined gain-of-function mutations in the C terminus of TAC1: an N972D amino acid substitution, results in the highest impact expression data, clinical isolate 5674, which carries the Tac1p encoded a cell wall protein (Table 5). As judged from the SOD5 membrane proteins, and OPT6 phosphatidylinositol-anchored protein of unknown function), and included regulated in all four matched clinical isolate sets. These inmutations in Tac1p (85). We also found 12 genes to be downther directly or indirectly, as a consequence of gain-of-function (Table 4), including the known Tac1p targets to be up-regulated in all four matched clinical isolate sets included in a final data set (Tables 4 and 5). We found 31 genes by the and whose average change (n-fold) was statistically significant by the test. By use of these criteria, there were 222 genes up-regulated and 150 genes down-regulated in C56 compared to C43, 104 genes up-regulated and 63 genes down-regulated in Gu5 compared to Gu2, 126 genes up-regulated and 92 genes down-regulated in 17 compared to 3, and 327 genes up-regulated and 243 genes down-regulated in 5674 compared to 5457 (see Table S1 in the supplemental material). Genes which met these criteria for all four matched isolates and whose average change (n-fold) was statistically significant by the test in at least two of the four matched isolates were included in a final data set (Tables 4 and 5). We found 31 genes to be up-regulated in all four matched clinical isolate sets (Table 4), including the known Tac1p targets CDR1, CDR2, IFU5, HSP12, and RTA3 (16). Other up-regulated genes included GPX1 (putative glutathione peroxidase), CHK1 (histidine kinase), LCB4 (sphingoid long-chain base kinase), NDH2 (NADH dehydrogenase), SOU1 (sorbose dehydrogenase), orf19.3047 (transcription cofactor), and orf19.4531 (ABC (sorbose dehydrogenase), orf19.3047 (transcription cofactor), and orf19.4531 (ABC (sorbose dehydrogenase), orf19.3047 (transcription cofactor), and orf19.4531 (ABC (sorbose dehydrogenase), orf19.3047 (transcription cofactor), and orf19.4531 (ABC transport). As well as TAC1 itself, consistent with our previous proposition that TAC1 transcription is autoactivated, either directly or indirectly, as a consequence of gain-of-function mutations in Tac1p (85). We also found 12 genes to be downregulated in all four matched clinical isolate sets. These included FTR1 (iron transporter), IHD1 (putative glycosylphosphatidylinositol-anchored protein of unknown function), and OPT6 (oligopeptide transporter), all of which encode integral membrane proteins, and SOD5 (superoxide dismutase), which encodes a cell wall protein (Table 5). As judged from the expression data, clinical isolate 5674, which carries the Tac1p N972D amino acid substitution, results in the highest impact on gene expression among the isolates tested, as 38 out of the

### RESULTS

#### Global gene expression profile

As a means to identify genes that are coregulated with the CDR1 and CDR2 multidrug efflux pumps and thus potentially new transcriptional targets of Tac1p, we performed gene expression profiling analyses of four matched sets of azole-susceptible and -resistant C. albicans clinical isolates in which the acquisition of azole resistance is associated with CDR1 and CDR2 overexpression (Table 1). Three of these azole-resistant isolates possess previously defined gain-of-function mutations in the C terminus of TAC1: an N972D amino acid substitution in isolate C56 (also known as DSY296) (15), an N972D amino acid substitution in isolate 5674 (85), and an A736C amino acid substitution in one allele and an L962 N969 deletion in the second allele of TAC1 in isolate 17 (14). Isolate 17 is the last in a series of 17 clinical isolates taken from a single human immunodeficiency virus-infected patient (80, 83). Relative to the parental isolate 2, isolate 3 exhibits increased expression of MDR1 whereas isolate 17 exhibits increased expression of CDR1 and CDR2 as well as MDR1 (80, 83). We therefore compared isolate 17 to isolate 3 to identify the genes coregulated with CDR1 and CDR2. The other azole-resistant isolate, Gu5, overexpresses CDR1 and CDR2 and presumably also contains gain-of-function mutations in Tac1p. The eight strains did not show major chromosomal rearrangements or aneuploidies, as determined by comparative genomic hybridization analyses (data not shown and J. Berman, personal communication).

Three independent RNA samples per strain were hybridized to custom-designed Affymetrix C. albicans microarrays and the data analyzed as described in Materials and Methods. Genes were initially considered to be differentially expressed if (i) their average change (n-fold) in expression was ≥1.5 for each matched isolate, (ii) their expression changed by at least 1.5-fold in at least two of the three experiments for each matched isolate, and (iii) the average change (n-fold) was statistically significant by the t test. By use of these criteria, there were 222 genes up-regulated and 150 genes down-regulated in C56 compared to C43, 104 genes up-regulated and 63 genes down-regulated in Gu5 compared to Gu2, 126 genes up-regulated and 92 genes down-regulated in 17 compared to 3, and 327 genes up-regulated and 243 genes down-regulated in 5674 compared to 5457 (see Table S1 in the supplemental material). Genes which met these criteria for all four matched isolates and whose average change (n-fold) was statistically significant by the t test in at least two of the four matched isolates were included in a final data set (Tables 4 and 5). We found 31 genes to be up-regulated in all four matched clinical isolate sets (Table 4), including the known Tac1p targets CDR1, CDR2, IFU5, HSP12, and RTA3 (16). Other up-regulated genes included GPX1 (putative glutathione peroxidase), CHK1 (histidine kinase), LCB4 (sphingoid long-chain base kinase), NDH2 (NADH dehydrogenase), SOU1 (sorbose dehydrogenase), orf19.3047 (transcription cofactor), and orf19.4531 (ABC transport). As well as TAC1 itself, consistent with our previous proposition that TAC1 transcription is autoactivated, either directly or indirectly, as a consequence of gain-of-function mutations in Tac1p (85). We also found 12 genes to be downregulated in all four matched clinical isolate sets. These included FTR1 (iron transporter), IHD1 (putative glycosylphosphatidylinositol-anchored protein of unknown function), and OPT6 (oligopeptide transporter), all of which encode integral membrane proteins, and SOD5 (superoxide dismutase), which encodes a cell wall protein (Table 5). As judged from the expression data, clinical isolate 5674, which carries the Tac1p N972D amino acid substitution, results in the highest impact on gene expression among the isolates tested, as 38 out of the

#### Microarray data accession number

Data files of each scanned chip were submitted to the Gene Expression Omnibus database (GEO; www.ncbi.nlm.nih.gov/geo/). The accession number for the series is GSE8727.

![Table 3. Primers used for quantitative real-time PCR binding assays](image-url)

| Promoter | Primer/probe sequence (5′-3′)a | Amplicon locationb |
|----------|--------------------------------|-------------------|
| CDR1     | F, GGTGCACACACACACACACACACACAC | –435 → –341       |
|          | R, TTAGGCTCCCACACTCGATCCCTTA   |                   |
|          | P, TaqMan probe CGCCCTCACTGCTTGTCATACAAAT |                   |
| PDR16    | F, GAAAAGAAAAAGAAATGGGACAT    | –424 → –501       |
|          | R, TGGACACGTTTCTTCCATCAC      |                   |
|          | P, Universal ProbeLibrary probe no. 77 (catalog no. 04689003001) GGTGGTG | |
| TAC1     | F, CACGAAGATAAAAAATGTGTGAGC  | –1024 → –948      |
|          | R, AATTTGCTGATATTTAATGTGGGT |                   |
|          | P, Universal ProbeLibrary probe no. 18 (catalog no. 0468918001) CAGCAGGA | |
| FUR1     | F, GGTGCTTTTTGGAAGTAAGGA  | –987 → –913       |
|          | R, CTTCCTCAAACAAAAACTGCAA   |                   |
|          | P, Universal ProbeLibrary probe no. 27 (catalog no. 04687582001) GCTGCGTG | |
| SPS4     | F, TACAGTTGCCCCAGTCAACA    | –636 → –574       |
|          | R, TGTCTTGGGAAAGCGAAAACCTCA |                   |
|          | P, Universal ProbeLibrary probe no. 15 (catalog no. 04685148001) TCCTGCTC | |

a F, forward; R, reverse; P, probe. The TaqMan probe was from IDT, and the Universal ProbeLibrary probes were from Roche.
b Position according to the ATG start codon.
We selected four genes of interest for confirmation of differential expression by real-time RT-PCR: \textit{CDR1}, \textit{CDR2}, \textit{TAC1}, and \textit{PDR16}. \textit{PDR16} was selected as it has been shown to be coregulated with \textit{CDR1} and \textit{CDR2} in association with azole resistance; yet, this is one of a limited number of genes not represented on the microarray used in this study (19, 66). As expected, all four genes were confirmed to be up-regulated in all four isolate sets (Fig. 1). Likewise, up-regulation of all four of these genes in isolate 5674 was ablated in strain \textit{SZY31} (Fig. 1).

**Identification of Tac1p-binding sites in vivo.** To determine if the \textit{TAC1}-dependent genes that were differentially expressed in the clinical isolates have their promoters bound in vivo by Tac1p and to identify additional direct targets of Tac1p, we

| Systematic name\(^a\) | CGD name\(^b\) | CandidaDB name\(^c\) | GO annotation (molecular function)\(^d\) | Fold change in expression\(^e\) in isolates: |
|------------------------|----------------|----------------------|----------------------------------------|---------------------------------------|
| orf19.6000             | CDR1          | CDR1                 | Multidrug transporter activity         | C56 vs C43  Gu5 vs Gu2  17 vs 3  5674 vs 5457  SZY31 vs 5457 |
| orf19.5958             | CDR2          | CDR2                 | Transporter activity                   | 1.7  3.0  2.4  4.4  -1.0                 |
| orf19.896              | GPX1          | GPX1                 | Glutathione peroxidase activity        | 50.6  14.1  17.1  50.5  -1.3               |
| orf19.896              | HK1           | HK1                  | Protein histidine kinase activity      | 2.3  3.0  2.3  4.0  -1.2                  |
| orf19.3160             | HSP12         | HSP12                |                                           | 2.5  1.9  1.8  4.5  1.3                     |
| orf19.157              | IFA24.3       | IFA24.3              |                                           | 5.3  2.9  3.1  16.7  -1.7                   |
| orf19.2568             | IFU5          | IFU5                 |                                           | 3.3  3.0  3.7  5.4  1.5                     |
| orf19.5257             | LCB4          | LCB4                 | d-Erythro-sphingosine kinase activity   | 2.2  1.7  2.2  4.0  1.1                     |
| orf19.2896             | SOU1          | SOU1                 | Sorbose dehydrogenase activity         | 3.1  1.8  1.9  5.2  -1.1                   |
| orf19.3104             | YDC1          | YDC1                 |                                           | 5.3  3.1  2.9  15.3  1.2                   |
| orf19.2726             | PPI10262      | PPI10262             |                                           | 2.1  1.8  1.7  1.7  1.0                     |
| orf19.4459             | PPI11849      | PPI11849             |                                           | 1.8  2.8  1.9  6.2  -2.9                   |
| orf19.2244             | PPI12897      | PPI12897             |                                           | 2.0  1.7  1.5  4.4  -1.7                   |
| orf19.344              | PPI1514       | PPI1514              |                                           | 15.5  4.7  4.0  28.9  1.9                   |
| orf19.4907             | PPI17283      | PPI17283             |                                           | 3.2  1.6  2.3  2.0  1.1                     |
| orf19.5313             | PPI19810      | PPI19810             |                                           | 36.0  29.4  18.1  143.1  1.7                  |
| orf19.5777             | PPI19961      | PPI19961             |                                           | 2.0  1.7  2.2  4.2  -1.3                   |
| orf19.5777             | PPI3535       | PPI3535              |                                           | 1.8  2.3  1.9  2.3  -1.4                   |
| orf19.3047             | SIP3          | SIP3                 | Transcription cofactor activity         | 1.5  1.5  1.7  3.0  1.3                     |
| orf19.2531             | CSP37         | CSP37                | ER\(^f\)-to-Golgi vesicle-mediated transport | 2.5  1.8  5.5  6.8  -1.3                   |
| orf19.7310             | PPF5981       | PPF5981              |                                           | 4.9  2.7  2.0  16.8  1.2                     |
| orf19.1887             | PPF6464       | PPF6464              |                                           | 2.3  1.8  1.7  4.5  1.2                     |
| orf19.2790             | PPF7260       | PPF7260              | Histone-lysine N-methyltransferase activity | 1.8  1.6  1.6  2.8  -1.0                   |
| orf19.4531             | PPF7530       | PPF7530              | ATPase activity                         | 2.7  3.8  3.5  4.0  -1.6                   |
| orf19.4898             | PPF8950       | PPF8950              |                                           | 2.4  1.6  1.8  3.4  1.1                     |
| orf19.3188             | TAC1          | TAC1                 |                                           | 2.7  2.8  2.8  4.2  -11.7                   |
| orf19.3644             | TAC1          | TAC1                 |                                           | 2.5  1.5  2.1  3.4  1.1                     |

\(^a\) orf19 nomenclature according to the assembly 19 version. Systematic names in bold designate overlap with ChIP-chip experiments.
\(^b\) Gene name at CGD (http://www.candidagenome.org/).
\(^c\) Gene name at CandidaDB (http://genolist.pasteur.fr/CandidaDB/).
\(^d\) GO annotation found at CGD (http://www.candidagenome.org/).
\(^e\) Gene expression values in bold designate statistical significance (\(P < 0.05\)) in all three experiments.
\(^f\) ER, endoplasmic reticulum.

43 genes modulated in a \textit{TAC1}-dependent manner had the highest modulation (\(n\)-fold) in this strain (Tables 4 and 5).

In order to determine which of these genes require Tac1p for their constitutive differential expression in these isolates, the gene expression profile of strain \textit{SZY31}, a \textit{tac1}\(\Delta/tac1\Delta\) derivative of the azole-resistant strain 5674 (Table 1), was compared with that of the azole-susceptible parental strain 5457 (85). Genes whose expression was modulated in strain 5674 compared to strain 5457 and whose expression in strain \textit{SZY31} was returned to levels similar to those observed for strain 5457 were determined to be dependent upon Tac1p for their up- or down-regulation. As shown in Tables 4 and 5, the majority of commonly differentially expressed genes were found to be Tac1p dependent.
conducted genome-wide location analyses (ChIP-chip), a procedure combining ChIP with hybridization to DNA intergenic microarrays (60). For this purpose, Tac1p was tagged at its C terminus with a triple HA (HA3) epitope by use of homologous recombination at the TAC1 chromosomal locus (Fig. 2). First, we adapted the S. cerevisiae PCR epitope-tagging vector pMPY-3×HA (72) for its use in C. albicans by substituting the S. cerevisiae URA3 marker with the C. albicans URA3 gene (see Materials and Methods for details). The resulting vector (pCaMPY-3×HA) contains the C. albicans URA3 marker flanked by direct repeats of the HA3 epitope. The TAC1-tagging cassette was amplified with 100-bp primers (16 bp of vector sequence and 84 bp from the gene to be tagged), allowing tagging of Tac1p at its C terminus (Fig. 2A). After transformation of the CAI4 strain and integration of the cassette by homologous recombination, the marker was excised via recombination between the two directly repeated HA3 tags and the excision mutants were selected on 5-FOA medium (Fig. 2A). The resulting preexcision (strain SZY51) and postexcision (strain SZY63) strains were characterized by Southern hybridization using HindIII and EcoRV double-digested genomic DNA and the TAC1 probe (Fig. 2B). As predicted (Fig. 2B), the probe detected two fragments, one of 3.3 kb and one of 1.4 kb, in the CAI4 strain, corresponding to the wild-type TAC1-1 (orf19.3188) and TAC1-2 (orf19.10700) alleles, respectively (Fig. 2C, lane 1). Integration of the HA3-tagging cassette occurred at the TAC1-2 allele in the preexcision strain SZY51, as the 1.4-kb band was shifted to the 3.1-kb band (Fig. 2C, lane 2). Excision of the C. albicans URA3 marker in strain SZY63 was confirmed by the presence of the 1.6-kb band (Fig. 2C, lane 3). To detect the Tac1p-HA3 fusion protein, Western blotting was performed with strains CAI4 and SZY63 by use of an anti-HA monoclonal antibody (Fig. 2D). A signal corresponding to the Tac1p-HA3 fusion protein was detected in the HA-tagged SZY63 strain but not in strain CAI4 (control) (Fig. 2D). The size of this band was approximately 120 kDa, consistent with the predicted molecular size of the Tac1p-HA3 fusion protein (118 kDa). Because the SZY63 integrant is heterozygous for the HA3 tag and carries a wild-type copy of the TAC1 gene (TAC1-1), it was not possible to test the functionality of the Tac1p-HA3 fusion protein in this background. Using growth on sorbose-containing medium, which induces the loss of one copy of chromosome 5 in which TAC1 is located (64), we constructed strains carrying only the HA3 epitope-tagged allele of Tac1p (see Fig. S1 in the supplemental material). Functional analysis of these strains by Northern blotting (see Fig. S1C in the supplemental material) demonstrated that introduction of the HA3 tag at the C terminus of Tac1p did not alter Tac1p function (i.e., did not result in a loss- or gain-of-function mutation).

ChIP-chip experiments were conducted using the CAI4 and SZY63 strains and intergenic DNA microarrays containing 70-mer oligonucleotides covering the promoter regions of the C. albicans genome (see Materials and Methods). Using a P value of <0.001 (enriched binding ratio of ≥1.5), we identified 37 promoters bound by Tac1p (Table 6). These included the promoters of genes known to be regulated by Tac1p, CDR1 (10.5), IFU5 (3.0), PDR16 (2.1), RTA3 (1.8), and CDR2 (1.7) (16.85), thus validating our ChIP-chip procedure and showing that Tac1p binds in vivo to the promoters of these genes to regulate their expression. HSP12, also known to be regulated by Tac1p, had a binding ratio of 1.2 (P = 0.09) and thus was not bound significantly by Tac1p. These experiments also identified several new targets, including genes involved in lipid metabolism and oxidative stress response as well as genes of unknown function (see Discussion). On two occurrences,
Tac1p binding was found at promoters of two genes located on opposite strands (thus sharing the same probe on the chip): *UGA33* (orf19.7317) and *SUC1* (orf19.7319) as well as *LPE10* (orf19.3455) and *KIC2* (orf19.3456) (Table 6). While the expression data did not allow us to discriminate which of the *LPE10* or *KIC2* genes is regulated by Tac1p, we found that *SUC1* was up-regulated in a *TAC1*-dependent manner in three out of the four clinical isolates tested by expression microarrays (see Table S1 in the supplemental material) while *UGA33* expression was unchanged in three out of the four clinical isolates, suggesting that *SUC1*, a sucrose uptake zinc cluster regulator (37), is a target of Tac1p. The identification of several promoters bound in vivo by Tac1p under nonactivating conditions (i.e., in the absence of drugs or gain-of-function mutations) demonstrates that Tac1p is at least constitutively bound to its targets and suggests the possible involvement of postbinding mechanisms for Tac1p-mediated transcriptional activation (see Discussion).

Using quantitative real-time PCR, we confirmed the binding of Tac1p to the *CDR1* and *PDR16* promoters, with enrichment ratios of 29.1 ± 4.0 and 4.1 ± 0.2, respectively (Fig. 3). As a control, we investigated binding of Tac1p to the promoter of the *FUR1* gene, which was neither enriched in the ChIP-chip experiments nor modulated in the azole-resistant clinical isolates, and found no significant enrichment of that promoter by Q-PCR (1.1 ± 0.2), confirming the validity of the data obtained in the ChIP-chip experiments (Fig. 3).

Although our results suggested that the expression of the
TAC1 gene is controlled by Tac1p in an autoregulatory loop, the ChIP-chip data showed no enrichment for Tac1p at its own promoter (see Table S2 in the supplemental material), supporting an indirect autoregulation mechanism. However, the fact that the TAC1 promoter contains a DRE-like motif located between positions −1051 and −1060 relative to the ATG translation start codon (Table 7) (see below) and the TAC1 oligonucleotide printed on the intergenic chips is located at position −480 may have prevented us from detecting Tac1p binding at the TAC1 promoter. Therefore, we used for Q-PCR a set of TaqMan probe and primers hybridizing close to this DRE motif (Table 2). Under these conditions, we observed a strong enrichment of the TAC1 promoter (179.6 ± 25.2) (Fig. 3). These results strongly support a direct autoregulatory loop controlling TAC1 expression (see Discussion).

Identification of potential Tac1p-binding motifs. Tac1p belongs to the fungal-specific family of zinc cluster transcription factors that contain a DNA-binding domain formed by six cysteines coordinating two zinc atoms (48). Zinc cluster factors can bind as homodimers to CGG triplets with various orientations (everted, inverted, or direct repeats) and spacings (48). The CDR1 and CDR2 DRE consists of a direct CGG repeat with 4 intervening nucleotides (CGGAA/TATCGG), a configuration also found in the promoters of the other Tac1p-regulated genes RTA3 and IFU5 (16, 20). We thus looked for a DRE motif in the promoters of the 37 genes identified by the ChIP-chip experiments, using 1.5 kb of promoter sequence and the motif definition CGGN4CGG, which allows for complete degeneracy for the 4 nucleotides between the CGG repeats. In addition to CDR1, CDR2, RTA3, and IFU5, this analysis iden-

FIG. 2. Chromosomal tagging of Tac1p. (A) PCR epitope-tagging strategy for C-terminally tagging transcription factor Tac1p with the triple HA epitope. (Top) Primers (100 nucleotides) (see Materials and Methods) were designed such that the 5’ 84 bases of the forward (FWD) and reverse (REV) primers are homologous to sequences of the TAC1 gene and the 3’ 16 bases are complementary and in-frame to unique sequences (open boxes) in the tagging cassette which contains the C. albicans URA3 marker (CaURA3, light-gray box) flanked by direct repeats of the HA3 encoding sequences (HA, black boxes). The Tac1p stop codon is indicated by the asterisk. (Middle) PCR amplification results in a fragment whose ends include the primer sequences, allowing integration by homologous recombination of the tagging cassette upstream of the TAC1 3’-untranslated region (thick horizontal line). The orientation of the TAC1 ORF (dark-gray box) is indicated by the arrow. (Bottom) C. albicans URA3 marker excision results in the final product, TAC1-HA3. (B) Schematic representations of the TAC1-1 (orf19.3188) and TAC1-2 (orf19.10700) alleles (gray boxes) in strain CAI4. Sizes of the HindIII (HIII)/EcoRV (RV) double-digested fragments detected by the TAC1 probe (top, thick line) used for the Southern blot experiment are given for the TAC1-2 allele following integration of the HA3-tagging cassette (open box) and excision of the C. albicans URA3 marker through HA3 recombination. (C) Southern blot analysis of genomic DNA from the CAI4 strain and its URA3 preexcision (SZY51) and postexcision (SZY63) derivatives, digested with EcoRV and HindIII and hybridized with the TAC1 probe shown in panel B. Marker sizes are indicated on the left. (D) Western blot analysis of protein extracts from strains CAI4 and the Tac1p-HA3 integrant SYY63 with an anti-HA monoclonal antibody. Molecular size markers are indicated on the left.
### Table 6. List of Tac1p-enriched promoters (P < 0.001)

| Systematic name<sup>a</sup> | CGD/NRC name<sup>b</sup> | CandidaDB name<sup>c</sup> | S. cerevisiae ortholog<sup>d</sup> | GO terminology<sup>e</sup> | BR<sup>f</sup> | P value<sup>e</sup> | E 5674/5457<sup>g</sup> | E SY31/5457<sup>h</sup> |
|-----------------------------|--------------------------|---------------------------|-----------------------------|--------------------------|--------|----------------|------------------|------------------|
| orf19.6000                  | CDR1                     | CDR1                      | PDR5                        | Transporter activity     | 10.5   | 0.0000        | 4.4*             | −1.0             |
| orf19.6809                  | AST2                     | IPF8041                   | AST2                        | Peptidase activity       | 4.1    | 0.0000        | 1.6              | 1.9              |
| orf19.7669                  | IPE1                     | IPF3931                   | BDH2                        | 3.3  | 0.0000        | 5.4*             | 1.5              |
| orf19.2568                  | IFU5                     | IFU5                      | WWM1                        | 3.0  | 0.0000        | 5.4*             | 1.5              |
| orf19.86                     | GPX2                     | GPX1                      | HR1                         | Glutathione peroxidase activity | 2.8    | 0.0000        | 4.0*             | −1.2             |
| orf19.3406                  | IPF9989                  | YHL008C                   | YGR110W                     | 2.5  | 0.0000        | 2.7*             | −1.5             |
| orf19.7166                  | IPF2186                  | YGR108W                   | 2.8  | 0.0000        | 5.0              | 1.2              |
| orf19.7042                  | IPF3080                  | YGR110W                   | 2.1  | 0.0000        | 5.5*             | 1.3              |
| orf19.93                     | PDR16                    | IPF14895                  | MIC17                       | Phosphatidylinositol transporter activity | 2.1    | 0.0000        | 1.5              | −1.1             |
| orf19.1027                  | PDR17                    | IPF1548                   | PDR1                        | 2.1  | 0.0000        | 20.0*            | 1.2              |
| orf19.3395                  | IPF9983                  | YHR1048W                  | 2.0  | 0.0000        | 2.8*             | 1.2              |
| orf19.5877                  | ATV1                     | IPF1837                   | ATV1                        | 2.0  | 0.0000        | 2.8*             | 1.2              |
| orf19.6627                  | IPF2557                  | YGR110W                   | 1.9  | 0.0000        | 2.6*             | −1.0             |
| orf19.23                     | RTA3                     | IPF19998                  | LPE10                       | Phospholipid-translocating ATPase activity | 1.8    | 0.0000        | 41.0*            | 1.0              |
| orf19.6501                  | IPF3931                  | YJU3                      | 1.8  | 0.0000        | 4.6*             | 1.1              |
| orf19.5037                  | IPF7054                  | YJU3                      | 1.8  | 0.0000        | 7.7              | 2.1              |
| orf19.4898                  | IPF8950                  | FMP52                     | 1.8  | 0.0000        | 3.4              | 1.1              |
| orf19.1267                  | CAAJ                     | IPF10278                  | CAAJ                        | 1.7  | 0.0001        | 2.5              | −1.2             |
| orf19.1887                  | IPF6464                  | YEH1                      | 1.7  | 0.0001        | 4.5              | 1.2              |
| orf19.691                   | GPD2                     | IPF6464                   | GPDI                        | 1.7  | 0.0001        | 1.1              | 1.1              |
| orf19.2175                  | IPF9998                  | AIF1                      | 1.7  | 0.0001        | 3.3              | 1.3              |
| orf19.1444                  | EMT2                     | IPF755.3                  | EMT2                        | 1.7  | 0.0001        | 1.5              | 1.1              |
| orf19.5958                  | CDR2                     | IPF9983                   | LPE10                       | Magnesium ion transporter activity | 1.6    | 0.0003        | 1.7              | −1.7             |
| orf19.3458<sup>i</sup>     | LPE10<sup>i</sup>        | IPF9785<sup>i</sup>       | LPE10                       | 1.6  | 0.0003        | 1.7              | −1.7             |
| orf19.1089                  | PEX11                     | YGR110W                   | 1.6  | 0.0003        | 1.8*             | −2.4             |
| orf19.1665                  | MNT1                     | MNT1                      | KTR1                        | 1.6  | 0.0003        | 2.1              | 1.1              |
| orf19.7306                  | MNT1<sup>h</sup>         | MNT1<sup>h</sup>          | KTR1                        | 1.6  | 0.0003        | 5.1*             | 1.2              |
| orf19.7310<sup>h</sup>     | SUC1<sup>h</sup>         | YPR127W                   | MAL13                       | 1.6  | 0.0003        | 2.1              | 1.1              |
| orf19.7310<sup>h</sup>     | SUC1<sup>h</sup>         | YPR127W                   | MAL13                       | 1.6  | 0.0003        | 5.1*             | 1.2              |
| orf19.1718                  | ZCF8                     | IPF9769                   | 1.7  | 0.0004        | 1.7              | 1.8              |
| orf19.406                   | ERG1<sup>i</sup>         | ERG1<sup>i</sup>          | ERG1                        | 1.7  | 0.0005        | 1.6              | −1.2             |
| orf19.7603                  | IPF660                   | YMR244C-A                 | 1.6  | 0.0005        | 1.6              | −1.2             |
| orf19.5005                  | OSM2                     | OSM2                      | OSM1                        | 1.6  | 0.0005        | 2.0*             | 1.2              |
| orf19.577                   | IPF7353                  | YDL057C                   | d-Erythro-sphingosine kinase activity | 1.6  | 0.0006        | 2.3              | 1.5              |
| orf19.5257                  | LCB4                     | LCB4                      | LCB4                        | 1.6  | 0.0007        | 4.0*             | 1.1              |
| orf19.5525                  | IPF4328                  | YMR315W                   | 1.5  | 0.0008        | 2.3*             | 1.1              |
| orf19.4476                  | IFD6                     | YPL088W                   | 1.6  | 0.0009        | 2.2              | 1.2              |
| orf19.951                   | IPF1548                  | YPL088W                   | 1.6  | 0.0009        | 1.5              | 1.1              |

<sup>a</sup>or19 nomenclature according to the assembly 19 version. Systematic names in bold are modulated in the four sets of isolates, whereas those underlined are modulated at least in strain 5674.

<sup>b</sup>Gene name according to CGD (http://www.candidagenome.org/) or the NRC Candida albicans database (http://candida.bri.nrc.ca/candida/index.cfm?page=CgGeneSearch).

<sup>c</sup>Gene name according to CandidaDB (http://genolist.pasteur.fr/CandidaDB).

<sup>d</sup>S. cerevisiae ortholog or best hit according to CGD (http://www.candidagenome.org/).

<sup>e</sup>GO terminology according to CGD.

<sup>f</sup>BR, binding ratio. Probes were spotted in duplicate on the ChIP-chip arrays (H. Hogues, H. Lavoie, A. Sellam, M. Mangos, T. Roemer, E. Purisima, A. Nantel, and M. Whiteway, submitted for publication). The binding ratio with the most significant P value is shown.

<sup>g</sup>P value of the corresponding binding ratio.

<sup>h</sup>orf19.3455, expression (n-fold) of the gene in strain 5674 relative to that in strain 5457. An asterisk (*) indicates that expression (n-fold) is statistically significant (see Table S1 in the supplemental material).

<sup>i</sup>orf19.3455 probe is shared by LPE10 and KIC2; the orf19.3719 probe is shared by SUC1 and UGA33.
tified eight new genes containing the CGGN_4CGG sequence, AST2, ATF1, PEX11, IFD6, and four ORFs of unknown function (orf19.7166, orf19.7042, orf19.6627, and orf19.4898), for a total of 15 matches in 12 genes (Table 7). As a control, we searched the CGGN_3CGG motif in 1.5 kb of promoter sequence from 6,068 ORFs and found an average of 1.5 matches per 37 promoters, yielding a 10-fold enrichment for the presence of the CGGN_3CGG sequence in the Tac1p-bound promoters. We also searched the 37 enriched promoters for the motif CGGN_3CGG, since our unpublished data indicated that a CGGATTCCGG sequence in the PDR16 promoter is involved in its transcriptional activation by Tac1p. This analysis identified seven genes (including PDR16), one of them with three CGGN_3CGG motifs (Table 7). Searching the 6,068 promoter sequences yielded an average of 2.2 genes per 37 promoters, resulting in a 3.2-fold enrichment for that sequence in the Tac1p-bound promoters.

Finally, we examined whether the CGGN_3CGG and CGG NCGG motifs appear in the promoter regions of the genes whose expression was modulated in all four resistant isolates (31 up-regulated and 12 down-regulated) compared to their matched parent strains. We found seven up-regulated genes containing the CGGN_3CGG motif, five of which were also identified by the ChIP-chip experiments (CDR1, CDR2, IFU5, RTA3, and orf19.4898) (the two others being TAC1 and orf19.3447), as well as two up-regulated genes with the CGG NCGG motif (TAC1 and orf19.3447) (Table 7). Finally, a search of the down-regulated genes identified one gene with the CGGN_3CGG motif (SOD5) and one gene with the CGG NCGG motif (OPT6).

Expression and location data mining. When merging the expression and location data, we identified eight genes whose promoters were bound by Tac1p in vivo and which were up-regulated in the four azole-resistant clinical isolates in a TAC1-dependent manner, thus qualifying these genes as bona fide Tac1p targets. These genes were CDR1 and CDR2, GPX1 (putative glutathione peroxidase), LCB4 (putative sphenophile kinase), and RTA3 (putative phospholipid flippase), as well as three genes of unknown function: IFU5, orf19.1887, and orf19.4898 (Tables 4 and 6). We also identified nine genes whose promoters were bound by Tac1p in vivo and which were significantly modulated in a TAC1-dependent manner in at least strain 5674, the isolate displaying the strongest several-fold change in gene expression. These genes were orf19.5877 (ATF1, alcohol acetyltransferase), orf19.1089 (PEX11, fatty acid oxidation), orf19.5005 (OSM2, fumarate reductase), and orf19.7319 (SUC1, sucrose metabolism), as well as five ORFs of unknown function (orf19.6627, orf19.7042, orf19.5525, orf19.3406, and orf19.6501) (Table 6; see also Table S1 in the supplemental material). These results suggest that, in addition to its function in azole drug resistance, Tac1p regulates other cellular functions, such as lipid metabolism and oxidative stress response (see Discussion).

FIG. 3. In vivo enrichment of Tac1p binding at the CDR1, PDR16, and TAC1 promoters, determined using Q-PCR. The CA14 and SZY63 strains were submitted to ChIP (three biological replicates), and the recovered DNA samples were analyzed by Q-PCR using Universal Probe-Library probes (Roche) for the PDR16, TAC1, SPS4, and FUR1 promoters or a TaqMan probe (IDT) for the CDR1 promoter. Enrichments (n-fold) are presented in log scale: 3.8 for the PDR16 promoter (95% confidence interval of 4.0, 5.0), 28.8 for the CDR1 promoter (95% confidence interval of 21.4, 38.9), 189.3 for the TAC1 promoter (95% confidence interval of 128.6, 278.6), and 1.1 for the FUR1 promoter (95% confidence interval of 0.9, 1.3), which was used as a negative control. Error bars denote standard deviations.

DISCUSSION
To identify the Tac1p regulon, we combined genome-wide expression and location analyses, two technologies with complementary strengths and limitations. Expression profiling is extremely valuable since it can identify all of the changes in transcript abundance associated with the perturbation of a specific transcriptional regulator; however, it cannot distinguish between direct and indirect effects at individual target promoters. Location profiling is a very powerful tool to identify all of the direct targets of a specific transcription factor; however, it does not allow the determination of whether the bound factor acts as an activator or a repressor of these targets. Each method also gives rise to false positives and false negatives. Merging the results from the two approaches thus generates data that are most complete and cross-validated.

While merging the expression and location data sets for Tac1p, we found that, out of 37 genes whose promoters were bound by Tac1p, 20 (64%) did not have their expression modulated (Table 6). Possible technical explanations for this limited overlap are that some of these genes did not pass the stringent criteria applied to the expression microarray data or that they represent false positives. However, a biological explanation could be that the expression of the bound promoters is not modulated in response to gain-of-function mutations in Tac1p but rather in response to other potential Tac1p-activating signals. We also found that, out of 43 modulated genes, 35 (81%) were not identified in the location profiling experiments, suggesting that these genes are indirect targets of Tac1p (i.e., controlled by Tac1p-regulated transcription factors and/or other transcriptional regulators) or that they are not bound by Tac1p under nonactivating conditions (see below). It is also possible that the detection of Tac1p binding to some of these targets may have been prevented by the fact that their Tac1p-binding site is too far from the oligonucleotide sequence printed on the intergenic chip, as we found for TAC1.

Last, some of these genes could have been missed by use of a P value of <0.001 as the cutoff in the location array experi-
ments. This seems to be the case for the Sip3 gene, whose expression was up-regulated in the four azole-resistant isolates in a TAC1-dependent manner and which had a binding ratio of 1.6 but a P value of 0.0014. Taken together, combining the expression and location profiling data allowed us to identify many new genes which unambiguously belong to the Tac1p regulon and thereby gain new insights into the biological functions of Tac1p.

Previously, we examined the gene expression profiles of matched isolates 2, 3, 15, and 17 by using an earlier-generation microarray (61). In that analysis, we identified five genes as matched isolates 2, 3, 15, and 17 by using an earlier-generation microarray (61). In that analysis, we identified five genes as

| Motif definition | Systematic name | CGD name | Motif | Strand | Position |
|------------------|-----------------|----------|-------|--------|----------|
| CGGN,CGG         | orf19.6000      | CDR1     | CGGAATACGG | S       | 457      |
|                  | orf19.6869      | AST2     | CGGCTAACGG | A       | 262      |
|                  | orf19.2568      | IFU5     | CGGAATACGG | A       | 235      |
|                  | orf19.7166      |          | CGGTAACGG  | S       | 292      |
|                  | orf19.7042      |          | CGGGAACGG  | S       | 285      |
|                  | orf19.5877      | ATFI     | CGGATACGG  | S       | 731      |
|                  | orf19.6627      |          | CGGATACGG  | A       | 632      |
|                  | orf19.23        | RTA3     | CGGAATACGG | S       | 595      |
|                  | orf19.4898      |          | CGGTTACGG  | A       | 232      |
|                  | orf19.5958      | CDR2     | CGGAAATCGG | A       | 220      |
|                  | orf19.1089      | PEX11    | CGGGAACGG  | A       | 340      |
|                  | orf19.4476      | IFD6     | CGGTTGTCGG | A       | 339      |
|                  | orf19.10790     | TAC1     | CGGAGCACGG | A       | 1054     |
| CGGN,CGG         | orf19.3406      |          | CGGCAACGG  | A       | 332      |
|                  | orf19.1027      | PDR16    | CGGATACGG  | S       | 558      |
|                  | orf19.5037      |          | CGGTTCCGG  | A       | 358      |
|                  | orf19.2175      |          | CGGAAACGG  | A       | 351      |
|                  | orf19.3455      | LPE10    | CGGAGACGG  | A       | 250      |
|                  | orf19.1718      | ZCF8     | CGGGTACGG  | S       | 167      |
|                  | orf19.6869      | AST2     | CGGATACGG  | S       | 246      |
|                  | orf19.10790*    | TAC1     | CGGAAACGG  | A       | 1040     |

a orf19 nomenclature according to the assembly 19 version.
b Gene name at CGD (http://www.candidagenome.org/).
c The underlined motifs have been described previously (16). Consensus sequences were obtained using the AlignX program (component of Vector NTI Advance 10.1.1; Invitrogen) and the WebLogo application (http://weblogo.berkeley.edu/) (17). The consensus sequence for CGGN,CGG motifs is CGGATAACGG, with nucleotide frequencies of 1.0, 1.0, 1.0, 0.6, 0.6, 0.7, 0.5, 1.0, 1.0, and 1.0, respectively. The consensus sequence for CGGN,CGG motifs is CGGAATACGG, with nucleotide frequencies of 1.0, 1.0, 1.0, 0.5, 0.5, 0.4, 1.0, 1.0, and 1.0, respectively.
d S, sense strand; A, antisense strand.
e Nucleotide position from the ATG translation start site.
f Position in contig19.20170, upstream of orf19.10700; position in contig.10170, upstream of orf19.3188, is −1051 due to sequence polymorphisms.
g Present only in contig.20170 due to a sequence polymorphism (underlined) (CGGAAACGG).
additional genes whose expression is modulated in azole-resistant strains, dependent upon Tac1p.

Known Tac1p targets, such as CDR1, CDR2, IFU5, and RTA3, are induced in a TAC1-dependent manner upon exposure of the cells to fluphenazine or estradiol or upon expression of a gain-of-function TAC1 allele in a tac1Δ/tac1Δ background (16, 20). This indicates that the activation of Tac1p by such inducers or by gain-of-function mutations is required for Tac1p-mediated transcriptional regulation. Our ChiP-chip experiments were done with cells grown in rich media under uninduced conditions (45). We found that, under these conditions, Tac1p binds to its target promoters, indicating that this binding is constitutive or at least partially constitutive since it cannot be excluded that Tac1p binding increases in the presence of an inducer or an activating mutation. Our functional characterization of the epitope-tagged Tac1p strain showed that Tac1p binding to its targets is not due to an activating effect of introducing the HA tag at the C terminus of Tac1p (see Fig. S1 in the supplemental material). It was reported recently that the S. cerevisiae zinc cluster regulator Pdr1p, which controls the expression of the multidrug transporters PDR5, SNQ2, and YOR1 (6), also binds constitutively to its target promoters in vivo (24). Likewise, the S. cerevisiae zinc cluster transcription factor War1p, which controls the expression of the ABC transporter Pdr12p in response to weak acid stress, has been shown to constitutively bind to the PDR12 promoter in vivo (43). Thus, the mechanisms by which Tac1p activates transcription in response to drugs or to gain-of-function mutations appear to be similar to those already documented for zinc cluster factors in S. cerevisiae and most likely involve postbinding mechanisms, such as loss of interaction with a repressor protein, as proposed for Upc2p and Ecm22p (18), or recruitment of coactivator complexes (SAGA, Mediator, SWI/SNF), as shown for Pdr1p (30).

Our analyses of the Tac1p-bound promoters for the presence of the DRE-like motif CGGN₂CGG allowed us to identify eight new Tac1p targets, in addition to CDR1, CDR2, RTA3, and IFU5, containing this sequence (Table 7). The fact that 25 of the Tac1p-bound promoters do not contain this motif suggests that Tac1p may recognize additional configurations of the CGG triplets, including monomeric CGG triplets or CGG triplets with different spacings and/or orientations (48). The latest proposition is supported by our unpublished data that a CGGATTCCGG sequence in the PDR16 promoter is involved in its transcriptional activation by Tac1p and the enrichment of the CGGN₂CGG motif in the Tac1p-bound promoters (Table 7). Alternatively, Tac1p may bind upstream or downstream of the DNA sequences analyzed (1.5 kb of upstream sequences). Finally, it is also possible that Tac1p binds to some of its targets indirectly through its association with other DNA-binding proteins. For instance, the S. cerevisiae zinc cluster proteins Rsc30p and Rsc3p, which are part of the chromatin remodelling complex, have been shown to bind indirectly to DNA (2). Whether or not the DRE motifs identified in the Tac1p-bound promoters are functional as well as the sequences/factors mediating Tac1p binding to its target promoters in the absence of a classical DRE remains to be determined experimentally.

We showed previously that strain 5674 overexpresses the TAC1 gene and that introduction of a TAC1 allele carrying the N972D gain-of-function mutation in a tac1Δ/tac1Δ strain leads to the constitutive upregulation of the TAC1 transcript, which suggested that Tac1p is positively autoregulated, directly or indirectly (85). We show in the present study that (i) TAC1 is upregulated in three additional azole-resistant isolates in which the Tac1p pathway is activated (Table 4), (ii) the TAC1 promoter contains a DRE motif, and (iii) Tac1p binds in vivo to its own promoter (Fig. 3). Taken together, our findings support the proposition that TAC1 expression is controlled by a direct positive autoregulatory loop. This situation is similar to that of S. cerevisiae Pdr3p, which binds in vivo to two pleiotropic DREs located in the PDR3 promoter to transactivate its own expression (27). It appears that direct self-regulatory loops are a common feature among zinc cluster transcription factors in yeast, including Pdr3p and Yrr1p (pleiotropic drug resistance), Hap1p (respiration), and Sbt5p (pentose phosphate pathway) (48). Studies of budding yeast have shown that transcription factor autoregulation is necessary to respond to environmental stresses. As examples, autoregulation of PDR3 in a pdr1Δ background is crucial for growth on a medium containing cycloheximide (27) and autoregulation of the basic leucine zipper transcription factor Hac1p, which controls the unfolded protein response, is required to protect the cells from prolonged endoplasmic reticulum stress (55). Similarly, autoregulation of Candida glabrata AMT1, a copper-sensing transcription factor, is necessary to protect the cells upon exposure to high environmental copper levels (84). Thus, one possible outcome of Tac1p autoregulation would be an amplifiable production of the Tac1p protein necessary for rapid and sustained response to drugs and yet-unknown activating signals (45).

Three previously identified Tac1p targets (CDR1, CDR2, and PDR16) have been shown to play a role in azole drug resistance (59, 66, 68). Thus, Tac1p confers azole resistance by activating different effectors, each contributing to some extent to the overall azole resistance of the cells. Additional Tac1p targets identified by our studies and coregulated with CDR1, CDR2, and PDR16 may also play a protective role against toxic injuries. For instance, orf19.4531 is found among the group of genes differentially expressed under the gene ontology (GO) molecular function term “ATPase activity coupled to movement of substances,” including CDR1, CDR2, and RTA3 (Table 4). orf19.4531 encodes a putative ABC transporter of the PDR subfamily (to which CDR1 and CDR2 belong). Whether this transporter impacts azole resistance, alone or in conjunction with Cdr1p and Cdr2p, remains to be determined. This efflux pump may also protect the cell from other toxic compounds. As another example, orf19.86 (GPX1), which encodes a putative glutathione peroxidase, is an integral component of the glutathione and glutathione-dependent enzyme system, which has been implicated in the resistance of tumor cells to anticancer agents (7, 22). Increased activity of this enzyme system is often observed in conjunction with increased activity of the ABC transporter P glycoprotein in drug-resistant human cancer cells (9).

Although not found among the 37 promoters observed to be bound by Tac1p, CHK1 was among the 31 genes that were consistently coregulated with CDR1 and CDR2 in all four matched isolate sets. Its up-regulation in these isolates is at least influenced by Tac1p, as deletion of TAC1 in isolate 5674 reduced its expression to normal levels. Chk1p is a histidine
kinase involved in a two-component signaling pathway, along with the response regulator Ssk1p, which regulates cell wall biosynthesis (10, 11). Interestingly, it was shown recently that strains of \textit{C. albicans} lacking either of these signal transduction proteins are hypersensitive to FLC (13). Up-regulation of \textit{CHK1} in association with \textit{CDR1-} and \textit{CDR2}-mediated azole resistance suggests that, in addition to its requirement for baseline azole tolerance in azole-susceptible cells, this protein may contribute to azole resistance in clinical isolates. In the related fungal species \textit{C. glabrata}, the ATPase activity of the ABC transporter Cdr1p and the drug efflux activity of the ABC transporter Pdh1p (Cdr2p) are regulated by phosphorylation (77, 78). Likewise, phosphorylation has been shown to modulate transcriptional activity of the transcription factor Gal4p (65) and has been suggested to regulate the activity of the transcriptional regulator Pdr3p (49). It is therefore tempting to speculate that Chk1p is involved in the phosphorylation and activity of the efflux pumps Cdr1p and Cdr2p or possibly Tac1p itself. Further investigation into the contribution of this signaling pathway to azole resistance is required to address this question.

Our genome-wide location experiments revealed that Tac1p binds to the promoters of a group of genes involved (or predicted to be involved) in lipid metabolism. These genes are \textit{CDR1}, \textit{CDR2}, \textit{PDR16}, \textit{RTA3}, \textit{ATF1}, \textit{ERG1}, \textit{LCB4}, orf19.6501, orf19.7166, and orf19.1887. With the exception of \textit{ERG1}, all of these genes were significantly up-regulated in a \textit{TAC1}-dependent manner at least in one out of the four clinical isolates tested by expression microarrays, strongly suggesting (i) that these genes are direct transcriptional targets of Tac1p and (ii) that there is a role for Tac1p in lipid metabolism. It has been shown that Rsb1p, the \textit{S. cerevisiae} orthologue of \textit{C. albicans} Rta3p, plays an essential role in the translocation of long-chain bases across the plasma membrane (39), suggesting a role for Rsb1p, and thus potentially Rta3p, in regulating the sphingolipid composition of the plasma membrane. Interestingly, \textit{S. cerevisiae} \textit{LCB4} encodes a major sphingolipid-long-chain-base kinase required for synthesis of long-chain-base phosphates and for the rapid incorporation of long-chain bases from the culture medium into sphingolipids (29). We also found that orf19.3104, the orthologue of \textit{S. cerevisiae} \textit{YDC1} encoding an alkaline dihydroceramidase, is up-regulated in a \textit{TAC1}-dependent manner in all four sets of clinical isolates (Table 4), although binding of Tac1p to the promoter of this gene was not detected. Ydc1p hydrolizes dihydroceramides to free fatty acid and dihydrosphingosine, the substrate of \textit{LCB4} (50). Taken together, these findings suggest a role for Tac1p in the synthesis and translocation of sphingolipids into the plasma membrane.

Likewise, \textit{C. albicans} Cdr1p and Cdr2p have been shown to function as plasma membrane energy-dependent translocators of phospholipids, mediating their in-to-out movement (flop-pases) (75). Thus, Cdr1p and Cdr2p would act in concert with Rta3p to establish the asymmetry of membrane lipids in the plasma membrane. Interestingly, Tac1p binds to the promoter of the \textit{S. cerevisiae} \textit{AST1} homologue, orf19.6869 (\textit{AST2}). \textit{S. cerevisiae} \textit{Ast1p} was shown to be required for the raft association and restoration of surface delivery of a mutant of the major plasma membrane proton ATPase, Pma1p, that was mistargeted to the vacuole (5). Elsewhere, it has been shown that Cdr1p is a lipid raft-associated protein (34). An attractive model would be that Tac1p contributes to the raft association and proper routing of its target gene products Cdr1p, Cdr2p, and Rta3p to the plasma membrane by regulating \textit{AST2} expression. Recent studies have demonstrated that both sphingolipids and sterols are important determinants of surface localization of Cdr1p, as reduced membrane localization of Cdr1p was associated with increased susceptibility of \textit{C. albicans} to ketoconazole in mutants defective in ergosterol biosynthesis (53). Interestingly, we found that Tac1p binding was enriched at the \textit{ERG1} (1.7-fold, \textit{P} value of 0.0005) and \textit{ERG2} (1.6-fold, \textit{P} value of 0.0011) promoters. Moreover, \textit{ERG2} gene expression was significantly up-regulated in a \textit{TAC1}-dependent manner in isolates C56, Gu5, and 17 (see Table S1 in the supplemental material), suggesting a role of Tac1p in ergosterol biosynthesis. \textit{PDR16}, another target of Tac1p, is the functional homologue of the \textit{S. cerevisiae \textit{PDR16}}, encoding a lipid transfer protein of the Sec14 family (58, 66). \textit{S. cerevisiae} \textit{Pdr16p} was shown to localize to lipid particles and to transport phosphatidylinositol (71). There is strong evidence showing that lipid transfer proteins exchange lipid molecules between organelle membranes and plasma membrane at membrane contact sites (33). Thus, by controlling the expression of the \textit{PDR16} gene, Tac1p would act as a regulator of phosphatidylinositol transfer within membrane contact sites, further supporting the role of Tac1p in membrane lipid traffic.

Consistent with the potential role of Tac1p in membrane lipid metabolism, we found that the Tac1p targets orf19.7166, orf19.6501, and orf19.1887 encode three putative steryl ester hydrolases or triglyceride lipases, as (i) a Pfam analysis detected an \(\alpha/\beta\) hydrolase fold in their primary sequences (Pfam entries PF04083.6 for orf19.7166 and PF00561 for orf19.1887 and orf19.6501), (ii) their primary sequences contain the lipase consensus sequence motif GXSXG (21), and (iii) Kyte-Doolittle hydrophathy plots revealed hydrophobic regions in the primary sequences, suggesting that these proteins are potentially membrane anchored to lipid particles (data not shown). Steryl esters and triacylglycerols are neutral lipids stored in lipid particles and serve as an energy source as well as a rapid fatty acid source needed upon lipid depletion conditions (79). The orf19.1887 protein is highly homologous to \textit{S. cerevisiae} \textit{Yeh1p}, a steryl ester hydrolase localized to lipid particles (41, 42), whereas the orf19.7166 and orf19.6501 proteins display moderate homology to the products of \textit{S. cerevisiae} \textit{YGR110W} and \textit{YJU3} genes. \textit{Yju3p} was shown to be localized in lipid particles as well (3, 54). Another Tac1p target whose product was shown to be localized in lipid particles in \textit{S. cerevisiae} \textit{ATF1}, encoding an alcohol acetyltransferase (76). This gene was significantly up-regulated in a \textit{TAC1}-dependent manner in strain 5674 (see Table S1 in the supplemental material). The fact that \textit{Atf1p} localizes to lipid particles and functions in the esterification process suggests that it is involved in the metabolism of lipids probably by transferring acetyl groups to free hydroxyl groups of fatty acids. Thus, in addition to being a potential regulator of membrane lipid traffic, Tac1p appears to be an important regulator of lipid mobilization in \textit{C. albicans}, a function needed for rapid restoration of membrane lipids upon lipid depletion conditions (79).

Our studies identified two Tac1p target genes, \textit{GPX1} and \textit{SOD5}, which have been shown to be involved in the oxidative
stress response. We provide strong evidence that Tac1p directly regulates the expression of GPX1, which is implicated in response to oxidative stress (44). Interestingly, although the C. albicans genome encodes two other putative glutathione peroxidases (encoded by orf19.85 and orf19.87) with high homology to Gpx1p, only Gpx1p was found to be overproduced upon treatments with diamide or hydrogen peroxide (44), suggesting that, among the three putative glutathione peroxidases, GPX1 plays an important role in the oxidative stress response. It was also shown that S. cerevisiae GPX1 encodes a phospholipid hydroperoxide glutathione peroxidase which protects the cell against phospholipid hydroperoxides during oxidative stress (4).

SOD5, a copper, zinc superoxide dismutase found in the cell wall of C. albicans, has been shown to be important in protection against osmotic and oxidative stress (51). Since SOD5 seems to play an important role in this process, it is interesting that the gene is in fact down-regulated in all fourazole-resistant clinical isolates studied. Martchenko et al. showed that the gene is in fact down-regulated in all four azole-resistant mutants studied. Martchenko et al. showed that while deleting SOD5 does not decrease cell viability, susceptibility to hydrogen peroxide under nutrient-poor conditions is increased (51). Indeed, we have shown previously that azole-resistant isolate 5674, while resistant to diamide, is hypersusceptible to hydrogen peroxide (31). While this hypersusceptibility to hydrogen peroxide could be explained by the down-regulation of SOD5, resistance to diamide could be attributed to the up-regulation of the CDR2 gene by Tac1p since CDR2 expression was shown to confer diamide resistance in S. cerevisiae (31). Taken together, these observations indicate that TAC1 differentially protects the cell against different oxidative stresses.

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