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Genome-wide expression profiling reveals genes associated with amphotericin B and fluconazole resistance in experimentally induced antifungal resistant isolates of *Candida albicans*

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***Objectives*: The aim of this study was to identify changes in the gene expression profile of *Candida albicans* associated with the acquisition of experimentally induced resistance to amphotericin B and fluconazole.**

***Methods*: *C. albicans* SC5314 was passed in increasing concentrations of amphotericin B to generate isolate SC5314-AR. Susceptibility testing by Etest revealed SC5314-AR to be highly resistant to both amphotericin B and fluconazole. The gene expression profile of SC5314-AR was compared with that of SC5314 using DNA microarray analysis. Sterol composition was determined for both strains.**

***Results*: Upon examination of MICs of antifungal compounds, it was found that SC5314-AR was resistant to both amphotericin B and fluconazole. By microarray analysis a total of 134 genes were found to be differentially expressed, that is up-regulated or down-regulated by at least 50%, in SC5314-AR. In addition to the cell stress genes *DDR48* and *RTA2*, the ergosterol biosynthesis genes *ERG5*, *ERG6* and *ERG25* were up-regulated. Several histone genes, protein synthesis genes and energy generation genes were down-regulated. Sterol analysis revealed the prevalence of sterol intermediates eburicol and lanosterol in SC5314-AR, whereas ergosterol was the predominant sterol in SC5314.**

***Conclusion*: Along with changes in expression of these ergosterol biosynthesis genes was the accumulation of sterol intermediates in the resistant strain, which would account for the decreased affinity of amphotericin B for membrane sterols and a decreased requirement for lanosterol demethylase activity in membrane sterol production. Furthermore, other genes are implicated as having a potential role in the polyene and azole antifungal resistant phenotype.**

Keywords: microarrays, polyenes, azoles, antifungal resistance, *C. albicans*

Introduction

Candida albicans and other *Candida* species are opportunistic fungal pathogens in humans, causing both mucosal and invasive bloodstream infections.[1–2] Amphotericin B, a polyene, and fluconazole, an azole, are two of the most commonly used drugs for the treatment of *Candida* infections. These agents both ultimately exert their effects on the fungal cell membrane, but there are distinct differences between them.

Amphotericin B, a fungicidal agent, has activity against a number of pathogenic fungi.[3] Its mode of action is thought to involve binding to ergosterol in the fungal cell membrane, which results in the formation of pores and ultimately leakage of cellular components. Although *C. albicans*, *Candida krusei* and *Candida*

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glabrata infrequently exhibit resistance to amphotericin B, species such as *Candida lusitaniae* and *Candida guilliermondii* are intrinsically resistant to the drug.[4,5] Acquired resistance to amphotericin B has been reported in *C. albicans*, often in conjunction with resistance to the azole antifungal agents.[6]

Fluconazole and other azoles are fungistatic and inhibit the cytochrome P450 enzyme lanosterol demethylase (encoded by the *ERG11* gene), a key enzyme in ergosterol biosynthesis.[7] Additionally, C-22 sterol desaturase, another cytochrome P450 enzyme encoded by the *ERG5* gene, also appears to be a target of azole antifungal compounds.[8] There are several well-documented examples in the literature of acquisition of azole resistance in clinical isolates of *C. albicans*.[9–13] Moreover, many non-*albicans* species of *Candida* are either intrinsically resistant to fluconazole or acquire a resistant phenotype at a greater frequency.[14,15]

Several studies have focused on identifying mechanisms of resistance to amphotericin B and fluconazole in *C. albicans*.[9,11,12,16–20] To date, resistance to fluconazole has been shown to involve two general mechanisms: increased expression of drug efflux pumps and alterations in genes that encode enzymes in the ergosterol biosynthesis pathway. Drug efflux pumps demonstrated to be overexpressed in some resistant isolates are ATP-binding cassette transporters encoded by *CDR1* and *CDR2* and a major facilitator encoded by *MDR1* (also known as *BMR1*) in *C. albicans*.[9,11,1,12,16] A commonly altered ergosterol biosynthesis enzyme is the azole target Erg11p. This experiences amino acid substitutions that result in lowered affinity of azoles for the enzyme or is overexpressed at levels that overwhelm the drug's ability to inhibit growth.[16,21]

Another mechanism by which antifungal resistance can occur is through changes in other components of the ergosterol biosynthesis pathway that result in the accumulation of sterol intermediates.[22] In clinical isolates of *C. albicans*, combined resistance to both amphotericin B and azoles has been associated with accumulation of ergosta-7,22-dienol. This is consistent with reduced activity of C-5 desaturase, encoded by *ERG3*.[6,23,24] Such changes in *ERG3* are also thought to reduce the conversion of episterol to potentially toxic metabolites that accumulate in the cell membrane during azole exposure.[22]

In the present study, we generated a strain of *C. albicans* that is resistant to both amphotericin B and fluconazole by serially passaging *C. albicans* strain SC5314 in increasing concentrations of amphotericin B until resistance was achieved. Gene expression in the resulting resistant strain SC5314-AR and its parent strain SC5314 was compared for over 6000 genes by microarray analysis. This analysis identified the differential expression of genes involved in ergosterol biosynthesis, cell stress and resistance to other inhibitors of the ergosterol biosynthesis pathway. Analysis of sterol content in the strains confirmed the hypothesis that resistance to fluconazole and amphotericin B was the result of accumulation of sterol intermediates consistent with inactivation of lanosterol demethylase, and potentially due, in part, to the increased expression of several ergosterol biosynthesis genes.[27]

Materials and methods

C. albicans isolates

Cultures of *C. albicans* SC5314 were passed in increasing concentrations of amphotericin B. Specifically, the culture of SC5314 was initiated by adding 100 μL of glycerol stock to 15 mL of 0.25 mg/L amphotericin B in YPD broth (1% yeast extract, 2% peptone, 2% dextrose). Cultures were grown at 30°C in an environmental shaking incubator, and cells were passed when cultures were turbid (1–3 days between passages). With each passage, the concentration of amphotericin B in YPD broth was doubled until the concentration used for the final passage was 128 mg/L. Aliquots of the parent strain and the final strain SC5314-AR were stored at −70°C as glycerol stocks.

Determination of stability of the antifungal resistant phenotype

Stability of the resistant phenotype was tested by growing the SC5314-AR strain in 10 mL of YPD broth (without drug) for a total of ~60 doublings. Once every 24 h, an aliquot of the previous day's saturated culture was added to 10 mL of fresh YPD broth to an OD_{600} of 0.2, the culture was grown to an OD_{600} of 0.6–0.8, an aliquot was taken to prepare a glycerol stock and the remainder of the culture was allowed to grow to saturation until the next day. The MICs of amphotericin B and fluconazole were then determined for each of the glycerol stocks collected.

Susceptibility testing of C. albicans isolates

MICs were determined with fluconazole [(FLU) 0.016–256 mg/L] and amphotericin B [(AMB) 0.002–32 mg/L] Etest strips (AB Biodisk North America Inc., Piscatway, NJ, USA), with solidified (1.5%) 100 mm RPMI + MOPS agar plates serving as the medium. A standardized cell suspension (0.5 McFarland) in sterile 0.85% NaCl was prepared by transferring three to four colonies from a 24 h culture onto potato dextrose agar (Remel, Lenexa, KS, USA). Plates were then inoculated by pouring 5 mL of the standardized cell suspension onto the agar. After allowing the suspension to achieve a uniform distribution, moisture was aspirated with a vacuum pipette and the plates allowed to dry at ambient temperature for 15 min. Antifungal strips were then placed onto the agar. Plates were then inoculated at 37°C and MICs recorded at 24 h. The susceptibility endpoint for fluconazole was read at the intersection of the first discernable growth-inhibition ellipse, whereas that of amphotericin B was read at the intersection of the scale with the first completely clear ellipse.

RNA isolation

For each of two independent sets of cultures, an aliquot of glycerol stock from each isolate was diluted in YPD broth and grown overnight at 30°C in an environmental shaker. Cultures were diluted to an OD_{600} of 0.1–0.2 in 200 mL of fresh YPD (1% yeast extract, 2% peptone, 2% dextrose) and grown at 30°C for 3 h for subsequent RNA isolation. RNA was isolated using the hot phenol method.[25] Briefly, cells were collected by centrifugation and snap-frozen in liquid nitrogen. Frozen cells were then resuspended in 12 mL of AE buffer (50 mM sodium acetate pH 5.2, 10 mM EDTA) at room temperature, after which 800 μL 25% SDS and 12 mL of acid phenol (pH 4.5–5.5; Fisher Scientific) were added. The cell lysate was then incubated for 10 min at 65°C with vortexing each min, snap-cooled on ice for 5 min and subjected to centrifugation for 15 min at 11 952 **g**. Supernatants were transferred to new tubes containing 15 mL of chloroform, mixed and subjected to centrifugation at 200 **g** for 10 min. RNA was precipitated from the resulting aqueous layer by transferring that portion to new tubes containing one volume isopropanol and 0.1 volume 2 M sodium acetate pH 5, mixing well, and subjecting the mixture to centrifugation at 17 211 **g** for 35 min

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at 4°C. The supernatants were removed, the pellet resuspended in 10 mL of 70% ethanol and RNA collected by centrifugation at 17 211 **g** for 20 min at 4°C. Supernatants were again removed, and RNA was resuspended in 0.5–1 mL of diethylpyrocarbonate (DEPC)-treated water. Absorbance was measured at 260 and 280 nm and integrity of RNA was visualized by subjecting 2–5 μL of the sample to electrophoresis through a 1% agarose–MOPS gel.

Microarray design and preparation

The *C. albicans* microarray was manufactured by Eurogentec SA in collaboration with the European Galar Fungail Consortium (www.pasteur.fr/recherche/unites/Galar_Fungail/). Primers for each of the 6039 putative ORFs in the *C. albicans* genome were designed to amplify a specific region of each ORF. Amplicons were an average length of 300 bp and were spotted in duplicate, along with 27 control genes, using a ChipWriter Pro robotic array printer.

Probe preparation and microarray hybridization

Ten mg of total RNA sample was added to a mixture of T20VN and Oligo(dT) primer mix; dNTPs including Cy3- or Cy5-dCTP; and DTT in a buffer containing Tris-HCl, KCl and $MgCl_2$. The reaction mixture was denatured at 65°C for 5 min and incubated at 42°C for 5 min, after which Rnasin and Superscript II reverse transcriptase (RT) were added to the mixture. The reaction proceeded at 42°C for 1 h, after which additional Superscript II RT was added, and the reaction mixture incubated at 42°C for an additional hour. To stop the reaction, EDTA and NaOH were added, the mixture was incubated at 65°C for 20 min and acetic acid was added. Five microlitres each of the Cy3- and Cy5-labelled probes were mixed with heat-denatured salmon sperm DNA, incubated at 95°C for 2 min and snap-cooled. The mixture was added to hybridization buffer and applied to the array slides under glass coverslips. Hybridization was performed at 37°C overnight in a humidified chamber. To wash the slides, the coverslip was removed and the slide incubated at room temperature in 0.2 × SSC (20 × SSC stock consists of 3 M sodium chloride, 0.3 M sodium citrate) + 0.1% SDS for 5 min, rinsed at room temperature with 0.2 × SSC for 5 min and spin-dried for 5 min. Slides were scanned using a ChipReader microarray scanner.

Data analysis

GenePix 1.0 software was used for image analysis. The local background values were calculated from the area surrounding each feature and subtracted from the total feature signal values. These adjusted values were used to determine differential gene expression for each feature. A normalization factor was applied to account for systematic differences in the probe labels by using the differential gene expression ratio to balance the Cy5 signals. Only features with a mean balanced differential expression ratio ≥1.5 or ≤−1.5 (increased or decreased by 50%) for both features representing a given cDNA on the array in two independent experiments were considered to be differentially expressed. DNA sequences were annotated on the basis of BLAST*n* searches using the Stanford database (http://www-sequence.stanford.edu/group/candida), GenBank, and the CandidaDB database (http://www.pasteur.fr/Galar_Fungail/CandidaDB/).

cDNA synthesis and RT-PCR

cDNA was synthesized using the protocol and reagents from Invitrogen Corp. (Carlsbad, CA, USA). Briefly, 2 μg of total RNA from samples not used in microarray hybridization was mixed with 2 μL random hexamers (50 ng/mL) in 10 μL of 1 mM dNTP (equimolar solution of dATP, dCTP, dGTP and TTP) solution. The mixture was incubated at 65°C for 5 min then snap-cooled on ice. A reaction mixture was added to the denatured RNA to give the following final concentrations: RT buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 5 mM $MgCl_2$, 0.01 M dithiothreitol (DTT), and 2 U RNaseOUT Recombinant Ribonuclease Inhibitor. After a 2 min incubation at 25°C, 50 U Superscript II RT was added and the reaction incubated at 25°C for an additional 10 min. The reaction was then allowed to proceed at 42°C for 50 min followed by termination at 70°C for 15 min and snap-cooling on ice. Finally, 2 U RNase H was added and the reaction incubated for 20 min at 37°C.

Table 1. Sequences and melting temperatures of primers used in RT-PCR

Primer name	Primer sequence	Tm (°C)
DDR48-F	5′-TTTCGGTTTCGGTAAAGACG-3′	54.1
DDR48-R	5′-TGAGTCGGTCTTGGAGGAAC-3′	57.2
ERG5-F	5′-GGCTCACCAATCACCACTTT-3′	56.6
ERG5-R	5′-TTAAAGAACGGCGGTAATCG-3′	54.0
ERG6-F	5′-CTGCTTCTGTTGCTGCTGAG-3′	57.3
ERG6-R	5′-CAATGGCATAAACAGCATCG-3′	53.5
ERG25-F	5′-TGGATTGGCAGCAGAATATG-3′	54.0
ERG25-R	5′-TTTGGACCAGCTTCGGTATC-3′	55.7
FTR1-F	5′-TATCATTGCCACGGTCTTGA-3′	55.2
FTR1-R	5′-GGACCAGAACCGTTTTCAGA-3′	55.9
RTA2-F	5′-AAGAGCCACACAAGCGATTT-3′	56.6
RTA2-R	5′-TCCCGTGAATAACCACCAAT-3′	55.2
UBI4-F	5′-CGAATCTTCTGACACCATCG-3′	54.0
UBI4-R	5′-CGGCGAAAATCAATCTTTGT-3′	53.0
18S rRNA-F	5′-AAACGGCTACCACATCCAAG-3′	56.3
18S rRNA-R	5′-CCAAGCCCAAGGTTCAACTA-3′	56.1

PCR was performed by mixing 1 μL of the appropriate dilution of cDNA (empirically determined for each gene to give product in the linear range), 0.5 μg of each forward and reverse primer, 2.5 U *Taq* polymerase (Sigma, St. Louis, MO, USA) and 0.1% Triton X-100 in EasyStart Micro50 PCR tubes, and subjecting the reaction mixture to the following reaction conditions: one repetition of 94°C for 5 min; 32 repetitions of 94°C for 1 min, gene-specific annealing temperature for 1 min and 72°C for 2 min; and one repetition of 72°C for 5 min. The amount of cDNA used per reaction was based upon the volume of each cDNA sample required to normalize the intensity of 18S rRNA PCR products. Equivalent volumes of PCR product were applied to a 3% agarose gel and separated by gel electrophoresis in 1 × TAE (50 × TAE stock consists of 2 M Tris-acetate, 0.05 M EDTA). Primer sequences used in PCR are listed in Table 1.

Sterol analysis

Sterols were isolated as previously described and analysed by gas chromatography.[26] Sterol analysis was performed using a DB-5 capillary column (15 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA) and an HP5890 Series II gas chromatograph equipped with Hewlett-Packard CHEMISTATION software. The gas chromatograph was programmed from 195–280°C (1 min at 195°C, then an increase at 20°C/min until 240°C and from 240–280°C at 2°C/min). The linear velocity was 30 cm/s using nitrogen as the carrier gas. All injections were run in the spitless mode.

Gas chromatography/mass spectrometry (GC/MS) analyses of sterols were performed using a Thermoquest Trace 2000 gas chromatograph interfaced to a Thermoquest Voyager mass spectrometer.

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The GC separations were performed on a DB-5MS fused silica column (20 m × 0.18 mm × 0.18 μm film thickness; J&W Scientific). The injector temperature was 190°C, whereas the oven temperature was programmed to remain at 100°C for 1 min followed by a temperature ramp of 6.0°C/min to a final temperature of 300 °C. The temperature was held at 300°C for 25 min. Helium was the carrier gas with a linear velocity of 50 cm/s in the spitless mode. The mass spectrometer was operated with the following settings: electron impact ionization mode at an electron energy of 70 eV, scanning from 40–850 atomic mass units at 0.6 s intervals, and an ion source temperature of 150°C.

Results

MICs for the parent isolate as well as the final isolate collected from serial passage are shown in Figure 1. Surprisingly, isolate SC5314-AR exhibited high-level fluconazole resistance in addition to resistance to amphotericin B. Further characterization of the strains revealed the doubling time for isolate SC5314-AR was 181 ± 19 min as compared with 97 ± 3 min for isolate SC5314.

Strain SC5314-AR was serially passaged in the absence of drug for ~60 doublings and its resistance to amphotericin B and fluconazole was found to be stable for ~28 doublings. This may reflect a mixed population of both susceptible (revertant) and resistant cells present in the culture where, after 28 doublings in the absence of drug, the susceptible cells in the culture are able to outgrow the resistant cells.

Microarray analysis revealed 133 genes differentially expressed by at least 50% in SC5314-AR, with 27 genes up-regulated and 106 genes down-regulated (Tables 2 and 3). Additionally, differential expression of several genes of interest found in the data set (*ERG5*, *ERG6*, *ERG25*, *DDR48*, *RTA2*, *UBI4*, and *FTR1*) was validated by RT-PCR and is shown in Figure 2.

Because several ergosterol biosynthesis genes were differentially expressed, sterol composition of both SC5314 and SC5314-AR was analysed and compared with each other. As shown in Table 4, ergosterol was the major sterol in SC5314, whereas SC5314-AR contained sterol intermediates, mainly lanosterol and 24-methylene lanosterol (eburicol).

Discussion

In the present study, we have identified *C. albicans* genes that are differentially expressed in association with experimentally induced resistance to amphotericin B and fluconazole (Tables 2 and 3). Particularly noteworthy is the overexpression of the ergosterol biosynthesis genes *ERG5*, *ERG6* and *ERG25*. These genes encode enzymes that represent critical steps in this pathway at points where alternate sterol production may be facilitated. As shown in Table 2, *ERG6* and *ERG25* are overexpressed in SC5314-AR. This may allow for increased conversion of lanosterol to eburicol and 14-methyl fecosterol at the expense of conversion to 4,4-dimethyl-ergostera-8,14,24-trienol (illustrated in Figure 3). By altering the pathway at this particular point, the cell would lose susceptibility to the effects of both fluconazole and amphotericin B. Overexpression of these enzymes also facilitates the conversion of episterol to ergosta-7,22-dienol and ultimately ergosta-7-enol at the expense of ergosta-5,7,24 (28) trienol. Furthermore, overexpression of the *ERG5* gene product would allow for enhanced conversion of this sterol ultimately to ergosterol, especially in the presence of fluconazole. Indeed, production of these sterol intermediates has been demonstrated in *erg11* and *erg11/erg3* mutants that exhibit high-level azole and amphotericin B resistance.[27] Likewise, our sterol analysis of SC5314-AR and SC5314 (Table 4) shows a similar sterol profile suggestive of impaired function of lanosterol demethylase in SC5314-AR leading to sterols other than ergosterol as the predominant sterol species.

In a study by De Backer *et al.*,[29] comparing the 24 h treatment of *C. albicans* strain CAI4 with 10 μM itraconazole to untreated cells by microarray analysis, several ergosterol biosynthesis genes including *ERG5*, *ERG6* and *ERG25* were found to be up-regulated. Other genes found to be differentially expressed in the present study, such as *DDR48* and *TYE7*, are similarly differentially expressed in that study as well. This relatively long-term exposure, encompassing several population doublings at such a high dose of azole should effectively shut down ergosterol biosynthesis by inhibiting the *ERG11* gene product. Comparing the results of De Backer *et al.*[29] with those presented here emphasizes the probable inactivation of *ERG11* in SC5314-AR, resulting in the gene expression profile and sterol analysis observed in the present study.

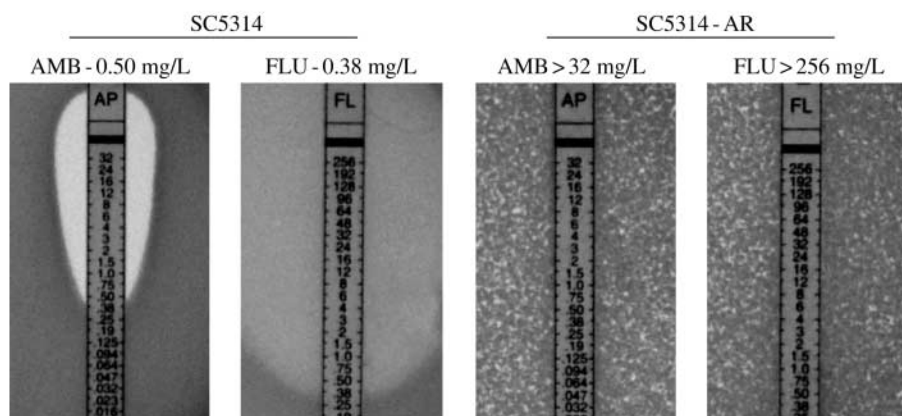

Figure 1. MICs (in mg/L) for isolates used in this study. MICs of AMB and FLU for SC5314 and SC5314-AR were determined as described in Materials and methods.

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Table 2. Genes up-regulated in SC5314-AR compared to SC5314

Gene name	*S. cerevisiae* homologue name	Function	Change in fold expression
Cell wall maintenance			
PHR2	*YMR301W*	pH-regulated protein 2	1.7
Lipid, fatty acid, sterol metabolism			
ERG6	*YML008C*	sterol transmethylase	3.4
ERG25	*YGR060W*	C-4 sterol methyl oxidase	1.9
ERG5	*YMR015C*	C-22 sterol desaturase	1.9
Other			
IFU5	*YFL010C*	unknown	1.7
PEX3	*YDR329C*	peroxisomal membrane protein	1.7
APR1	*YPL154C*	other	1.6
Other metabolism			
ACH1	*YBL015W*	acetyl-CoA hydrolase	1.7
CTA1	*YDR256C*	catalase A	1.7
Small molecule transport			
IPF3282	*YFL011W*	hexose transporter	2.5
RTA2	*YOR049C*	unknown	2.1
FTR1	*YER145C*	high affinity iron permease	2.0
FET34	*YMR058W*	iron transport multicopper oxidase	1.8
RBT2	*YOR381C*	repressed by TUP1p 2	1.8
Unknown			
DDR48	none	stress protein	7.4
RBT5	none	repressed by TUP1p 5	4.8
IPF6518	*YNR018W*	unknown	4.2
IPF12297	none	mycelial surface antigen	2.2
HGT12	*YFL040W*	hexose transporter	2.1
IPF20161	none	unknown	2.1
IPF20169	none	unknown	2.1
HGT11	*YFL040W*	hexose transporter	2.0
IPF3964	none	unknown	2.0
IPF12101	none	mycelial surface antigen	1.9
IPF3277	none	unknown	1.7
CRH11	*YGR189C*	probable membrane protein	1.6
IPF19801	none	unknown	1.6

In addition to *ERG5*, *ERG6* and *ERG25*, the stress response gene *DDR48*, iron transport genes *FTR1* and *FET34*, the hexose transporters *IPF3282*, *HGT11* and *HGT12*, *RBT5* and *RTA2* are among those found to be up-regulated in SC5314-AR. The role of these genes in either azole or amphotericin B resistance remains unclear; however, the *Saccharomyces cerevisiae* homologue of *RTA2*, *ScRTA1*, has been shown to confer resistance to 7-aminocholesterol, which exerts its activity by inhibiting the *ERG2* and *ERG24* gene products.[28] Previous studies from our laboratory examining gene expression profiles of azole-resistant clinical isolates of *C. albicans* found *DDR48*, a gene putatively involved in stress response, to be up-regulated.[19,20] Similarly, *RTA3*, which shares the same *S. cerevisiae* homologue to *RTA2* found in the present study, is up-regulated in azole resistance in *C. albicans*.[19,20] Further study of these genes in the context of antifungal resistance is warranted.

Of the 106 genes down-regulated in SC5314-AR, the large majority are involved in protein synthesis (Table 3). These protein synthesis genes, the histone genes *HTB1*, *HHT21*, *HHF21*, *HHF22* and the energy generation genes *ATP7*, *COX9*, *TIM11*, *IPF11271* and *IPF14452* are probably observed to be down-regulated due to the fact that SC5314-AR grows at a much slower rate than SC5314. Interestingly, the differences in growth rate are consistent with mutation, inactivation or deletion of *ERG11* in SC5314-AR. Sanglard *et al.*[27] documented much slower growth rates for *erg11* deletion strains than for *erg3* deletion and wild-type strains. Similar to our findings, they showed profound changes in sterol composition in the deletion mutants compared with the wild-type strain, as well as resistance to both fluconazole and amphotericin B in the mutant strains.

Other down-regulated genes in the present study include stress response genes *GPX2* and *CRD2*, heat shock proteins *HSP90*, *SSA1* and *SSE1*, and the polyubiquitin gene *UBI4*. *CRD2* and *GPX2*-related gene *GPX1* have been shown in previous studies to be differentially expressed in azole resistance; however, unlike this study, each is up-regulated.[19,20] *UBI4*,

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Table 3. Genes down-regulated in SC5314-AR compared to SC5314

Gene name	*S. cerevisiae* homologue name	Function	Change in fold expression
Cell stress			
SSA1	*YER103W*	heat shock protein 70 family	0.5
UBI4	*YLL039C*	polyubiquitin	0.4
Chromatin/chromosome structure			
HTB1	*YDR224C*	histone H2B	0.5
NHP6A	*YBR089C-A*	non-histone prot. related to *HMG1*	0.4
HHF21	*YNL030W*	histone H4	0.3
HHF22	*YNL030W*	histone H4	0.3
HHT21	*YNL031C*	histone H3	0.3
DNA repair			
RPS3	*YNL178W*	ribosomal protein S3	0.2
Energy generation			
ATP7	*YKL016C*	F1F0-ATPase complex subunit	0.5
COX9	*YDL067C*	cytochrome c oxidase	0.5
IPF11271	*YPL077W-A*	homologous to *ATP19*	0.5
IPF16566	*YHR001W-A*	homologous to *QCR1*	0.5
CYC1	*YJR048W*	cytochrome c isoform 1	0.4
GPM1	*YKL152C*	phosphoglycerate mutase	0.4
TIM11	*YDR322C-A*	mito. F1F0-ATPase subunit e	0.4
TYE7	*YOR344C*	basic HLH transcription factor	0.3
Nucleotide metabolism			
YNK1	*YKL067W*	nucleoside diphosphate kinase	0.4
Other			
UBI4	*YLL039C*	polyubiquitin	0.4
Other metabolism			
ACS2	*YLR153C*	acetyl CoA synthetase	0.5
GPX2	*YIR037W*	glutathione peroxidase	0.5
Pol II transcription			
MBF1	*YOR298C-A*	multiprotein bridging factor	0.5
NHP6A	*YBR089C-A*	non-histone prot. related to *HMG1*	0.4
TYE7	*YOR344C*	basic HLH transcription factor	0.3
Pol III transcription			
NHP6A	*YBR089C-A*	non-histone prot. related to *HMG1*	0.4
Protein complex assembly			
ATP7	*YKL016C*	F1F0-ATPase complex subunit	0.5
Protein degradation			
IPF10558	*YKR094C*	ubiquitin (*RPL40B*)	0.3
Protein folding			
HSP90	*YMR186W*	heat shock protein	0.5
SSA1	*YER103W*	heat shock protein 70 family	0.5
EGD1	*YPL037C*	*GAL4* DNA-binding enhancing protein	0.4
SSE1	*YPL106C*	heat shock protein 70 family	0.4
EGD2	*YHR193C*	nascent polypeptide assoc. complex prot.	0.3
Protein synthesis			
TIF11	*YMR260C*	translation initiation factor eIF1a	0.5
CAM1	*YPL048W*	translation initiation factor eEF1 gamma	0.4
IPF3584	*YER165W*	homologous to *PAB1*	0.4
RPA1	*YDL081C*	60S ribosomal protein	0.4
RPL17B	*YJL177W*	ribosomal protein L17	0.4
RPL21A.3	*YBR191W*	ribosomal protein	0.4
RPL3	*YOR063W*	ribosomal protein L3	0.4
RPP1B	*YDL081C*	ribosomal protein L44	0.4
RPP2	*YOL039W*	acidic ribosomal protein	0.4
RPP2B	*YDR382W*	acidic ribosomal protein	0.4
RPS15.3	*YOL040C*	ribosomal protein S15	0.4
RPS25B	*YLR333C*	cytosolic ribosomal protein	0.4
RPS30.3	*YOR182C*	ribosomal protein S30	0.4
RPS5	*YJR123W*	ribosomal protein S5	0.4
YST1	*YGR214W*	ribosomal protein	0.4
IPF10558	*YKR094C*	ubiquitin (*RPL40B*)	0.3
RPL10	*YLR075W*	ribosomal protein L10	0.3

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Table 3. (*Continued*)

Gene name	*S. cerevisiae* homologue name	Function	Change in fold expression
RPL10A	*YPL220W*	ribosomal protein L10A	0.3
RPL12	*YEL054C*	ribosomal protein	0.3
RPL13	*YDL082W*	ribosomal protein	0.3
RPL14B.3	*YKL006W*	ribosomal protein L14B	0.3
RPL16A	*YNL069C*	ribosomal protein	0.3
RPL18	*YOL120C*	ribosomal protein L18B	0.3
RPL19A	*YBR084C-A*	ribosomal protein L19	0.3
RPL2.3	*YIL018W*	ribosomal protein L8	0.3
RPL23B.3	*YER117W*	ribosomal protein L23	0.3
RPL24A	*YGL031C*	ribosomal protein L24	0.3
RPL26A.3	*YLR344W*	ribosomal protein	0.3
RPL27A	*YHR010W*	ribosomal protein L27	0.3
RPL28.3	*YGL103W*	ribosomal protein	0.3
RPL29	*YFR032C-A*	cytosolic ribosomal protein	0.3
RPL30.3	*YGL030W*	RNA binding	0.3
RPL32	*YBL092W*	ribosomal protein L23	0.3
RPL34B.3	*YIL052C*	ribosomal protein L34	0.3
RPL35.3	*YDL191W*	ribosomal protein L35A	0.3
RPL37B	*YDR500C*	ribosomal protein	0.3
RPL38	*YLR325C*	ribosomal protein L38	0.3
RPL39.3	*YMR194W*	ribosomal protein L39	0.3
RPL42.3	*YNL162W*	ribosomal protein L36A	0.3
RPL43A.3	*YPR043W*	ribosomal protein	0.3
RPL6.3	*YLR448W*	ribosomal protein	0.3
RPL7A.3	*YPL198W*	ribosomal protein L7A	0.3
RPL81	*YLL045C*	ribosomal protein L7a.e.B	0.3
RPL82	*YLL045C*	ribosomal protein L7a.e.B	0.3
RPS10	*YML063W*	ribosomal protein 10	0.3
RPS12	*YOR369C*	ribosomal protein S12	0.3
RPS13.3	*YDR064W*	ribosomal protein	0.3
RPS14B	*YJL191W*	ribosomal protein	0.3
RPS17.3	*YDR447C*	ribosomal protein S17	0.3
RPS18	*YML026C*	ribosomal protein S18	0.3
RPS21	*YGL123W*	ribosomal protein	0.3
RPS22	*YHL015W*	ribosomal protein	0.3
RPS22.exon2	*YJL190C*	ribosomal protein S15A	0.3
RPS23	*YPR132W*	ribosomal protein S23	0.3
RPS24	*YIL069C*	ribosomal protein S24	0.3
RPS26A	*YGL189C*	ribosomal protein S26	0.3
RPS27	*YKL156W*	ribosomal protein S27	0.3
RPS28B.3	*YLR264W*	ribosomal protein S28B	0.3
RPS31	*YLR167W*	ubiquitin fusion protein	0.3
RPS4A	*YJR145C*	ribosomal protein S4	0.3
RPS7A	*YOR096W*	ribosomal protein	0.3
RPS8A	*YER102W*	ribosomal protein	0.3
RPS9B	*YPL081W*	ribosomal protein	0.3
TIF51.3	*YEL034W*	translation initiation factor eIF5A	0.3
RPL11	*YPR102C*	60S ribosomal protein	0.2
RPL15B	*YLR029C*	ribosomal protein L15	0.2
RPL20B	*YOR312C*	ribosomal protein	0.2
RPL25.3	*YOL127W*	ribosomal protein L23A	0.2
RPL33.3	*YPL143W*	ribosomal protein L35A	0.2
RPL5	*YPL131W*	ribosomal protein	0.2
RPL9B	*YNL067W*	ribosomal protein L9	0.2
RPS10.3	*YOR293W*	ribosomal protein	0.2
RPS19A.3	*YOL121C*	ribosomal protein S19	0.2
RPS21B.3	*YJL136C*	ribosomal protein S21	0.2
RPS22A	*YJL190C*	ribosomal protein S15A	0.2
RPS3	*YNL178W*	ribosomal protein S3	0.2

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Table 3. (*Continued*)

Gene name	*S. cerevisiae* homologue name	Function	Change in fold expression
RPS6A	*YPL090C*	ribosomal protein S6	0.2
Protein translocation			
SSA1	*YER103W*	heat shock protein 70 family	0.5
RNA processing/modification			
RPL30.3	*YGL030W*	RNA binding	0.3
Unknown			
IPF14452	none	unknown	0.5
CRD2	none	unknown	0.4
IPF11725	none	unknown	0.4
IPF277	*YKL056C*	unknown	0.4
QCR9	none	unknown	0.4
IPF11625	none	unknown	0.3

previously found to be down-regulated in azole-resistant *C. albicans* isolates, is down-regulated in the present study as well.[19,20]

The strain SC5314-AR was passaged for ~60 doublings in the absence of drug to determine whether the strain was stably resistant to amphotericin B and fluconazole. Upon measuring susceptibility of cells tested at each passage, it was determined that the strain lost its phenotype sometime after the third passage (~28 doublings). However, in shorter-term cultures in the absence of drug—such as those from which RNA was obtained for the initial microarray experiments and follow-up RT-PCR, the MICs of the antifungals were obtained, and the sterol analysis was performed—the MICs remained stable.

This study examines changes in the gene expression profile of *C. albicans* in association with experimentally induced amphotericin B and fluconazole resistance. Whereas many of these changes appear to be associated with the cell growth rate, others may be more directly involved in the fluconazole- and amphotericin B-resistant phenotype of, and are consistent with the altered sterol profile obtained for, SC5314-AR. Although an experimentally induced resistant strain is used in this study, comparison of SC5314-AR to its parent strain by microarray and sterol analyses offers insight into the adaptations the organism is capable of making in order to achieve a resistant phenotype.

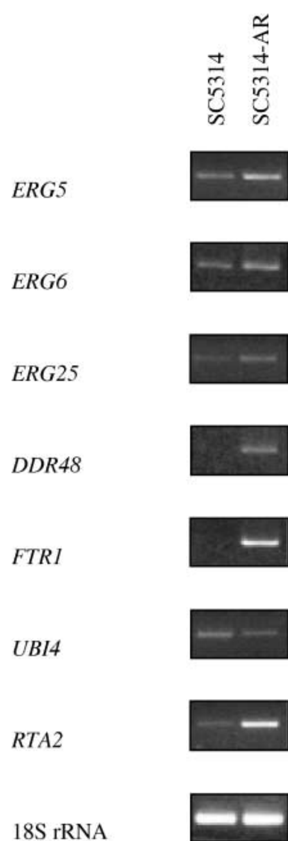

Figure 2. Independent validation of differential expression of several genes of interest by RT-PCR. Complementary DNA from RNA not used for microarray analysis was utilized for the purpose of validating differential gene expression data in SC5314 and SC5314-AR for *ERG5*, *ERG6*, *ERG25*, *DDR48*, *FTR1*, *UBI4* and *RTA2*. 18S rRNA expression was used to normalize the volume of cDNA used in all other PCR.

Table 4. Sterol content of SC5314 and SC5314-AR

	Percentage content	
Sterols present:	SC5314	SC5314-AR
Squalene	3.03	0.8
Zymosterol	3.46	0.0
Ergosterol	75.5	trace
Fecosterol	7.10	0.0
Episterol	4.30	0.0
Lanosterol	1.65	14.03
4,4-Dimethylzymosterol	3.10	0.0
14-Methylfecosterol	0.0	1.62
Obtusifoliol	0.0	7.86
24-Methylene lanosterol (eburicol)	0.0	75.0

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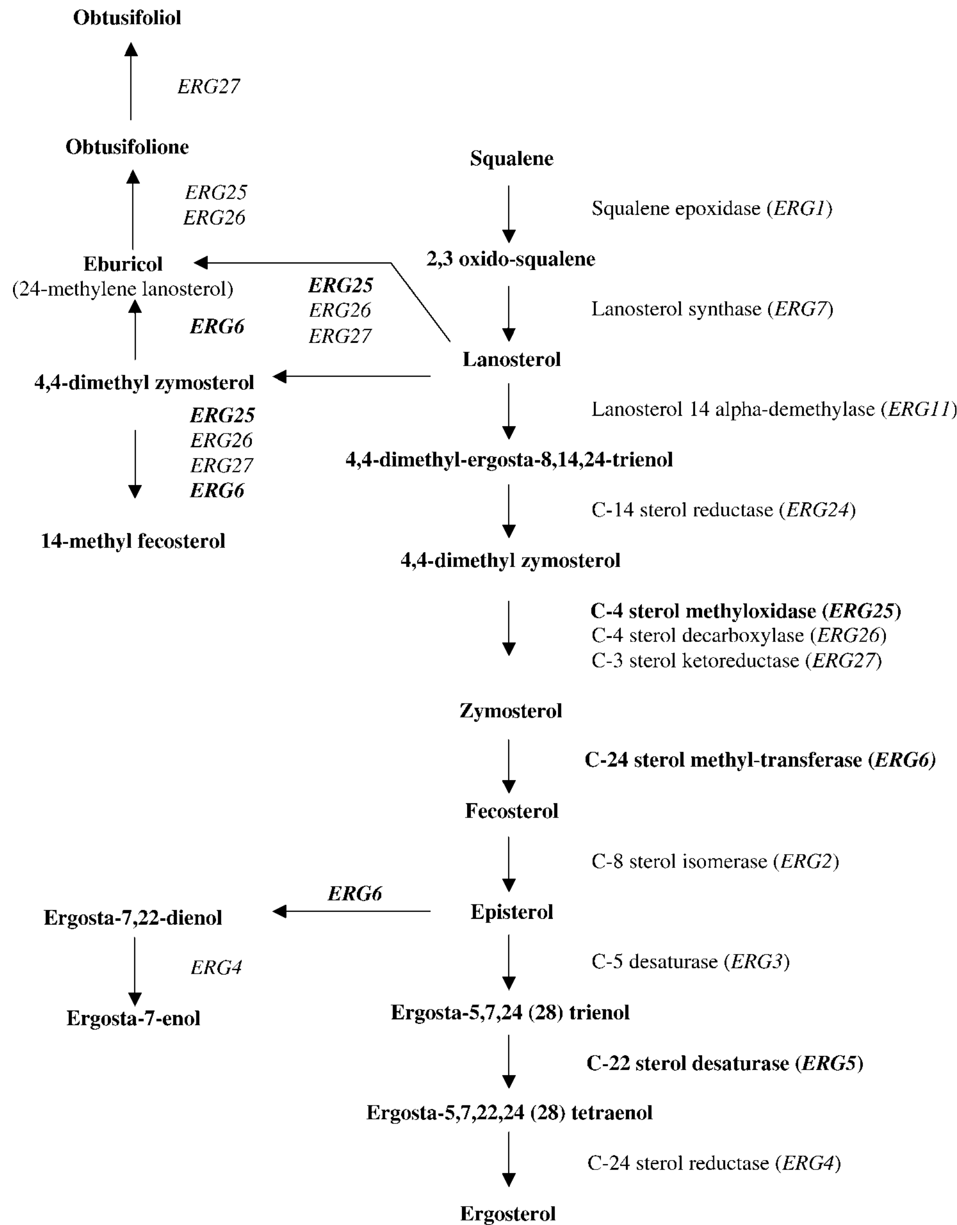

Figure 3. Enzymes of the sterol biosynthesis pathway. Genes that are bolded are those found to be up-regulated in strain SC5314-AR as compared with strain SC5314.

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