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Proteomic Analysis of Azole Resistance in *Candida albicans* Clinical Isolates

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Changes in protein expression within a matched set of *Candida albicans* isolates representing the acquisition of azole resistance were examined by two-dimensional polyacrylamide gel electrophoresis and peptide mass fingerprinting. Proteins differentially expressed in association with azole resistance included Grp2p, Ifd1p, Ifd4p, Ifd5p, and Erg10p, a protein involved in the ergosterol biosynthesis pathway.

Candida albicans is a cause of mucosal, cutaneous, and systemic infections including oropharyngeal candidiasis (OPC), the most frequent opportunistic infection among AIDS patients (8, 12). The azole antifungal agents have proven effective for the management of OPC. The repetition and lengthy duration of therapy for OPC in this patient population has led to an increased incidence of treatment failures secondary to the emergence of azole resistance in this pathogenic fungus (11, 14, 18, 25). Several mechanisms of azole resistance have been described for *C. albicans*, including point mutations in the gene encoding lanosterol demethylase (*ERG11*), as well as increased expression of *ERG11* and the genes encoding the multidrug efflux pumps, *CaMDR1*, *CDR1*, and *CDR2* (9, 10, 13, 19–21, 23, 24). In the present study, we examine changes in the *C. albicans* proteome of a well-characterized matched set of clinical isolates representing the acquisition of azole antifungal resistance.

C. albicans isolates 2-79 (fluconazole MIC, 0.25 μg/ml) and 12-99 (fluconazole MIC, ≥64 μg/ml) were used in this study. Isolate 12-99 has been shown to overexpress *ERG11*, *CaMDR1*, *CDR1*, and *CDR2* (16, 17, 23) and to have loss of allelic variation and a point mutation in *ERG11* (24). For each of three independent experiments, an aliquot of glycerol stock from each isolate was diluted in YPD broth (1% yeast extract, 2% peptone, 1% dextrose) and grown overnight at 30°C in an environmental shaking incubator. Cultures were diluted to an optical density at 600 nm of 0.2 in 0.5 liters of fresh YPD and grown as before to logarithmic phase (4.5 h) to an equivalent optical density. Cytosolic proteins were extracted, subjected to isoelectric focusing, and separated by polyacrylamide gel electrophoresis. Coomassie blue-stained gels were scanned (300-dpi resolution), and gel images were analyzed with PDQuest version 7.0 (Bio-Rad Laboratories). Spots were considered to represent differentially expressed proteins if they were up- or down-regulated ≥1.5-fold in three independent experiments. Differentially expressed proteins were selected for identification.

Spots of interest were excised and subjected to trypsinization. Peptides were extracted and analyzed using matrix-assisted laser desorption ionization–time of flight mass spectrometry. PROWL software (formerly Proteometrics, Inc.) was used to search a custom database constructed from the CandidaDB database of *C. albicans* open reading frame DNA sequences (http://genolist.pasteur.fr/CandidaDB/). A probability score for the match was attained in PROWL, with an accompanying Z score that represents a goodness of fit of the probability score for the search result. A Z score of 1.65 ranks the search result in the 95th percentile of nonrandom matches of the mass data set to the specific open reading frame.

We identified 17 proteins that were reproducibly differentially expressed in isolate 12-99 compared to isolate 2-79. Among these were 13 up-regulated proteins and 4 down-regulated proteins in isolate 12-99 (Fig. 1, Table 1). Technical limitations complicate the accurate quantification of protein abundance from staining intensities. We therefore used a semiquantitative scoring system to represent changes in protein abundance.

The protein isolation technique employed in the present study is inclusive of cytosolic proteins but is deficient in water-insoluble proteins such as those found in cell and organelle membranes. It is therefore not surprising that we did not detect the membrane-associated multidrug transporters Cdr1p, Cdr2p, and Mdr1p in this study. Of particular interest, however, was the finding of up-regulation of Grp2p, Ifd1p, Ifd4p, Ifd5p, and Ifd6p in association with azole resistance. The genes encoding all but one of these have been shown to be differentially expressed in this and other matched series of isolates (4, 16, 17). Ifd1p, Ifd4p, Ifd5p, and Ifd6p are members of a family of homologs of the *Saccharomyces cerevisiae YPL088W* gene product, a putative alcohol dehydrogenase-oxidoreductase.

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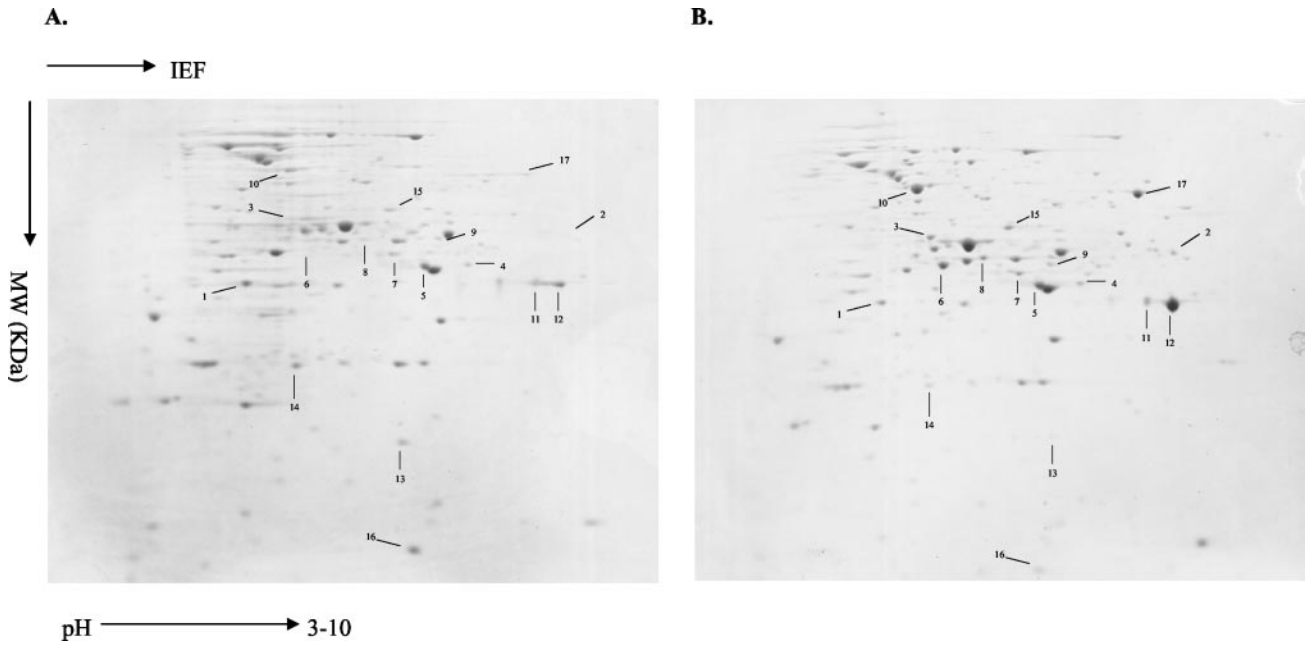

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis separation of total protein extract from *C. albicans* isolates 2-79 (A) and 12-99 (B). The numbered spots represent up- or down-regulated proteins between these two isolates identified by peptide mass fingerprinting (Table 1). IEF, isoelectric focusing.

The *YPL088W* gene and gene product have been shown to be up-regulated in microarray and proteomic studies of azole-resistant *S. cerevisiae* isolates with gain-of-function mutations of the transcription factor *PDR3* (7, 15). A *C. albicans* homolog of *YPL088W* has been shown to contain a drug response element in its promoter which leads to induction of mRNA expression upon estradiol treatment (6). This drug response element was recently shown to be shared by other genes, including *CDR1* and *CDR2* (M. Karababa, A. Coste, B. Rognon, J. Bille, and D. Sanglard, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-401, 2003). Likewise, Grp2p is a homolog of ScGre2p, which encodes a putative reductase with similarity to plant dihydroflavonol-4-reductases. It appears to have a role in protecting cells from the toxic effects of methylglyoxal (3). ScGre2p has also been observed to be up-regulated in microarray and proteomic studies of azole-resistant *S. cerevisiae* isolates with constitutive activation of the transcription factor *PDR3* (7, 15). While *ScGRE2* is responsive

TABLE 1. Proteins identified by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization–time of flight mass spectrometry as being differentially expressed between isolates 2-79 and 12-99

Spot	Protein	pI	Molecular mass (kDa)	Estimated *Z* score[b]	Protein coverage (%)	Fold change (12-99/2-79)[c]
1	Ilv5p (ketol-acid reducto-isomerase)	6.2	44.83	2.37	59	−
2	Pot14p (Erg10p) (acetyl-coenzyme A acetyl transferase)	6.5	41.92	1.51	24	++
3	Sah1p (*S*-adenosyl-L-homocysteine hydrolase)	5.4	49.05	2.23	31	+
4[a]	Grp2p (reductase)	6.0	37.62	2.15	57	+
5[a]	Grp2p (reductase)	6.0	37.62	2.32	43	+
6[a]	Ifd1p (aryl-alcohol dehydrogenase)	5.6	39.14	2.39	24	+++
7[a]	Ifd4p (aryl-alcohol dehydrogenase)	6.0	38.26	2.38	41	++
8[a]	Ifd5p (aryl-alcohol dehydrogenase)	5.4	39.20	2.27	39	++
9[a]	Ifd6p (aryl-alcohol dehydrogenase)	5.9	39.06	2.35	29	+++
10	Pdc11p (pyruvate decarboxylase)	5.4	62.42	2.38	40	+
11	Gap1p (glyceraldehyde-3-phosphate dehydrogenase)	6.6	35.81	2.4	72	+
12	Gap1p (glyceraldehyde-3-phosphate dehydrogenase)	6.6	35.81	2.4	72	+
13	Aat1p (aspartate aminotransferase)	8.7	48.87	2.36	33	−−
14	Pmm1p (phosphomannomutase)	5.5	29.00	2.24	29	−
15	Gnd1p (6-phosphogluconate dehydrogenase)	6.1	56.89	2.01	46	++
16	Ynk1p (nucleoside diphosphate kinase)	6.1	16.87	2.35	53	−
17	Cdc19p (pyruvate kinase)	6.5	55.43	2.38	32	++

[a] Also found previously to be differentially expressed in DNA microarray analysis of this set of isolates (17). Homolog of a *S. cerevisiae* protein found to be differentially expressed in PDR1-3 gain-of-function mutants that exhibit azole resistance (15).

[b] A probability score of 1.0 was determined for each spot.

[c] + or −, 1.5- to 3-fold; ++ or −−, 3.1- to 10-fold; +++, more than, 10-fold. The plus and minus represent increase and reduction, respectively.

to osmotic, ionic, oxidative, and heat stresses, its function is unknown.

Sulfur amino acid biosynthesis is peripherally linked to ergosterol biosynthesis. Both aspartate and homocysteine influence the biosynthesis of *S*-adenosylmethionine, which is required for the ability of sterol C-24 methyltransferase to convert zymosterol to fecosterol (5). Up-regulation of *S*-adenosyl–L-homocysteine hydrolase (Sah1p) and down-regulation of aspartate aminotransferase (Aat1p) may therefore impact the ergosterol biosynthesis pathway in azole resistance. Also of interest is the up-regulation of acetyl-coenzyme A acetyltransferase (Pot14p or Erg10p). This enzyme represents the first step in ergosterol biosynthesis. Its expression may be regulated by membrane ergosterol content, as the *ERG10* gene is responsive to ergosterol depletion by itraconazole treatment (5).

Other changes in protein abundance in association with azole resistance included the up-regulation of proteins involved in carbohydrate metabolism. These were glyceraldehyde-3-phosphate dehydrogenase (Gap1p), pyruvate decarboxylase (Pdc11p), pyruvate kinase (Cdc19p), and phosphogluconate dehydrogenase (Gnd1p). Phosphomannomutase (Pmm1p), an enzyme involved in mannose and GDP-mannose metabolism (22), was also up-regulated in the resistant isolate. Ketol-acid reductoisomerase (Ilv5p), an enzyme central to leucine, isoleucine, and valine biosynthesis, as well as mitochondrial DNA stability (2), was down-regulated in the azole-resistant isolate, as was the nucleoside diphosphate kinase Ynk1p. In *S. cerevisiae*, Ynk1p plays an important role in cellular homeostasis of nucleoside triphosphates and nucleoside diphosphates and is also thought to function as a signaling molecule (1).

We have demonstrated the differential expression of proteins whose genes have been previously shown to be differentially expressed in both experimentally induced and clinically acquired azole resistance, as well as proteins whose differential expression was found for the first time to be associated with this process. Proteomic analysis of multiple matched sets of azole-susceptible and -resistant *C. albicans* isolates will further our understanding of the mechanisms underlying azole antifungal resistance.

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