Gene Expression and Evolution of Antifungal Drug Resistance^{▽†}

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Permanent changes in gene expression result from certain forms of antifungal resistance. In this study, we asked whether any changes in gene expression are required for the evolution of a drug-resistant phenotype in populations. We examined the changes in gene expression resulting from the evolution of resistance in experimental populations of the yeast *Saccharomyces cerevisiae* with two antifungal drugs, fluconazole (FLC) in a previous study and amphotericin B (AmB) in this study, in which five populations were subjected to increasing concentrations of AmB, from 0.25 to 128 $\mu\text{g/ml}$ in twofold increments. Six genes, YGR035C, *YOR1*, *ICT1*, *GRE2*, *PDR16*, and YPLO88W, were consistently overexpressed with resistance to AmB reported here and with resistance to FLC involving a mechanism of increased efflux reported previously. We then asked if the deletion of these genes impaired the ability of populations to evolve resistance to FLC over 108 generations of asexual reproduction in 32 and 128 $\mu\text{g/ml}$ FLC, the same conditions under which FLC-resistant types evolved originally. For each of three deletion strains, *YOR1*, *ICT1*, and *PDR16* strains, extinctions occurred in one of two replicate populations growing in 128 $\mu\text{g/ml}$ FLC. Each of these three deletion strains was mixed 1:1 with a marked version of the wild type to measure the relative ability of the deletion strain to adapt over 108 generations. In these assays, only the *PDR16* deletion strain consistently became extinct both at 32 and at 128 $\mu\text{g/ml}$ FLC. The deletion of *PDR16* reduces the capacity of a population to evolve to resistance to FLC.

Certain forms of antifungal drug resistance are accompanied by alterations in gene expression that persist even in the absence of the agent to which the resistance evolved (1, 8, 10, 16, 18, 19). Are any of these changes in gene expression required for populations to acquire and support a drug-resistant phenotype? It is well established that the expression of *HSP90* is a necessary precondition for the evolution and maintenance of certain forms of antifungal drug resistance in a phylogenetically broad array of fungi (6, 7). In this study, we examined the postconditions of resistance, specifically, the downstream gene expression changes resulting from the evolution of a resistant phenotype from a susceptible ancestor. We identified changes in gene expression common to resistance to two important antifungal drugs, fluconazole (FLC) and amphotericin B (AmB). We then asked if a deletion of these genes impaired the ability of populations to evolve resistance to FLC. The rationale was to include agents causing membrane stress by completely different mechanisms in order to search for genes whose expression had the broadest possible effect on the evolution of resistance.

Most prominent among the resistance mechanisms causing widespread changes in gene expression include gain-of-function mutations in transcription factors, such as *PDR1* and *PDR3* in *Saccharomyces cerevisiae*, leading to increased levels of expression of efflux proteins, and a loss of function in other genes, such as *ERG3*, causing changes in sterol metabolism (8, 16, 18, 19). FLC exerts a fungistatic effect on susceptible strains

by inhibiting lanosterol demethylase, a key enzyme in the biosynthesis of ergosterol, while AmB exerts a fungicidal effect by binding directly to ergosterol in membranes and thereby destroying membrane integrity. FLC resistance may involve increased efflux or changes in sterol metabolism, while AmB resistance generally involves changes in sterol metabolism. For all of these types of resistance, gene expression changes are extensive. The goal of these experiments was to test whether the failure of gene expression changes downstream of the original mutation for drug resistance would affect the ability of a population of cells to evolve a drug-resistant phenotype.

In this study, replicate populations of *Saccharomyces cerevisiae* evolved in increasing concentrations of AmB over hundreds of generations to yield resistant types, among which there were permanent changes in gene expression. We then compared these changes with those identified in replicate populations that evolved FLC resistance in a previously reported study (3). The central question was whether targeting of such genes whose altered expression is widely associated with resistance might impair the ability of fungal populations to evolve resistance in the first place. Our test of the ability to evolve resistance involved FLC but not AmB because resistance to FLC occurs with high frequency in wild-type populations and, unlike resistance to AmB, is easily assayed in a matter of days (4). We observed that the deletion of certain genes whose levels of expression permanently increased with the evolution of resistance to FLC and AmB impaired the ability of populations to evolve resistance to FLC. In these cases, the reduction in population size upon the initial exposure to FLC was not sufficient to explain the failure to evolve resistance.

MATERIALS AND METHODS

A single *MATa* strain was the progenitor of all evolution experiments described here. In an earlier study, the *URA3* locus of this strain was replaced with

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FIG. 1. Evolution of resistance measured as MIC_{50} in three replicate yeast populations over 1,100 generations, during which the concentration of AmB was doubled every 100 generations. Circles, AmB concentration in the medium; diamonds, triangles, and squares, MICs of the three evolving populations, respectively. MICs of mass cultures always equaled those of single-colony isolates.

KanMX4, encoding G418 resistance, and with unique 20-base bar codes on either end as markers of identity in contamination checks during and after the evolution experiments (3, 11). Corresponding strains with nourseothricin (NAT) resistance were also constructed previously by switching the G418 resistance cassette with NAT resistance (11).

AmB was obtained from Sigma Chemical Co. (catalog number A4888; 5-mg/ml stock in dimethyl sulfoxide), and FLC was a gift from Pfizer Canada Inc. (5.12-mg/ml stock in sterile distilled water). Evolution experiments were started with 100 μ l of a culture grown overnight that was derived from a single colony inoculated into 10 ml of 0.5 $\times$ yeast-peptone-dextrose (YPD) medium containing 0.25 μ g/ml AmB. Five different populations evolved in the presence of AmB in two different regimens, long and short. The rationale for choosing different evolution regimens was to increase the chance of recruiting different mechanisms of AmB resistance among all of the replicates. In the long regimen, three populations started at 0.25 μ g/ml AmB, and this concentration was doubled every 100 generations for a total of 1,100 generations and a final AmB concentration of 128 μ g/ml.

In the short regimen, two populations were also started at 0.25 μ g/ml AmB but were given as much time as necessary to grow to a high density, usually 2 to 4 days. The populations were then transferred into the same AmB concentration a second time. At the next transfer, the AmB concentration was doubled for another two growth cycles, and so on, until the populations had been through two transfers in 128 μ g/ml AmB.

The MIC_{50} of FLC was measured as described previously, using 0.5 $\times$ YPD medium (3). The MIC_{50} of AmB was measured in the same way in the same medium.

Microarray experiments measuring genome expression genome wide were performed exactly as described previously (2), with the same standard quality control filters. To be accepted as a significant departure from the expression level in the ancestor, the mean deviation was required to be 1.5-fold up or down, and all four spots in the two replicate arrays for each population were required to show the same directionality in change.

Knockout strains were purchased from Open Biosystems. Although aneuploidy occurs in about 8% of the strains in the knockout collection (14), the *pdr16* Δ strain, which was the strain that was the most consistently impaired in its ability to evolve FLC resistance (see Results and Discussion), appeared to be of normal ploidy. For this strain, we used PCR to verify (i) that the *PDR16* gene was missing and (ii) that the insert cassette encoding G418 resistance was in the correct position (PCR primers are available upon request). The G418 resistance of this knockout strain segregated normally (2:2) in a cross with a wild-type, unmarked strain. Finally, the chromosome-sized DNAs on pulsed-field agarose gels did not show any obvious copy number alterations for the *pdr16* Δ strain (data not shown).

In our assay system, the ancestral yeast strains and all gene deletion strains had MIC_{50} s of FLC of 16 μ g/ml. Although the *pdr16* Δ strain was reported to be more

FIG. 2. Evolution of resistance measured as MIC_{50} in two replicate populations over 57 transfers, during which the concentration of AmB was doubled every other transfer. Circles, AmB concentration in the medium; diamonds, triangles, and squares, MICs of the two evolving populations, respectively. MICs of mass cultures always equaled those of single-colony isolates.

susceptible than the wild type to a variety of agents (20), it was not included in our assay. The difference between previous (20) and present observations may be attributed to differences in the media and the actual agents tested.

To test their abilities to evolve resistance to FLC, deletion mutants were batch transferred in FLC at 0, 32, or 128 μ g/ml for a total of 18 days. These experiments were conducted using 24-well plates with 1 ml of 0.5 $\times$ YPD medium and a daily transfer size of 0.1 ml. The culture density was monitored by measuring the optical density at 600 nm.

Competitive adaptation experiments were done to compare the potentials of gene deletion strains marked with G418 resistance and the wild type marked with NAT resistance to evolve resistance to FLC in the same environment. Under these conditions, the wild type consistently evolved resistance to FLC; the null expectation for each competitive adaptation experiment was that the deletion strains would keep pace by evolving resistance at the same rate as that of the wild type.

In these experiments, cultures of a gene deletion mutant and the wild type grown overnight were mixed 1:1, and 100 μ l was inoculated into 10 ml of 0.5 $\times$ YPD medium containing 32 or 128 μ g/ml FLC. Each population was then serially transferred at the same dilution rate for 16 cycles or 108 generations. At the end, the cell concentrations of the deletion and wild-type strains were determined by dilution plating onto YPD medium containing G418 or NAT, respectively. Samples of survivors were tested for their MICs of FLC.

Microarray data accession number. The complete data set reported here can be accessed under Gene Expression Omnibus accession number GSE12055 (<http://www.ncbi.nlm.nih.gov/geo/>).

RESULTS AND DISCUSSION

In previous studies, replicate populations evolved in the presence of FLC, and their gene expression was profiled (2, 3); these expression data were used in comparisons with strains that evolved AmB resistance in this study to identify consensus changes. All populations initially subjected to a high concentration (256 μ g/ml) became extinct, and no AmB resistance evolved (data not shown); this is unlike resistance to FLC, in which wild types subjected to a high concentration (256 μ g/ml) initially did survive and evolved resistance. Here, five different populations were evolved in the presence of AmB under two different regimens, long (Fig. 1) and short (Fig. 2). Under both regimens, cell division kept pace with the repeated dilution of cultures, and no extinctions occurred. Under the long regimen, the MIC_{50} remained equal to or greater than the ambient

FIG. 3. Gene expression in the five evolved populations resistant to AmB measured in the absence of the drug. Replicate bar graphs facing upwards represent increased levels of expression relative to that of the ancestor. Graphs with bars facing downward represent decreased expression. Only changes >1.5-fold up or down in all five populations are shown. Genes are listed in the same order as they occur on the chromosomes.

AmB concentration. Under the short regimen, the MIC remained equal to or less than the ambient AmB concentration. In all five populations from the long and short regimens, in which the mass culture and three randomly selected single-colony isolates were assayed, the final MIC₅₀ of AmB was 128 or 256 µg/ml.

Next, a representative of each AmB-resistant population was assayed for alterations in patterns of gene expression in the absence of any drug. Figure 3 depicts the consensus genes whose levels of expression were altered 1.5-fold greater or lesser in each of the five populations. Numerous other changes were also detected among each of the five AmB-resistant strains; the individual genes and their expression values are listed in Table S1 in the supplemental material. The numbers of persistently overexpressed genes identified by our criteria ranged between 172 and 316 among the five AmB-resistant strains; of these examples of overexpression, between 43 and

64% were unique to the respective strains. Similarly, the numbers of underexpressed genes identified ranged from 84 to 139 among the five strains; of these, between 49 and 70% were unique to the respective strains.

Next, we searched for genes whose levels of expression were stably altered with evolved resistance to AmB in this study and to FLC in a previously reported study (3). Six genes were overexpressed in all five AmB-resistant strains from this study and in the three FLC-resistant strains that arose in one regimen of selection for FLC resistance in the earlier study (3). In this regimen, the concentration of FLC increased in four increments from 16 µg/ml to 256 µg/ml over 400 generations, and each resistant type carried a dominant mutation in the regulatory gene *PDR1*. The other regimen was based on a single exposure to a high concentration of FLC, and each of three independently evolved resistant strains had a loss of function in *ERG3*. No genes were overexpressed in all five AmB- and six FLC-resistant strains from the two studies.

Each of the six genes identified above is known to be involved either with multidrug resistance or with cellular stress: YGR035C is a putative gene whose transcription is activated by transcription factors Yrm1p and Yrr1p along with genes associated with multidrug resistance (15), *YOR1* is a plasma membrane ABC transporter involved in drug resistance (9, 13), *ICT1* is a lysophosphatidic acid acyltransferase gene responsible for enhanced phospholipid synthesis during organic solvent stress and for tolerance of calcofluor white (12), *GRE2* is a 3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase gene that is stress induced (5, 17), *PDR16* is a phosphatidylinositol transfer gene controlled by the multiple-drug-resistance regulator Pdr1p that localizes to lipid particles and microsomes and controls levels of various lipids (20), and YPL088 is a putative aryl alcohol dehydrogenase gene whose transcription, like those of YGR035C and other genes, is activated by the paralogous transcription factors Yrm1p and Yrr1p (15).

Does the expression of any of these six nonessential genes contribute to the capacity to evolve drug resistance? The next experiments used the null mutants of each gene in an otherwise wild-type and FLC-sensitive background to initiate short-term evolution experiments in 32 µg/ml or 128 µg/ml of FLC, a concentration for which resistance is known to evolve rapidly in wild-type populations (3, 4), even those of very small sizes. It was not practical in this study to assay the ability to evolve resistance to AmB because of the much longer time required for resistance to appear in populations subjected to sublethal concentrations of this agent. In these experiments with FLC, cell density invariably decreased in 32 µg/ml or 128 µg/ml FLC, but not in the absence of FLC, over several days. Evidence of adaptation was (i) the recovery of cell density to levels comparable to those in the absence of drug, which occurred in some but not all replicates, and (ii) an MIC₅₀ of FLC of 128 or 256 µg/ml in those that did recover at the end of the growth period. The wild-type, ancestral strain always achieves resistance under these conditions. Similarly, three of the deletion strains achieved resistance in all replicate populations under both concentrations of FLC. Three additional deletion strains, the *pdr16Δ*, *pdr5Δ*, and *hsp90Δ* strains, were tested in this way; all these strains consistently evolved resistance using this test (data not shown). Although the nature of resistance was not

determined in these cases, these deletion strains might achieve resistance in the following ways. The *pdr1Δ* strain may accumulate gain-of-function mutations in *PDR3*, a transcriptional regulatory gene similar to *PDR1*. Although defective in efflux of FLC, the *pdr5Δ* strain might achieve resistance through alterations in sterol metabolism even though it is especially sensitive to FLC (2). Last, it is known that resistance via gain-of-function mutations in the relevant transcriptional regulators is not sensitive to conditions of low levels of Hsp90 (6).

Those deletion strains in which extinctions or near extinctions occurred, the *yor1Δ*, *ict1Δ*, and *pdr16Δ* strains, were selected as candidates for the next round of experiments, in which each deletion strain was competed against the wild-type ancestor over 108 generations in the presence of 0, 32, and 128 μg/ml FLC. Here, populations were completely dependent on the occurrence of mutations for resistance to avoid extinction. The criterion for "winning" the competition was not the initial fitness but rather the capacity for FLC-resistant mutations to arise and propagate in the population. Under these conditions, the mean fitness for each marked strain can be calculated over the entire experiment based on initial and final densities for each transfer as the total number of doublings.

As a control, G418-resistant and NAT-resistant versions of the wild-type progenitor were competed with one another (Table 1). As expected, there was no consistent bias in the resistant types appearing in the absence of FLC (mean fitness of G418 resistance compared to NAT resistance over 108 generations of 0.998 with 0.018 standard deviations [SD]; $n = 3$ measurements). Surprisingly, in FLC, there were strong marker effects on adaptation. In 32 μg/ml FLC, NAT resistance was consistently favored (mean fitness of G418 resistance compared to NAT resistance over 108 generations of 0.956 with 0.003 SD; $n = 3$ measurements), and in 128 μg/ml, G418 resistance was consistently favored (mean fitness of G418 resistance compared to NAT resistance over 108 generations of 1.024 with 0.004 SD; $n = 3$ measurements). These marker effects, while small in terms of fitness per generation, had a strong effect on final proportions after 108 generations of evolution and were therefore taken into account in interpreting the outcome of the remaining competitive adaptation experiments. The mechanism by which these marker effects are conditional regarding the FLC concentration is unknown.

The *YORI* deletion strain showed a clear growth disadvantage relative to the wild type in the absence of FLC (Table 1). The *yor1Δ* strain was also among the minority after 108 generations in each concentration of FLC but did not become extinct in any of the replicates. Of the minority *YORI* deletion types surviving 108 generations in FLC, all representatives tested were resistant. In 128 μg/ml FLC, the predominance of the wild type marked by NAT resistance was opposite to the direction of the marker effect in which G418 resistance is at an advantage. Under both FLC concentrations, the proportion of the *YORI* deletion strain roughly paralleled the growth disadvantage of this strain in the absence of FLC. Even with a severe reduction in population size in the *YORI* deletion populations, the ability to acquire resistance to FLC was not impaired.

The *ICT1* deletion strain showed mixed effects. There was little or no growth disadvantage of the deletion strain relative to the ancestor in the absence of FLC. The *ict1Δ* strain showed mixed results at 32 μg/ml, probably indicating rough parity

TABLE 1. Mean fitness of test strains marked with G418 resistance compared to that of the progenitor marked with NAT resistance over 108 generations of direct competition

Test strain marked with G418 resistance	Final concn (10 ⁸)		Proportion of test G418 resistance to reference NAT resistance	Mean fitness test of G418 resistance to reference NAT resistance
	Reference NAT resistance	Test G418 resistance		
No drug				
Wild type	1.3	0.6	0.46	0.993
	1.0	7.2	6.9	0.982
	1.0	1.0	1.0	1.018
<i>yor1Δ</i>	1.4	0.09	0.06	0.975
	1.2	0.07	0.06	0.974
	1.1	0.09	0.08	0.997
<i>ict1Δ</i>	1.3	1.0	0.79	0.998
	1.0	0.59	0.60	0.995
	0.71	0.82	1.2	1.001
<i>pdr16Δ</i>	0.03	1.4	48	1.037
	0.02	1.2	80	1.041
	0.03	1.4	47	1.036
32 μg/ml FLC				
Wild type	1.8	0.03	0.01	0.961
	1.6	0.01	0.01	0.956
	1.8	1.02	0.01	0.957
<i>yor1Δ</i>	1.1	0.57	0.53	0.994
	1.3	0.03	0.03	0.966
	1.5	0.15	0.10	0.979
<i>ict1Δ</i>	1.2	0.65	1.9	0.994
	1.6	0.00 ^a	0.00 ^a	NA ^a
	<0.0001	0.82	1.1 × 10 ⁴	1.090
<i>pdr16Δ</i>	1.8	0.00 ^a	0.00 ^a	NA ^a
	1.8	0.00 ^a	0.00 ^a	NA ^a
	1.8	0.00 ^a	0.00 ^a	NA ^a
128 μg/ml FLC ^b				
Wild type	0.06	0.60	10.9	1.022
	0.10	0.92	9.2	1.021
	0.08	1.4	18	1.028
<i>yor1Δ</i>	0.60	0.06	0.10	0.979
	0.54	0.12	0.23	0.986
	1.0	0.04	0.04	0.971
<i>ict1Δ</i>	0.01	0.52	39	1.034
	0.02	0.59	26	1.031
	0.01	0.50	40	1.035
<i>pdr16Δ</i>	0.89	0.00 ^a	0.00 ^a	NA ^a
	1.1	0.00 ^a	0.00 ^a	NA ^a
	1.3	0.00 ^a	0.00 ^a	NA ^a

^a The test strain became extinct. The mean fitness of the test strain is lower than that of the reference strain but cannot be estimated. NA, not applicable.

^b Note that total cell densities in 128 μg/ml FLC tended to be lower than those in 32 and in 0 μg/ml.

with the wild type in adaptive potential. Unexpectedly, however, the *ict1Δ* strain showed a clear evolutionary advantage over the wild type in 128 μg/ml. Like the deletion of *YORI*, the deletion of *ICT1* does not impede the evolution of resistance. Unlike the deletion of *YORI*, however, the deletion of *ICT1*

FIG. 4. Evolution of FLC resistance in deletion strains for the six genes overexpressed in the AmB resistance strains from this study and in the three FLC-resistant strains reported previously by Anderson et al. (3) evolved through gradual increases in the FLC concentration. Diamonds, 0 $\mu\text{g/ml}$ FLC; circles, 32 $\mu\text{g/ml}$ FLC; x's, 128 $\mu\text{g/ml}$ FLC.

actually enhances the ability of populations to acquire FLC resistance relative to the wild type.

The *PDR16* deletion strain showed the most consistent results in the competitive adaptation experiments. The *pdr16Δ* strain showed a marked advantage over the wild type in the absence of FLC. Under both concentrations of FLC, the *pdr16Δ* strain was driven to extinction in all replicates. In 128 $\mu\text{g/ml}$ FLC, this extinction is opposite of the direction of the marker effect in which G418 resistance is favored. At 32 $\mu\text{g/ml}$, the extinction of the *pdr16Δ* strain exceeded the expected marker effect where NAT resistance is favored over G418 resistance but not to the point of extinction. Despite its ability to evolve resistance to FLC in some replicate populations when grown alone (Fig. 4), the *pdr16Δ* strain is at a clear disadvantage to the wild type in a competition.

The rationale for this study was to test whether downstream gene expression changes found after resistance appears might actually be necessary for the appearance and/or maintenance of a resistant phenotype. The *PDR16* deletion showed the greatest impairment of the ability of populations to evolve resistance to FLC. *PDR16* is involved in lipid metabolism and is under the regulation of the *PDR1* transcriptional network. The deletion of *PDR16* is already known to confer increased drug susceptibility (20), although here, it was no more susceptible to FLC than the wild type. In explaining the evolutionary outcomes with the *PDR16* deletion, population size and evolutionary potential are always intertwined, as larger populations have a larger supply of mutations, some of which confer greater fitness and result in a greater chance for the population to escape extinction by spreading rapidly to a high frequency in

the presence of the drug. In the evolutionary competitions, however, small population size alone cannot explain the observed deficit of the *pdr16Δ* strain in its ability to adapt to the presence of FLC; when grown alone in the presence of FLC, one replicate population even went to apparent extinction only to develop resistance and then abruptly recover in size. The most likely explanation is that the *PDR16* deletion somehow reduces the capacity of a population to maintain a resistant state in the presence of FLC. FLC is known to cause membrane stress by creating a deficiency of ergosterol. The best general test of this hypothesis would be to reduce or eliminate the expression of *PDR16* of an already resistant strain in the presence of FLC. More specifically, the loss of *PDR16* expression may alter membrane characteristics such that the function of the relevant ABC transporters is impaired, influx rates are increased (20), or membranes are made less robust to changes in sterol content. Each of these three possibilities is amenable to experimental testing.

This study shows that the effect of the deletion of *PDR16* extends beyond drug susceptibility and actually reduces the evolutionary potential to develop FLC resistance. The effect of the *PDR16* deletion may extend to other drugs or conditions conferring membrane stress. *PDR16* may play a similar role in the clinically important pathogen *Candida albicans*, in which it is part of the transcriptional network regulating the ABC transporters commonly involved in resistance to FLC. A potential application of this finding is that *PDR16* could be considered as a cotarget for the prevention of resistance evolution (1). The general principle of cotargets tested here could also be ex-

tended to other drugs, genes, pathogens, and forms of resistance.

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