Using *Aspergillus nidulans* To Identify Antifungal Drug Resistance Mutations

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Systemic fungal infections contribute to at least 10% of deaths in hospital settings. Most antifungal drugs target ergosterol (polycenes) or its biosynthetic pathway (azoles and allylamines), or beta-glucan synthesis (echinocandins). Antifungal drugs that target proteins are prone to the emergence of resistant strains. Identification of genes whose mutations lead to targeted resistance can provide new information on those pathways. We used *Aspergillus nidulans* as a model system to exploit its tractable sexual cycle and calcofluor white as a model antifungal agent to cross-reference our results with other studies. Within 2 weeks from inoculation on sublethal doses of calcofluor white, we isolated 24 *A. nidulans* adaptive strains from sectoring colonies. Meiotic analysis showed that these strains had single-gene mutations. In each case, the resistance was specific to calcofluor white, since there was no cross-resistance to caspofungin (echinocandin). Mutation sites were identified in two mutants by next-generation sequencing. These were confirmed by reengineering the mutation in a wild-type strain using a gene replacement strategy. One of these mutated genes was related to cell wall synthesis, and the other one was related to drug metabolism. Our strategy has wide application for many fungal species, for antifungal compounds used in agriculture as well as health care, and potentially during protracted drug therapy once drug resistance arises. We suggest that our strategy will be useful for keeping ahead in the drug resistance arms race.

Fungal infection is a growing problem in the developed world, particularly over the last several decades. Fungi readily infect immunocompromised patients, and systemic infections typically cause high morbidity (1–4). In addition, reports of fungal infections in healthy populations are rising (1, 4), for example, due to the increasing virulence of pathogens such as *Aspergillus fumigatus* (4). Fungi are now as serious a threat to human health as are bacteria, viruses, and parasites (1). Fungi and animals have conserved metabolic pathways, which limits the options for drug targets. There are four major classes of antifungal drugs. Azoles and polycenes interfere with biosynthesis or distribution of ergosterol, the sterol in fungal membranes (5). Although these drugs have a wide spectrum of effects against fungal growth, the structural similarity between ergosterol in fungi and cholesterol in mammals leads to toxicity and limits human drug usage (6, 7). Azoles also have substantial application as agricultural fungicides, and there are cases of cross-resistance (8). The newest antifungal drug class to be released clinically, echinocandins, targets cell wall beta-1,3-glucan synthase (9). The human toxicity of echinocandins is low, but their narrow spectrum of activity and already emerging resistance were a severe disappointment to the community (10). Collectively, we are still searching for new antifungal drug targets, but progress is slow.

With new broad-spectrum drug targets being few, and drug development being expensive, studies exploring resistance mechanisms against our current antifungal agents are important. Cowen (7) notes that the effectiveness of all existing antifungal drugs is reduced by resistant strains. Especially for echinocandins, clinically resistant strains were isolated shortly after their launch (11, 12). The strong adaptation ability of fungi is well documented (13–16), so it was not surprising to see antifungal drug resistance emerging quickly. Mutation hot spots in FKS1, which encodes beta-glucan synthase, were detected in some of the resistant strains but not in all (10–12). Our strategy can efficiently identify mutations in the latter group.

*Aspergillus nidulans* is used as a model for adaptation studies (13, 14). Vegetative nuclei in a colony are mitotically derived from a single spore nucleus, so the only source of genetic variation in an *A. nidulans* colony is somatic mutation, which then is clonally propagated as a mycelial sector (14). In *A. nidulans*, the asexual nuclear duplication cycle is roughly comparable to a somatic generation in yeast. In growing *A. nidulans* hyphae, the nuclear duplication cycle is 100 min (17), and a typical hyphal growth rate is 1 μm/min (18).

We used calcofluor white (CFW) as an antifungal agent to cross-reference with earlier studies. We found that robust, heritable adaptation against CFW could rapidly be acquired in multiple ways, each requiring only one mutation based on analysis of meiotic progeny. Two adaptive strains were selected for next-generation sequencing (NGS), to determine if this would be sufficient to identify adaptive mutations. Potential mutation sites were confirmed by gene replacement in the parental strain. In this study, one adaptive strain was related to cell wall synthesis and the other was related to drug metabolism. We suggest that our strategy can help us stay ahead in the fungal drug resistance arms race.

**MATERIALS AND METHODS**

Strains, plasmids, and medium. All of the strains in this study were derived from *A. nidulans* A1149, which was the wild-type control for all assays in this paper. All strains, primers, and plasmids are listed in Table S1.
in the supplemental material. All strains were grown on complete medium (CM; 1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% Casamino Acids, 50 ml 20X nitrate salts, 1 ml trace elements, 1 ml vitamin solution, pH 6.5) or minimal medium (MM; 1% glucose, 50 ml 20X nitrate salts, 1 ml trace elements, 0.001% thiamine, pH 6.5) supplemented with nutrients for auxotrophs as required. Trace elements, vitamin solution, nitrate salt, and all nutrition stocks are described in reference 19. For transformation medium, 1 M sucrose was added as an osmoticum to MM. All strains were grown at 30°C, unless mentioned specifically. For adaptive strain induction, CFW stock solution (10 mg/ml in 25 mM KOH) was added as required into CM when it cooled to 60°C.

Strategies for gene deletion and gene replacement methods used a fusion PCR method (20, 21). Briefly, deletion constructs included 1 kb upstream, a selectable marker, and 1 kb downstream. Gene replacement constructs included 1 kb upstream, the mutated gene sequence, a selectable marker, and 1 kb downstream (for details, see Fig. S1A in the supplemental material). Constructs were transformed to A1149 protoplasts. Aspergillus fumigatus pyrG and pyroA were used as selectable markers (for details, see Fig. S1A and B). Mutations in replacement strains were confirmed by Sanger sequencing.

**Drug sensitivity test.** The disc diffusion assay was adapted from reference 22. Briefly, freshly harvested spores (10⁵/ml) were added to CM at 55°C and poured immediately into 9-cm-diameter petri plates. After the agar solidified, a sterilized paper disc was put at designated places, followed by 20 µl of drug stock solution CFW (10 mg/ml in 25 mM KOH). Congo red (CR; 10 mg/ml in H₂O), or caspofungin (20 mg/ml in H₂O). Plates were incubated at 30°C for 1 day.

For the drug sensitivity assay, drug stock solution was added to CM at 60°C at the designated concentration and poured immediately into 9-cm-diameter petri plates. Then, 10⁵ freshly harvested spores were spot inoculated on the medium after it solidified. Plates were incubated at 30°C for 2 days.

**Mating of Aspergillus nidulans.** Mating experiments were performed as described in the work of Kaminskyj (19). AXM5 and AXM20 are white-spore-color, morphologically wild-type, and CFW-sensitive strains. They were obtained from previous mating experiments in our lab. The genotypes of AXM5 and AXM20 are given in Table S1 in the supplemental material. For assessing the drug sensitivity of progeny, at least 100 ascospores from each outcrossed cleistothecium were selected and tested for growth sensitivity on 30 µg/ml CFW.

**Next-generation sequencing.** NGS was performed at the Beijing Genomic Institute as a commercial service, using platform Illumina HiSeq 2000. Two strains were sequenced in separate lanes. One gigabyte of raw data was generated from each run, and an average 31× depth of each nucleotide (genome size, 30 Mb) was gained. Sequence assembly and single nucleotide polymorphism (SNP) analysis were also done at the Beijing Genomic Institute.

**RESULTS**

**Aspergillus nidulans** has strong adaptive ability against sublethal levels of calcofluor white. Schoustra and colleagues (13, 16) showed that A. nidulans readily gained fitness against the antifungal agent fludioxinol under partially suppressed growth conditions. One to three mutations were sufficient to recover full fitness, with the first mutation having the greatest phenotypic effect (13, 16). Following this idea, we grew 10⁵ freshly harvested A. nidulans spores on CFW-containing medium (15 or 30 µg/ml). As expected, CFW suppressed A. nidulans growth in vitro: colony growth was restricted to ∼50% and ∼30%, respectively, of that on drug-free medium (Fig. 1A). Colonies that were grown on 30-µg/ml CFW plates developed rapidly growing sectors (Fig. 1B), which we called adaptive strains, in about a week after inoculation. We named two of the adaptive strains AXE5 and AXE8; these arose at 5 days and 8 days after inoculation, respectively. No adaptive sectors developed on the 15-µg/ml CFW plates during this experiment.

To test whether adaptive sectors would eventually emerge on 15 µg/ml CFW, and to increase the number of adaptive strains in our collection, we performed the inducing experiment with 20 replicates at each CFW concentration and extended the incubation time to 10 days (15 µg/ml) and 15 days (30 µg/ml), by which time the petri plates were completely covered. No adaptive sectors developed on 15 µg/ml CFW. However, as before, adaptive sectors emerged on 30 µg/ml CFW beginning at 5 days. Eventually, 22 adaptive strains were isolated from 20 30-µg/ml CFW plates (Table 1, AXE20 to AXE69), so that a total of 24 adaptive strains were generated in our experiment (Table 1). All of the adaptive strains were stably resistant to 30 µg/ml CFW after restreaking and also after storage at −80°C in glycerol (Fig. 2).

**Adaptation to CFW can be acquired by single mutations.** We hypothesized that the adaptive sectors were each due to the first mutation that increased hyphal growth rate on CFW. To test this, each adaptive strain was crossed with AXM5 and with AXM20, both of which are wild type for CFW sensitivity and have white spores. Mating with mutant strains is less consistent than with wild-type ones (21). We performed each mating experiment in duplicate, and unsuccessful mating experiments were repeated once more. Compared to wild-type strains, many matings with adaptive strains showed delayed cleistothecium formation and/or reduced ascospore production. Nevertheless, we had mating results from 19 adaptive strains. For each of these strains, the ratio of CFW-resistant to CFW-sensitive progeny was ∼1:1 (Table 1 and Fig. 3), consistent with single-gene defects and χ² goodness-of-fit analysis. Five strains from our collection failed to mate with either AXM5 or AXM20, producing at best only tiny cleistothecia. We dissected some of these, but they produced only white-spored colonies. Therefore, we were not able to assess the number of muta-
TABLE 1 Isolation day and meiotic progeny for each adaptive strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation day</th>
<th>Ratio of CFW-resistant/CFW-sensitive progeny</th>
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<tbody>
<tr>
<td>AXE5</td>
<td>5</td>
<td>81:85</td>
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<tr>
<td>AXE8</td>
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<td>35:65</td>
</tr>
<tr>
<td>AXE69</td>
<td>13</td>
<td>37:62</td>
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*Isolation date is represented by days postinoculation. Strains without successful mating results are shown as NA.

Adaptation to CFW is specific. To test whether any of our adaptive strains had cross-resistance to other wall-targeting agents, we assessed their response to Congo red (CR; which binds to cellulose fibers) and to caspofungin. For AXE5 and AXE8, which were isolated from our preliminary test, we used a disc diffusion method (22). Weak cross-resistance to CR was found for both strains, but there was no cross-resistance to caspofungin (Fig. 2A). For the remainder of the adaptive mutant strains, we used a more efficient method to compare drug sensitivities by testing their survival ability on drug-containing medium. A few strains (AXE37, -51, -62, -64, and -69) showed a weak resistance to 500 μg/ml CR (Fig. 2B), whereas all strains maintained the same sensitivity to 10 μg/ml caspofungin as did A1149 (Fig. 2B). We interpret this as showing that the adaptation in these mutants was specific to CFW compared to caspofungin.

Adaptation to CFW appears to be acquired by many different mutations. Calcofluor inhibits chitin crystallization during cell wall formation (23), which is likely to require many protein products. To estimate the number of different CFW-adaptive mutants in our collection, we assessed their phenotypes under a suite of growth conditions. Precise analysis would have required 552 pairwise matings.

Based on this information, the 24 strains were grouped into

FIG 2 Drug sensitivities of adaptive strains to CFW, CR, and caspofungin. (A) The disc diffusion method was used to test drug sensitivity for AXE5 and AXE8. Freshly harvested spores were added to CM at a final concentration of 10⁷/ml. After solidification, 10 μl of each drug solution (CFW, 10 mg/ml; CR, 10 mg/ml; caspofungin, 20 mg/ml) was added to sterile filter paper discs. Plates were incubated at 30°C for 2 days. Both strains showed strong resistance to CFW and a weak cross-resistance to CR but similar resistance to caspofungin. (B) The survival ability test was used to show the drug sensitivity of other adaptive strains. Freshly harvested spores from each strain (10⁷) were spot inoculated on CM, which was supplemented with 30 μg/ml CFW, 500 μg/ml CR, or 10 μg/ml caspofungin as indicated. Plates were incubated at 30°C for 2 days. All strains had stable resistance to CFW, and some strains showed weak resistance to CR, but all strains maintained the same sensitivity to caspofungin as did their wild-type parent.
five classes. Five strains (AXE8, -52, -62, -66, and -69) showed obvious growth defects on drug-free medium (see Fig. S2 in the supplemental material). Three strains (AXE5, -33, and -37) were temperature sensitive, since they could not conidiate at 37°C. Five strains (AXE29, -30, -35, -41, and -58) showed resistance only to 30 μg/ml CFW but not to 50 μg/ml CFW (see Fig. S2). Six strains (AXE35, -52, -58, -62, -64, and -69) showed growth defects on 1 M NaCl (see Fig. S2). Ten strains (AXE20, -22, -43, -44, -46, -49, -51, -54, -63, and -65) had no obvious growth defect under any condition tested (see Fig. S2). Within these groups, there were minor differences between strains. This suggested that they may share the same overall resistance mechanism (perhaps mutations in the same gene or genes in the same pathway) but not necessarily the same mutation (e.g., a nonconservative mutation versus a premature stop). For example, AXE5, AXE33, and AXE37 were all temperature sensitive, but their phenotypes at 37°C differed (see Fig. S2 and S3), suggesting that they are related but different mutations. Eventually, we found that only strains AXE20 and -22; AXE29 and -30; AXE35 and -58; AXE44 and -51; and AXE52, -62, and -69 could not be distinguished based on these criteria. We interpret this to mean that there were at least 18 different mutations in our adaptive strain collection.

Since many mutations appeared to be unique in our collection, our current screen for the CFW resistance mutation is not exhaustive. However, the goal of this study was to assess whether we could efficiently identify fast-emerging resistance mutations to an antifungal agent, not to discover new genes involved in CFW resistance, so we concentrated on two strains and archived the rest.

NGS revealed potential mutations in adaptive strains. Plasmid complementation is a well-established method for gene identification in A. nidulans mutants (for example, see reference 24) but is not optimal for drug resistance mutants, due to their relatively subtle phenotypes, and need for a reverse-selection strategy. Instead, we used next-generation sequencing (NGS) to identify candidate genes, a strategy that has been used in other organisms to identify single mutations (25–27). We selected AXE5 and AXE8 for whole-genome sequencing. Sequence assembly was based on the A. nidulans A4 genome (Aspergillus Comparative Sequencing Project, Broad Institute [www.broadinstitute.org]). Compared to the A4 reference strain, about 400 single nucleotide polymorphisms (SNPs) were detected in each of the adaptive strains (Table 2). These were roughly evenly distributed across the 17 scaffolds that represent the whole genome (see Fig. S4 in the supplemental material). Since the two adaptive strains were both induced from the A1149 parent, the SNP distributions were very similar. After removing common SNPs and SNPs that were not in a coding region, there were only 15 (AXE5) and 13 (AXE8) unique SNPs (Table 2; see also Table S2). In this way, we successfully narrowed our targets from genome scale to a limited number of genes. Based on two well-established Aspergillus genome databases (28; Aspergillus Comparative Sequencing Project, Broad Institute [www.broadinstitute.org]), we comprehensively analyzed each candidate gene (see Table S2). Two SNPs that caused stop truncations and had 99% confidence scores in genes that were likely to be relevant to CFW resistance drew our attention and were selected for closer examination.

**CFW resistance mutations were confirmed by mutation-re-introduction.** For the AXE5 gene sequence, a C1198T mutation was detected in ANID_10647, which created a premature stop codon in the predicted protein product. Lin and Momany (24) had previously characterized this gene and annotated it as a predicted cytochrome P450 protein (24). Their study had been based on a different genetic mutation (G1225T), which also introduced a premature stop codon near the 3′-end. All of the AXE5 pheno-

<table>
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<th>TABLE 2 Summary of single nucleotide polymorphisms in AXE5 and AXE8</th>
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<td>Strain</td>
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<tr>
<td>AXE5</td>
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<td>AXE8</td>
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types were consistent with those described in the work of Lin and Momany (24), including temperature sensitivity, resistance to CFW, and being osmotically remediable for temperature sensitivity (Fig. 2; see also Fig. S3 in the supplemental material).

For the AXE8 gene sequence, a G1081T mutation created a premature stop codon in ANID_03445. This is an uncharacterized gene in A. nidulans. However, CHS4 in Saccharomyces cerevisiae is the orthologue of ANID_03445 and has been characterized previously (29, 30). CHS4 encodes the activator of the major chitin synthase (CHS3) in S. cerevisiae, which positively regulates chitin formation (29, 30). Chs4p has been shown to physically interact with Chs3p (31), and this binding relies on the C-terminal 86 amino acids of Chs4p (31). Sequence analysis by ClustalW2 showed a 54% sequence identity between ANID_03445 and CHS4. The G1081T mutation in ANID_03445 created a premature stop codon in the middle of the predicted protein product, thereby truncating 371 amino acids from the C-terminal sequence. It is possible that the ANID_03445 G1081T mutation affects the binding between this activator and chitin synthase in A. nidulans. In turn, the lack of this chitin synthase activation could lead to reduced chitin in the A. nidulans cell wall and resistance to CFW.

To test whether these two specific mutations caused the CFW resistance, we first confirmed the sequence of the target genes in AXE5/ANID_10647 and AXE8/ANID_03445 by Sanger sequencing, revealing the same mutation as that with NGS. Using PCR-based methods, we reconstructed the mutations and separately replaced each gene in A1149 (see Fig. S1 in the supplemental material). The PCR constructs contained 1 kb of target gene upstream plus the whole mutated gene plus a nutrition marker with its native promoter (A. fumigatus pyroA in each case) plus 1 kb of target gene downstream.

All of the constructed ANID_10647 C1198T strains showed CFW resistance; however, this was only so for some ANID_03445 G1081T strains. We sequenced the constructed strains and found that only the CFW-resistant colonies from ANID_03445 G1081T transformation plates had the desired mutation, whereas the other colonies did not. This is likely because the mutation site in ANID_03445 G1081T is far (1,174 bp) from the nutrition marker in the PCR construct. Therefore, homologous recombination could happen after the mutated site. In that case, the selective marker would be introduced to the A1149 genome without the mutation site, and those transformants would not have CFW resistance. In contrast, the mutation in ANID_10647 C1198T is close (242 bp) to the 3′end of the gene, so there was a low possibility that homologous recombination could happen after this site.

The proper reconstructed strains showed the same drug resistance as did AXE5 and AXE8, respectively (Fig. 4). All other associated phenotypic changes in AXE5 (e.g., temperature sensitivity) (see Fig. S3 in the supplemental material) and AXE8 (e.g., growth defect on drug-free medium [Fig. 4]) were also present in our reconstructed strains. Therefore, the drug resistance mutations were confirmed. In addition, we deleted ANID_03445 and found that the deletion strain was phenotypically indistinguishable from AXE8 (Fig. 4). As expected, the premature stop mutation in AXE8 may prevent the interaction between this activator and its corresponding chitin synthase. Lack of this interaction may lead to less chitin deposition in the A. nidulans cell wall and in turn lead to CFW resistance as it did in S. cerevisiae (29, 30). Furthermore, AXE8 showed phenotype defects in the absence of CFW, which is consistent with changes in its wall composition. Lin and Momany had previously deleted ANID_10647 (24), so this was not repeated in our study.

**DISCUSSION**

Excitingly, our strategy enabled us to generate and isolate adaptive strains in A. nidulans that had single-gene mutations and to rapidly identify the mutated genes. Working with single-gene mutations was important to simplify NGS analysis. We expect that our strategy should be generally applicable to fungi regardless of whether they have a tractable sexual life cycle to test the number of mutations.

Is our single-gene mutation collection a special case for CFW? We think not. First, Schoustra and colleagues found that single-gene mutations were sufficient to create a high level of resistance
to fludioxonil (13, 16). They did not identify the mutated genes, probably due to factors mentioned above. Second, echinocandin resistance mutations are typically at single sites in FKS1 (10–12). Third, single mutations in Erg11 lead to resistance to azoles (32). Fourth, we were able to generate a single adaptive strain with resistance to 500 μg/ml CR. Mating results for that strain were consistent with a single-gene mutation (data not shown). Fifth, if multiple mutations were necessary for drug resistance, antifungal adaptation that required a particular combination of mutations would be expected to be extremely rare. Taken together, several lines of evidence besides our CFW study show that drug resistance based on protein function can emerge quickly given the appropriate selection pressure (11, 13, 33), consistent with single-gene mutations. In contrast, resistance to polyenes that target ergosterol distribution, rather than its synthesis, has been relatively durable, although even it has been overcome recently (33).

Based on phenotype analysis, we have evidence to suggest that there are at least 18 different single-gene mutations that cause resistance to 30 μg/ml CFW, suggesting that the screen is not exhaustive. Multiple resistance mutations against CFW have been reported for fungi (34, 35); however, at least one mutated gene (ANID_03445) that we identified had not been characterized in A. nidulans. The objective of our study was not to determine how many mutations lead to CFW resistance, and in addition, the protein basis for the mode of action of CFW is not fully understood. The ways that fungi can gain resistance will depend on the mechanism of a certain drug, which will also be related to the adaptation rate. However, as long as single mutations can lead to resistance, then we should expect to isolate them with NGS.

We were able to identify many adaptive sectors on the 30-μg/ml CFW treatment but none on the 15-μg/ml plates, suggesting that the degree of inhibition may be important for induction of adaptive mutations. An interesting additional phenotype for the colonies on 30 μg/ml CFW was the marked increase in Hülle cells (Fig. 1A). Schoustra et al. (15) proposed that mitotic recombination has the potential to accelerate adaptation rates in A. nidulans. We suggest that the presence of large numbers of Hülle cells could be a sign that the colony has become potentiated for cell fusion. Hülle cells are seen with increasing frequency in older A. nidulans colonies, which may be associated with cleistothecia. Hyphal fusion and subsequent nuclear migration could lead to enhanced spread of nuclei bearing a beneficial mutation and a more efficient formation of a fast-growing sector (17). We suggest that Hülle cell formation could be a harbinger of potential spread of adaptive mutations and is consistent with the differences in adaptation rates between the two CFW concentrations that we used.

Identifying a single critical mutation site in a whole genome is not trivial. Since the development of NGS in 2008 (36), its application in resolving questions of adaptation has been applied in many organisms (reviewed in reference 37). NGS still suffers from short reads and high cost, which reduce its commercial availability; nevertheless, the small (30-Mb) haploid genome of A. nidulans made NGS more practical. We had ~31× coverage of each nucleotide and had high accuracy for most sites. Most SNPs were shared between the two strains, as expected since they were derived from the same parent, A1149. Despite this, we were able to use the A4 genome, which is thoroughly annotated, as a reference strain. In the end, only a few useful SNPs were identified in each adaptive strain. In our experiment, two samples were sequenced in separated lanes in order to assemble an accurate genome sequence. However, if the purpose is to figure out the mutation sites, this is still not the optimal way for cost efficiency. Recently, it seems that a new type of “phenotype sequencing” would enable us to identify all potential causal genes when all collected mutants are pooled and sequenced together (38). This would provide a more cost-saving manner for such studies; however, stringent criteria must be met for this strategy.

The downstream work is straightforward for A. nidulans, since two well-established databases (28; Aspergillus Comparative Sequencing Project, Broad Institute [www.broadinstitute.org]) were available and all molecular techniques for gene manipulation had been developed (20). As a result, the target mutations were readily verified. For some fungal species, genome databases are less well established. Until genome resources are expanded, the metabolic conservation between fungi should enable us to apply most results to other species. Beginning with a model system such as A. nidulans should facilitate development.

In this proof-of-principle study, we developed a strategy using A. nidulans to demonstrate that first-arising adaptive sectors were due to single-gene mutations and using NGS to rapidly identify potential mutations for drug resistance to CFW, which otherwise are difficult to locate. Our strategy has wide applicability to all antifungal drugs. Especially, we suggest that this strategy could be used during drug development in order to predict where these new treatments will fail in the future. The value of this will be in having a combination therapy in anticipation of future need. In summary, we suggest that our strategy will be broadly useful in the drug resistance arms race between humans and pathogenic microorganisms.

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