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MECHANISMS OF RESISTANCE

CRISPR/Cas9 Genome Editing To Demonstrate the Contribution of Cyp51A Gly138Ser to Azole Resistance in *Aspergillus fumigatus*

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ABSTRACT A pan-azole-resistant *Aspergillus fumigatus* strain with the *cyp51A* mutations Gly138Ser and Asn248Lys was isolated from a patient receiving long-term voriconazole treatment. PCR fragments containing *cyp51A* with the mutations were introduced along with the Cas9 protein and single guide RNA into the azole-resistant/susceptible strains. Recombinant strains showed increased susceptibility via the replacement of Ser138 by glycine. Genetic recombination, which has been hampered thus far in clinical isolates, can now be achieved using CRISPR/Cas9 genome editing.

KEYWORDS azole drugs, antifungal resistance, Cyp51A, Cas9, CRISPR, genome editing

The filamentous fungus *Aspergillus fumigatus* is the most common opportunistic human fungal pathogen, with a wide range of clinical features, including invasive pulmonary aspergillosis, chronic progressive pulmonary aspergillosis (CPPA), and allergic bronchopulmonary aspergillosis (1). Triazole antifungal drugs are the most common treatment for *A. fumigatus* infection. Itraconazole (ITC) and voriconazole (VRC) are the only oral drug treatment options for aspergillosis, which may lead to long-term administration. Since the discovery of the first ITC-resistant isolate in 1997 (2), epidemiological reports of new triazole-resistant isolates have been increasing worldwide (3). Mechanisms of acquired azole resistance may be explained by extended periods of azole exposure in the host or by environmental exposure of *A. fumigatus* to agricultural fungicides.

The primary molecular mechanisms of triazole resistance in *A. fumigatus* isolates are mutations that alter the target protein Cyp51A and prevent its interaction with the drug (4). Mutations in *cyp51A* may be classified as single-nucleotide polymorphisms (SNPs) and/or tandem repeats in the promoter region (3). The major SNPs affecting Cyp51A are positioned at Gly54, Gly138, Met220, and Gly448. The clinical isolates with these SNPs demonstrate various azole susceptibility profiles; for example, isolates with SNPs at Gly54 show resistance to ITC and varied susceptibility to posaconazole (POS) and VRC, whereas isolates with SNPs at Gly138 show pan-azole resistance, including resistance to ITC, POS, and VRC. Another alteration is a tandem repeat in the promoter region that results in the overexpression of *cyp51A* with specific SNPs. Two major classes of such azole-resistant mutations are TR34-Leu98His and TR46-Tyr121Phe-Thr289Ala, which carry a 34-bp and a 46-bp sequence duplication, respectively, as well as amino acid

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TABLE 1 *Aspergillus fumigatus* strains used in this study and their *in vitro* antifungal susceptibility profiles against three triazoles according to the Etest method[a]

Strain(s)	Parent	Genotype[b]	MICs (μg/ml)[c]			Source
			VRC	ITC	POS	
NIID0345	clinical isolate		1.0	>8	0.5	Current study
NIID0345-mut1-2	NIID0345	mut1 mut2 *cyp51A hph*	1.5	>8	0.75	Current study
NIID0345-S138G	NIID0345	mut1 S138G mut2 *cyp51A hph*	0.5	1.0	0.25	Current study
NIID0345-K248N	NIID0345	mut1 K248N mut2 *cyp51A hph*	2	>8	0.5	Current study
NIID0345-S138G-K248N	NIID0345	mut1 mut2 *cyp51A hph*	0.5	1.0	0.25	Current study
AfS35	D141	*akuA*ΔloxP	0.094	0.5	0.125	Fungal Genetics Stock Center
AfS35-mut1-2	AfS35	mut1 mut2 *cyp51A hph*	0.125	0.5	0.125	Current study
AfS35-G138S	AfS35	mut1 G138S mut2 *cyp51A hph*	0.38	1.5	0.19	Current study

[a]See Fig. 1 and 3.
[b]mut1 and mut2 are silent mutations for Cas9-nuclease resistance.
[c]VRC, voriconazole; ITC, itraconazole; POS, posaconazole.

substitutions. Although many SNPs in *cyp51A* that may be linked to low susceptibility in azole-resistant isolates have been previously reported, few studies have been conducted to conclusively demonstrate the contribution of SNPs to decreased azole susceptibility in clinical isolates. One obstacle affecting the molecular analysis of clinical *A. fumigatus* isolates is the production of genetically manipulated mutants, as the efficiency of homologous recombination is extremely low.

Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 has been applied as a powerful genome editing tool in various organisms (5). By forming a ribonucleoprotein complex with an artificial single guide RNA (sgRNA) designed to target a cellular gene, the Cas9 nuclease efficiently introduces double-stranded breaks (DSBs) at the corresponding target locus (6). The sgRNA hybridizes to its cDNA sequence immediately upstream of the protospacer-adjacent motif (PAM), which consists of NGG for the *Streptococcus pyogenes* Cas9 variant (7). DSBs in the target genomic DNA can be repaired by either homology-directed repair or nonhomologous end joining (NHEJ) (5, 8, 9). DNA repair via homology-directed repair requires a homologous DNA template with sequence similarity to that of the adjacent region of the DSB locus, whereas NHEJ ligates the DSB, leading to indels in a template-independent manner. The CRISPR/Cas9 system has also been successfully applied to *A. fumigatus* (10–12).

The *A. fumigatus* strains used in the present study are listed in Table 1. The clinical isolate NIID0345 was obtained in 2016 from the sputum sample of a 74-year-old male patient with CPPA who had received VRC treatment for 3 years. The isolate was not susceptible to VRC, ITC, or POS (Fig. 1) but was susceptible to amphotericin B, micafungin, and caspofungin (data not shown). A comparison of *cyp51A* from the azole-resistant isolate (NIID0345) with those from azole-susceptible strains (Af293 and AfS35) revealed that NIID0345 carried two amino acid substitutions, Gly138Ser (GGC→AGC) and Asn248Lys (AAT→AAA). To verify which SNP is involved in azole resistance, we substituted the nucleotide sequences corresponding to amino acid

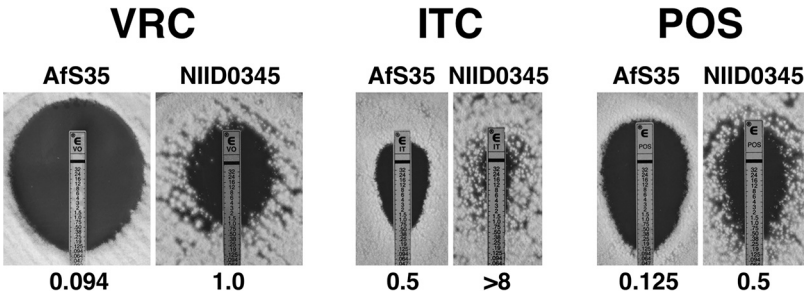

FIG 1 Antifungal susceptibility testing using Etest strips for voriconazole, itraconazole, and posaconazole in the azole-susceptible *Aspergillus fumigatus* strain AfS35 and clinical azole-resistant *A. fumigatus* strain NIID0345. The number below each photo represents the MIC (in μg/ml).

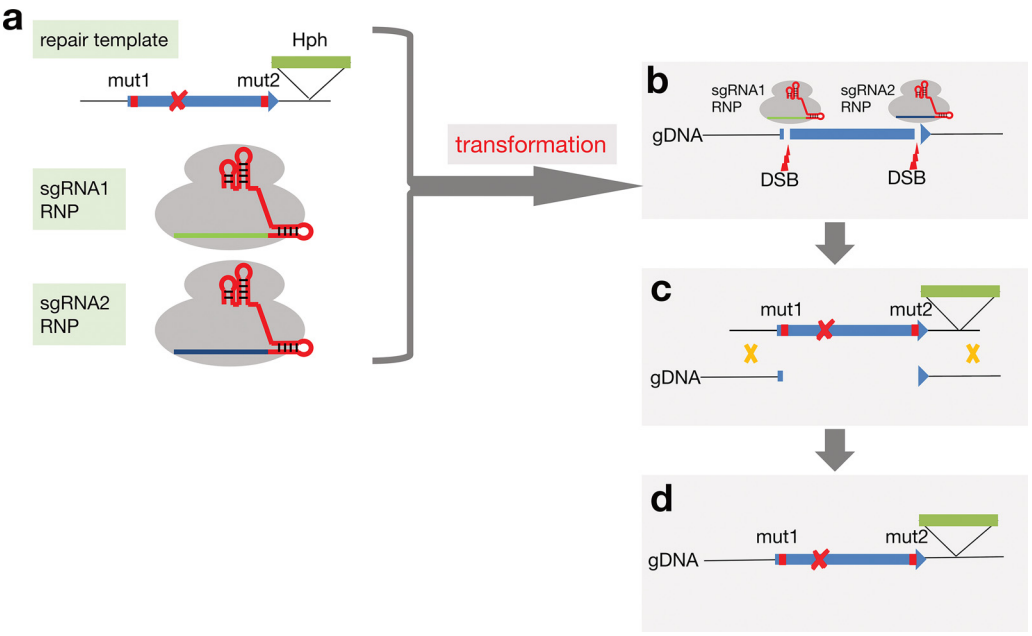

FIG 2 Overview of the genetic modification via CRISPR/Cas9-promoted homology-directed repair. (a) Cas9 protein and *in vitro*-synthesized sgRNAs were mixed to form two RNPs. The repair template and two RNPs were transformed into *Aspergillus fumigatus* protoplasts. (b) The dual Cas9-sgRNA complex introduced two double-stranded breaks at the N and C termini of *cyp51A* (c and d). The cleaved *cyp51A* on the genomic DNA is replaced by the repair template, resulting in the introduction of the desired mutations and *hph* marker. The silent mutations mut1 and mut2 on the repair template and the replaced genomic DNA (gDNA) cannot be cleaved by the RNP nuclease.

Ser138 and/or Lys248 in the clinical isolate NIID0345. We attempted to replace the genomic *cyp51A* gene locus by homologous recombination with a linear DNA fragment harboring mutations. For this purpose, Cas9/sgRNA ribonucleoproteins and the repair template were simultaneously transformed via the protoplast-polyethylene glycol method into the *A. fumigatus* azole-resistant clinical isolate (Fig. 2). For detailed methods for strain construction, see described in the text in the supplemental material.

Next, we examined azole susceptibility by using Etest strips on the constructed recombinant strains. The strains in which only nuclease-resistant silent mutations were introduced demonstrated an azole resistance profile similar to that of the parental strain, NIID0345 (Fig. 3A; Table 1), indicating that CRISPR/Cas9-mediated homologous recombination had no effect on azole susceptibility. Both of the recombinant strains with Ser138Gly and Ser138Gly/Lys248Asn amino acid substitutions showed increased susceptibility to all azoles tested, whereas the strain with only the Lys248Asn substitution showed an azole resistance profile similar to that of the parental clinical isolate. These results indicate that Lys248 is not associated with azole resistance and that Ser138 is responsible for azole resistance in this clinical isolate.

To verify whether Gly138 in Cyp51A is responsible for azole resistance, an amino acid substitution of Gly138 to serine was introduced into the azole-susceptible strain AfS35. The method for producing the recombinant strain was the same as that described above; however, highly efficient homologous recombination was expected because strain AfS35 is deficient in the NHEJ repair system. As expected, almost all transformants exhibited ideal recombination. Azole susceptibility testing of the recombinant strains with Etest showed a slight decrease in azole susceptibility when the Ser138 mutation was introduced into the azole-susceptible strain AfS35 (Fig. 3B; Table 1). The strain with only nuclease-resistant silent mutations demonstrated an azole susceptibility profile similar to that of parental strain AfS35. From these results, we elucidated the direct involvement of Gly138 in Cyp51A in azole resistance, which had previously been supported only by indirect epidemiological evidence.

The CRISPR/Cas9 genome editing technique used in this study has enabled site-

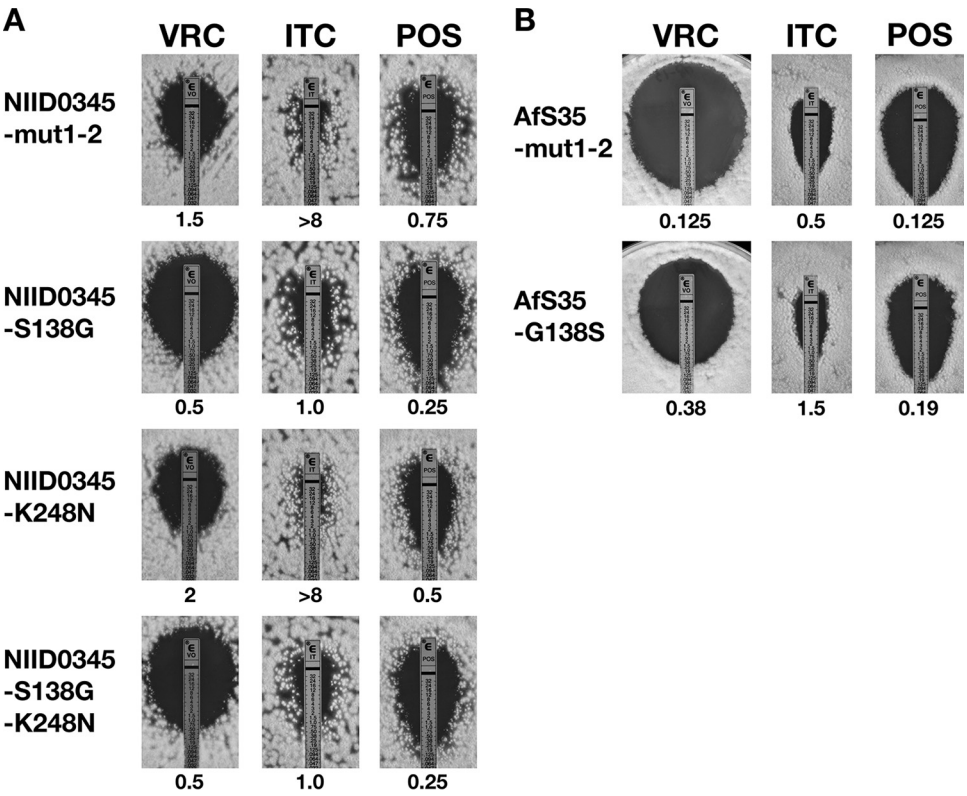

FIG 3 Antifungal susceptibility testing using Etest strips for voriconazole, itraconazole, and posaconazole for the strains generated via CRISPR/Cas9-promoted gene replacement from the strains NIID0345 (A) and AfS35 (B). The number below each photo represents the MIC (in μg/ml).

directed mutagenesis, altering Ser138 to glycine on the genomic Cyp51A locus in an azole-resistant clinical strain. This is, to our knowledge, the first example of site-directed mutagenesis performed in a clinical azole-resistant fungal isolate to elucidate whether azole susceptibility is altered by mutations in the genomic Cyp51A locus. Although genetically recombinant strains harboring mutations such as TR34-Leu98His (13), TR46-Tyr121Phe-Thr289Ala (14), Gly54Trp (13), and Thr301Ile (15) in the genomic Cyp51A locus have been reported, all these strains were constructed in the *akuB*^{KU80}-deficient strain as a recipient. It is well known that wild-type strains, such as clinical isolates, tend to exhibit low efficiency in homologous recombination, largely because of high NHEJ activity. To overcome this limitation, the gene encoding either KU70 or KU80, which are the components of NHEJ machinery, was knocked out, leading to a significant increase in the frequency of homologous recombination (16, 17). In contrast, our CRISPR/Cas9 method can facilitate efficient homologous recombination without the inactivation of the NHEJ pathway, which is supported by previous studies, concluding that the frequency of homologous recombination can be increased by the CRISPR/Cas9 system in *Candida glabrata* (18).

To build the CRISPR/Cas9 system in *A. fumigatus* clinical isolates, we incorporated several additional methods to improve the efficiency and accuracy of *cyp51A* gene replacement events. Improved efficiency of *cyp51A* replacement was achieved by introducing two DSBs via the design of two target sequences for sgRNA at sites close to the N and C termini, repressing homologous recombination within the *cyp51A* coding region. Additionally, to avoid digestion of the repair template and redigestion of the edited target after the homologous recombination event, nuclease-resistant silent mutations were introduced in two loci of three codons immediately upstream from the PAM sites of the repair template, preventing it from being targeted by CRISPR/Cas9 (19). To minimize the off-target effects from continuous DNA-based Cas9

and sgRNA expression (which should be considered whenever the CRISPR/Cas9 system is used for genome editing), we introduced ribonucleoproteins consisting of commercially available recombinant Cas9 protein and *in vitro*-synthesized sgRNAs directly into protoplasts of clinical isolates. As one means of minimizing off-target effects, directly transfected Cas9 protein reduces the off-target cleavage rate when compared with Cas9 expression by a plasmid or mRNA transfection in mammalian cells (20). One recent study demonstrated that direct delivery of Cas9-guide RNA ribonucleoprotein can facilitate genome editing in *A. fumigatus* (10). Based on these improvements, we produced a simple, efficient, and accurate site-directed mutagenesis system for investigating structure-phenotype relationships of the azole target Cyp51A. Since this system can be applied to numerous genes other than *cyp51A*, this method will accelerate the progress of many pathogenic fungal studies.

In conclusion, we have developed a simple, efficient, and accurate gene replacement system using CRISPR/Cas9 genome editing techniques and applied these techniques to investigate the mechanisms of azole resistance via Cyp51A alteration. We confirmed at the molecular level that the Gly138Ser mutation is one reason for azole resistance in a clinical isolate. There are many *cyp51A* mutations that may result in potential but unconfirmed amino acid changes conferring azole resistance. Further investigation of Cyp51A using our CRISPR/Cas9 system is required to verify whether the diverse SNPs reported are in fact responsible for azole resistance.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00894-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare no conflicts of interest.

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