



# *cyp51A* Mutations, Extolite Profiles, and Antifungal Susceptibility in Clinical and Environmental Isolates of the *Aspergillus viridinutans* Species Complex

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**ABSTRACT** The past decade has seen an increase in aspergillosis in humans and animals due to *Aspergillus viridinutans* species complex members. Azole resistance is common to these infections, carrying a poor prognosis. *cyp51A* gene mutations are the main cause of acquired azole resistance in *Aspergillus fumigatus*. This study aimed to determine if the azole-resistant phenotype in *A. viridinutans* complex members is associated with *cyp51A* mutations or extrolite profiles. The *cyp51A* gene of clinical and environmental isolates was amplified using novel primers, antifungal susceptibility was tested using the Clinical and Laboratory Standards Institute methodology, and extrolite profiling was performed using agar plug extraction. Very high azole MICs were detected in 84% of the isolates (31/37). The MICs of the newer antifungals luliconazole and olorofim (F901318) were low for all isolates. *cyp51A* sequences revealed 113 nonsynonymous mutations compared to the sequence of wild-type *A. fumigatus*. M172A/V and D255G, previously associated with *A. fumigatus* azole resistance, were common among all isolates but were not correlated with azole MICs. Two environmental isolates with nonsusceptibility to itraconazole and high MICs of voriconazole and isavuconazole harbored G138C, previously associated with azole-resistant *A. fumigatus*. Some novel mutations were identified only among isolates with high azole MICs. However, *cyp51A* homology modeling did not cause a significant protein structure change for these mutations. There was no correlation between extrolite patterns and susceptibility. For *A. viridinutans* complex isolates, *cyp51A* mutations and the extrolites that they produced were not major causes of antifungal resistance. Luliconazole and olorofim show promise for treating azole-resistant infections caused by these cryptic species.

**KEY WORDS** cryptic species, *Aspergillus viridinutans*, *Aspergillus felis*, *Aspergillus udagawae*, azole resistance, *cyp51A*

**A**spergillosis, most commonly due to *Aspergillus fumigatus*, can cause invasive and fatal disease in humans and animals. Triazole antifungal drugs (itraconazole, voriconazole, posaconazole, and, more recently, isavuconazole) target fungal ergosterol synthesis and are the mainstay of treatment of *Aspergillus* diseases in humans and

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animals (1). Since the late 1990s, aspergillosis treatment in humans has been complicated by the development of azole resistance, most commonly reported in *A. fumigatus* isolates, leading to excess mortality in patients with azole-resistant invasive aspergillosis (2, 3). Additionally, poor clinical outcomes have been associated with infection by other species in *Aspergillus* section *Fumigati*, including members of the *Aspergillus viridinutans* species complex (AVSC), that demonstrate decreased antifungal susceptibility *in vitro* and *in vivo*, reports of which have increased (4).

Azole drug resistance can be due to innate or acquired mechanisms (5). Acquired resistance in *A. fumigatus* clinical and environmental isolates has most frequently been associated with tandem repeats in the promoter region and single point mutations of the *cyp51A* gene. In the past decade, acquired antifungal resistance was reported initially in human isolates from patients in the United States (6) and then in the Netherlands, where it was associated with the use of triazole fungicides in agriculture (7–9). Azole resistance due to *cyp51A* mutations has since been reported globally among clinical and environmental isolates (10), reinforcing the need to apply the World Health Organization concept of a one health approach in the management of fungal diseases (4, 11).

More than 50 mutations and tandem repeat combinations in the *cyp51A* gene have been detected among *A. fumigatus* isolates (12). Those most frequently associated with environmentally acquired azole resistance include TR<sub>3,4</sub>/L98H and TR<sub>4,6</sub>/Y121F/T289 (13), while a number of mutations have been associated with therapeutic exposure to azole drugs (G54, G138, G448, M220) (14).

The AVSC contains 10 species (*A. arcoverdensis*, *A. aureolus*, *A. felis*, *A. frankstonensis*, *A. pseudoviridinutans*, *A. siamensis*, *A. udagawae*, *A. viridinutans*, *A. wyomingensis*, *A. acrensis*) (15). In 2014, *A. parafelis* and *A. pseudofelis* were described as novel species closely related to *A. felis*; however, analysis of a larger number of strains indicates that they are actually *A. felis* (15, 16). Since 2005, four members of the AVSC have been increasingly associated with infections in humans and animals, including *A. felis*, *A. pseudoviridinutans*, *A. udagawae*, and *A. wyomingensis* (4). These species can cause localized or disseminated infections that are difficult to treat, with many isolates being noted to have high MICs of triazole antifungals (4). Innate resistance mechanisms are thought to be responsible for the high levels of intrinsic resistance encountered among these species (5). However, whether mutations of the *cyp51A* gene could be involved has not yet been investigated.

This study aimed to determine if there was any correlation between the azole-resistant phenotype and the *cyp51A* genotype among clinical and environmental AVSC isolates (see Table S1 in the supplemental material) through amplification of the *cyp51A* gene, antifungal susceptibility testing, and *cyp51A* protein homology modeling. An additional aim was to determine potential innate virulence factors of AVSC isolates by determining their extralite production.

## RESULTS

**Antifungal susceptibility testing.** Of 37 AVSC isolates that sporulated adequately for antifungal susceptibility testing (see Table S2 in the supplemental material), 31 (83.8%) were defined as having high MICs of at least one azole, including itraconazole (20 of 37 [54.1%]; MIC, >1  $\mu\text{g/ml}$ ), voriconazole (31 of 37 [83.8%]; MIC, >1  $\mu\text{g/ml}$ ), isavuconazole (30 of 37 [81.1%]; MIC, >1  $\mu\text{g/ml}$ ), and posaconazole (1 of 37 [2.7%]; MIC, >0.5  $\mu\text{g/ml}$ ). Of these 37 isolates, 15 were of environmental origin, 14 were clinical isolates, and 2 were of unknown origin. A comparison of the geometric mean MIC, MIC ranges, MIC<sub>50</sub>, and MIC<sub>90</sub> results for environmental versus clinical AVSC isolates is presented in Table 1. Environmental isolates had higher MICs than clinical isolates; however, this was not statistically significant (for isavuconazole, itraconazole, and posaconazole,  $P \geq 0.2$ ; for voriconazole,  $P = 0.1$  to 0.2). All isolates were inhibited by very low luliconazole and olorofim (F901318) concentrations (for luliconazole, the geometric mean MIC was 0.002  $\mu\text{g/ml}$ , the MIC<sub>90</sub> was 0.002  $\mu\text{g/ml}$ , and the MIC range was 0.001 to 0.004  $\mu\text{g/ml}$ ; for olorofim, the geometric mean MIC was 0.008  $\mu\text{g/ml}$ , the

**TABLE 1** Comparison of CLSI testing results for clinical versus environmental AVSC isolates<sup>a</sup>

Antimicrobial	MIC ( $\mu\text{g/ml}$ )							
	Range		Geometric mean		50%		90%	
	Clinical	Environmental	Clinical	Environmental	Clinical	Environmental	Clinical	Environmental
ITC	0.125 to >16	0.5 to >16	2.76	5.5	1	>16	>16	>16
POS	0.031 to 0.25	0.125 to 0.5	0.16	0.26	0.25	0.25	0.25	0.5
VRC	0.25 to 8	2 to 16	2.4	4.69	2	4	4	8
ISA	0.5 to 4	2 to 8	2.4	3.79	2	4	4	8
LUL	0.001 to 0.004	0.001 to 0.004	0.002	0.002	0.002	0.002	0.002	0.004
OLO	0.002 to 0.016	0.002 to 0.016	0.008	0.007	0.008	0.008	0.016	0.008

<sup>a</sup>Data are for 17 clinical AVSC isolates and environmental AVSC isolates. Data for three isolates with an unknown origin (DTO 019-D8, DTO 316-F9, and DTO 342-I3) were excluded from this table. Luliconazole data were available for 16/17 AVSC environmental origin isolates (no data were available for *A. udagawae* DTO 006-A3). Abbreviations: ITC, itraconazole; POS, posaconazole; VRC, voriconazole; ISA, isavuconazole; LUL, luliconazole; OLO, olorofim (F901318).

MIC<sub>90</sub> was 0.016  $\mu\text{g/ml}$ , and the MIC range was 0.002 to 0.016  $\mu\text{g/ml}$ ). Seven AVSC isolates (four clinical isolates, two environmental isolates, and one isolate of unknown origin) had low MICs for all azole drugs tested.

**cyp51A sequencing.** A total of 204 synonymous and 113 nonsynonymous mutations were identified among the 56 AVSC isolates (Table S3). Of the nonsynonymous mutations, all AVSC isolates tested, regardless of azole phenotype, had the mutations W6L, V15M, K80R, D255G, C270S, I367L, H403Y, and L464I. At amino acid position M172, all isolates had a mutation of either M172V ( $n = 55$ ) or M172A ( $n = 1$ ).

For isolates demonstrating high azole MICs, 15 unique mutations that were not present in isolates with low MICs were identified (Table 2). This included G138C, found in two environmental *A. felis* isolates that were nonsusceptible to itraconazole at the highest concentration tested and that had high MICs of voriconazole and isavuconazole. One of these isolates also demonstrated a posaconazole MIC at the *A. fumigatus* epidemiological cutoff value (ECV), and the other had an intermediate posaconazole MIC (Table 2). Both these isolates also harbored a unique single nucleotide polymorphism (SNP), T215S (Table 2). Two *A. aureolus* environmental isolates harbored the nonsynonymous mutation V101L; isolate DTO 278-B7 had a MIC at the *A. fumigatus* ECV only for posaconazole, while DTO 331-G6 did not sporulate to be used in antifungal susceptibility testing. Both these isolates also harbored unique *cyp51A* SNPs, A103T, A234V, I360V, V428I, Q423D, F478V, and G505R (Table 2).

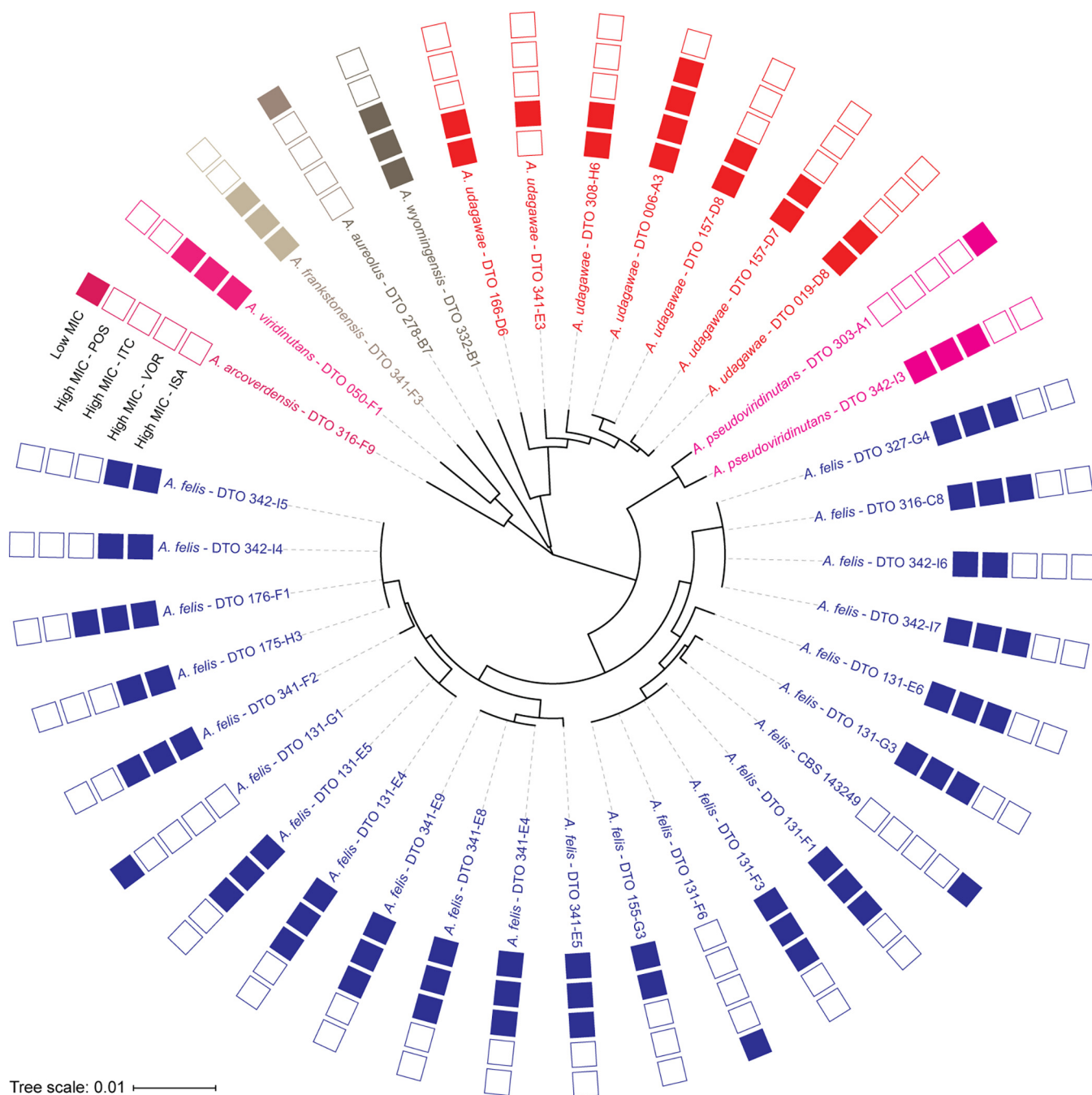
A phylogenetic tree developed from the *cyp51A* sequences grouped isolates of the same species together (Fig. 1).

**cyp51A homology modeling.** Five AVSC *cyp51A* homology models (models F to J) were constructed to compare the amino acid profiles of isolates with low azole MIC values (model F, *A. felis* DTO 131-G1) to those of isolates with high azole MIC values (model G, *A. viridinutans* DTO 050-F1; model H, *A. aureolus* DTO 278-B7; model I, *A. felis* DTO 341-E5; model J, *A. felis* DTO 131-E6) (Fig. S1). Docking scores and molecular mechanics-generalized Born surface area (MMGBSA) analysis results are presented in Table 3. Compared to model F, there were no significant changes to the overall protein structure to confer resistance in models G to J. For itraconazole, the most negative docking score value was obtained with model I ( $-11.03$  kcal/mol) and the most negative binding free energy [ $\Delta G(\text{binding})$ ] value was with low-azole-MIC model F

**TABLE 2** *cyp51A* mutations observed in high-azole-MIC-phenotype AVSC isolates that were not found in low-azole-MIC isolates<sup>a</sup>

Azole-resistant phenotype	Species (isolate no.)	<i>cyp51A</i> mutations
High multiazole MICs (ITC, ISA, VRC)	<i>A. felis</i> (DTO 341-E5)	T215S, G138C
High multiazole MICs (ITC, ISA, VRC)	<i>A. felis</i> (DTO 341-E4)	T215S, G138C
High multiazole MICs (ITC, ISA, VRC)	<i>A. felis</i> (DTO 131-E6)	S197C, Q340R
High-multiazole MICs (ITC, ISA, VRC)	<i>A. viridinutans</i> (DTO 050-F1)	A63S, L327P, V396A
Single azole MIC at the ECV for <i>A. fumigatus</i> (POS)	<i>A. aureolus</i> (DTO 278-B7)	A103T, V101L, A234V, I360V, V428I, G505R, Q423D, F478V

<sup>a</sup>MICs were determined by CLSI testing. See Table S3 in the supplemental material for a list of all mutations found. Abbreviations: ISA, isavuconazole; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ECV, epidemiological cutoff value.



**FIG 1** Phylogenetic tree of *Aspergillus viridinutans* species complex *cyp51A* sequences with corresponding low, high, or unknown triazole MICs. ISA, isavuconazole; ITC, itraconazole; VOR, voriconazole; POS, posaconazole.

( $-148.403$  kcal/mol), with a higher potential for azole binding being seen in model F than in models G to J. For posaconazole's interactions, the most negative docking score was obtained with model I ( $-11.54$  kcal/mol) and the most negative  $\Delta G(\text{binding})$  value was obtained with model G ( $-148.061$  kcal/mol). Conflicting results were found for voriconazole, with model H having the most negative docking score ( $-7.881$  kcal/mol) and the  $\Delta G(\text{binding})$  value ( $-64.09$  kcal/mol) having the highest potential for azole binding (Table 3). Root mean square deviation (RMSD) scores demonstrated high similarity among models G to J compared to the susceptible *A. felis* model (model F), with all models having a difference of less than  $0.2 \text{ \AA}$  (model I =  $0.2004 \text{ \AA}$ , model J =  $0.1929 \text{ \AA}$ , model G =  $0.0613 \text{ \AA}$ , model H =  $0.0570 \text{ \AA}$ ). Additionally, two *A. fumigatus*

**TABLE 3** Docking scores of the best conformers for posaconazole, itraconazole, and voriconazole and corresponding MMGBSA study values

Model <sup>a</sup> (species)	Ligand <sup>b</sup>	Docking score (kcal/mol)	Ligand strain energy (kcal/mol)	Receptor strain energy (kcal/mol)	$\Delta G(\text{binding})$ (kcal/mol)
F ( <i>A. felis</i> )	Posaconazole 2	-9.549	20.174	43.264	-145.031
	Itraconazole 2	-10.348	10.148	61.584	-148.403
	Voriconazole	-7.284	2.101	18.284	-56.913
G ( <i>A. viridinutans</i> )	Posaconazole 2	-9.457	17.582	20.2	-148.061
	Itraconazole 1	-7.972	11.809	48.485	-117.783
	Voriconazole	-7.13	2.618	23.192	-51.94
H ( <i>A. aureolus</i> )	Posaconazole 2	-10.21	11.18	62.408	-117.291
	Itraconazole 4	-8.732	8.419	29.881	-134.629
	Voriconazole	-7.881	10.41	16.574	-64.09
I ( <i>A. felis</i> ; mutations T215S/G138C)	Posaconazole 2	-11.541	13.355	34.947	-140.137
	Itraconazole 1	-11.031	10.476	36.487	-122.160
	Voriconazole	-7.220	6.929	20.331	-50.546
J ( <i>A. felis</i> ; mutations S197C/Q340R)	Posaconazole 2	-10.275	16.779	36.947	-141.408
	Itraconazole 3	-10.318	14.646	36.402	-112.306
	Voriconazole	-7.461	6.916	24.457	-50.255
B ( <i>A. fumigatus</i> )	Posaconazole 2	-10.385	11.936	34.031	-133.210
	Itraconazole 4	-9.543	11.503	10.276	-128.252
	Voriconazole	-6.991	2.048	-0.416	-49.793
K ( <i>A. fumigatus</i> model of model I mutations)	Posaconazole 2	-9.859	12.880	30.784	-145.495
	Itraconazole 4	-9.002	11.412	52.439	-131.727
	Voriconazole	-7.570	11.101	-0.489	-72.311
L ( <i>A. fumigatus</i> model of V101L/A103T/A234V/I360V/V428I/G505R/Q423D/F478V)	Posaconazole 1	-10.530	12.928	29.622	-122.594
	Itraconazole 2	-10.764	10.357	49.188	-148.968
	Voriconazole	-7.098	2.482	31.636	-49.211

<sup>a</sup>Models F to J are *Aspergillus viridinutans* species complex member-based models, and models B, K, and L are *A. fumigatus*-based models.

<sup>b</sup>Ligand column numbers preceding antimicrobial name refer to the conformation number of the antimicrobial structure relative to ground state conformation, generated by the Ligprep program (Schrodinger release 2017-3). Only one conformation number was generated for voriconazole.

*cyp51A* homology models (models K and L) were constructed to determine if the mutations reported in association with azole resistance in *A. fumigatus* (positions G138 and V101) altered the protein structure in a way similar to that of the mutations in AVSC-based models H and I. This confirmed the results of AVSC models H and I, with no major difference for docking and MMGBSA analysis with these mutations in the *A. fumigatus* model (Table 3).

**Extrolite profiling.** Table S5 provides the extrolite profiles of the AVSC species tested. *Aspergillus felis* and *A. udagawae* species shared the production of only fumagillin and were otherwise chemotaxonomically different. *Aspergillus felis* generally produced a large number of extrolites, including antafumicins/clavatols, aszonalenins, cytochalasin E, fumagillin, helvolic acid, kotanins, and viriditoxin, while *A. udagawae* produced fumigatins, fumitremorgins, fumagillin, pseurotin A, tryptoquivalines, and tryptoquivalones.

## DISCUSSION

High azole MIC values were commonly detected for AVSC clinical and environmental isolates. These were most frequently for isavuconazole, followed by voriconazole and itraconazole, while high MICs of posaconazole were not common. These results are similar to those of a recent study of AVSC isolates, which also found high MICs of voriconazole and itraconazole and generally low MICs of posaconazole (17).

As patients with aspergillosis due to AVSC members commonly have poor clinical outcomes and high *in vitro* MICs of azoles, we tested their susceptibility to two newer antifungal drugs, luliconazole and olorofim (F901318). Luliconazole is a commercially

available cytochrome P450 2C19 inhibitor imidazole antifungal. There are limited efficacy data for its use in the treatment of aspergillosis; however, *in vitro* antifungal susceptibility testing of *A. fumigatus* and *A. terreus* isolates and in *in vivo* animal models has demonstrated that it is a promising treatment alternative to currently used antifungals, has much lower MIC values than other antifungals (azoles, echinocandins, and polyenes), and results in improved survival rates (18–20). All AVSC isolates had luliconazole MICs in the same range reported for azole-susceptible *A. fumigatus* isolates (0.001 to 0.008  $\mu\text{g/ml}$ ) (19). The reference ranges were generally lower than those of the other azoles tested, consistent with the findings for other *Aspergillus* species (18, 20). Olorofim (F901318) is an orotomide antifungal drug that has proven *in vitro* activity against filamentous fungi with high MIC values of commonly used antifungal drugs or demonstrated azole resistance, including *Scedosporium*, *Lomentospora*, and *Aspergillus* species (21, 22). *In vivo* pharmacodynamics studies in animals with invasive pulmonary and sinopulmonary aspergillosis have demonstrated similar or improved survival outcomes in response to first-line triazole drugs, with similar survival rates reported for posaconazole-susceptible strains and improved survival reported in posaconazole-resistant strains, with pharmacodynamic targets being identified in phase 2 clinical trials (23–25). All our AVSC isolates had olorofim MICs below the range reported for azole-susceptible *A. fumigatus* isolates (0.031 to 0.125  $\mu\text{g/ml}$ ) (22). Although further evaluation is required, these antifungal drugs have promising potential as alternatives to the current azole therapeutics for *in vivo* therapeutic use against AVSC species.

Of the *cyp51A* SNPs identified only in isolates with high MICs, none were found to confer the same susceptibility phenotype (Table 2), so *cyp51A* modeling was used to further examine their significance, and the resulting homology models varied. Itraconazole interactions demonstrated the most consistent result, with model F having the strongest binding affinity to this drug compared to the rest of the models. This trend was also observed in posaconazole's interactions, and voriconazole demonstrated conflicting results, which is most likely due to its smaller size, as it does not require much energy to position itself into the correct binding mode. These results and the similarity between the protein structures, as demonstrated via RMSD score calculations, suggest that the *cyp51A* mutations in these isolates do not cause a significant change in the overall protein structure and thus do not directly interfere with azole drug binding to confer resistance. Future work investigating the genome sequences of susceptible and resistant AVSC isolates could shed light on these amino acid profile differences, which have the potential to determine strain lineage as well as identify other genetic causes of azole resistance. *Aspergillus felis* is heterothallic, and progeny resulting from direct mating of susceptible and resistant *A. felis* isolates could be used in segregate analyses.

Although nonsynonymous mutations at sites previously associated with azole resistance in *A. fumigatus* were found (M172A, M172V, and D255G), these were present in AVSC isolates with both high and low azole MICs and so are unlikely to be a cause of azole resistance, as has also been reported for *A. fumigatus* wild-type (WT) isolates (26–40). However, the combination of single point mutations may also be important when determining the ability to cause azole resistance, as recently demonstrated by a study investigating *A. fumigatus* isolates harboring mutations at these positions (M172V and D255E) in combination with either F46Y, G89G, L358L, and C454C or F46Y, G89G, N248T, L358L, E427K, and C454C, which were shown to have elevated azole MICs and were pathogenic in *Galleria mellonella* host virulence studies and of a cluster/lineage different from strains of WT *A. fumigatus* (41). However, in our study this was not the case for isolates with high azole MIC values that underwent *cyp51A* homology modeling. Future studies could examine the transfer of mutated *cyp51A* genes that confer resistance from *A. fumigatus* to AVSC strains with low azole MICs and, similarly, the transfer of *cyp51A* genes from a high-azole-MIC AVSC strain to wild-type *A. fumigatus* to observe if an increase in azole resistance occurs. This could be achieved via gene editing, for example, by cloning or using the CRISPR/Cas9 system, as has been described previously for the *cyp51A* gene for *Aspergillus* species (42–45). Our study found

the azole resistance-associated mutation G138C in two environmental *A. felis* isolates (DTO 341-E4, DTO 341-E5) from woodland soil in Australia. Interestingly, this mutation has previously been reported in *A. fumigatus* isolates of clinical origin and is thought to be acquired following therapeutic azole exposure and to confer resistance to itraconazole, voriconazole, posaconazole, and isavuconazole (40), similar to our phenotype results. However, this is not clear, as other single point mutations previously associated with in-host resistance development have also since been found in the environment (e.g., M220 and G54) (46, 47). Strains with these two mutations also harbored the T215S mutation, which has not previously been reported for *A. fumigatus* and which was not identified in any other isolates in this study. This mutation, in combination with G138C, may also contribute to acquired resistance; however, this was not supported by our *cyp51A* homology modeling of AVSC or *A. fumigatus* species. Additionally, it has previously been demonstrated via *A. fumigatus cyp51A* homology modeling that while G138C is closely positioned to the protein access channel opening, the mutation can actually increase stability for azole docking (48).

The mutation V101L was found in two *A. aureolus* isolates; however, only one of these sporulated adequately for antifungal susceptibility testing, demonstrating a posaconazole MIC at the *A. fumigatus* ECV. Mutations at this position in *A. fumigatus* have led to the change V101F, which, in association with M220I, confers multiazole resistance (itraconazole and posaconazole resistance with variable voriconazole resistance) (49). Mutations at M220 were not present in these or any other AVSC isolates. *cyp51A* homology modeling of these mutations in both the AVSC and *A. fumigatus* models did not confer changes consistent with azole resistance.

Nine *cyp51A* mutations present among all AVSC isolates (isolates with high and low MICs) compared to the sequence of the *A. fumigatus* WT (see Table S3 in the supplemental material) are likely nonfunctional in relation to azole-resistant phenotypes. Species-specific amino acid substitutions were also observed and could be interesting targets for identification purposes (at either the species or the complex level) or for the development of diagnostics, and the degenerate primers developed for this study have potential clinical application for other pathogenic, non-*fumigatus* *Aspergillus* species.

We found that isolates of *A. felis*, *A. frankstonensis*, *A. pseudoviridinutans*, *A. udagawae*, *A. viridinutans*, and *A. wyomingensis* were very competent in producing secondary metabolites (small-molecule extrolites). Some of these extrolites play a role in the infection process (50). Fumagillin and pseurotin A are also produced by *A. fumigatus*, and these two different types of extrolites are based on intertwined gene clusters (51) and may play a role in pathogenesis (50, 52, 53). While gliotoxin was not found in *A. felis* or *A. udagawae*, the latter species shared either helvolic acid, fumigatins, and fumitremorgins with *A. fumigatus* (52). There was a tendency for itraconazole-resistant strains to produce antafumicins and clavotols, at least within *A. felis*; however, two strains producing these extrolites were sensitive to itraconazole. Several studies have shown that the secondary metabolites produced by some filamentous fungi confer antifungal resistance (54). Here, we could find no correlation between the patterns of extrolites produced and the azole susceptibility of individual isolates. Additionally, none of the extrolites produced had a structure similar to that of the azoles. Nonetheless, the extrolites found could be further investigated, as previous studies looking at extrolite products in a limited number of isolates from the complex have demonstrated that they have unique properties, including antibacterial and anticancer activities (52, 55–58).

Other mechanisms of innate and acquired azole resistance should be further investigated as possible causes of AVSC resistance. Exploration of other resistance-associated genes in *A. fumigatus*, such as the *cyp51B* and genes involved in transcription, could help determine the cause of high azole MICs in AVSC isolates. The overexpression and induction of *cyp51B* were previously identified to be the most likely cause of azole resistance in two *A. fumigatus* isolates (30). Many other genes associated with reduced azole susceptibility to azole resistance in *A. fumigatus* could be explored in these species, including the gene for a truncated transcriptional regulator protein,

*afyap1* (59, 60), and *aldA* (60), as could loss of the protein encoded by *alga* and a mutation in *A. fumigatus* *cox10* resulting in decreased azole absorption (61). Additionally, overexpression of the drug efflux mediator ATP-binding cassette transporter gene *atrF*, identified in azole-resistant *A. fumigatus* isolates (62–64), could be explored. Other genes that could be investigated include the *hapE* gene with the P88L mutation (*hapE* encodes a binding transcription factor complex subunit of the CCAAT binding complex [CBC]), shown to interfere with the binding of CBC to the target site (65), and the *A. fumigatus* multidrug resistance efflux pump genes *MDR3* and *MDR4*, whose overexpression has been associated with the induction of itraconazole resistance (63, 66). The sterol regulatory element binding protein *SrbA*, a transcriptional regulator in connection with the inactivation of the CBC, has been associated with the TR<sub>3,4</sub> resistance mechanism in *A. fumigatus* by increasing sterol levels (65); however, it is less likely to be involved in the development of resistance in AVSC isolates, as TR<sub>3,4</sub> was not present.

The amplification of *cyp51A* in members of the AVSC identified a number of nonsynonymous mutations within the group compared to the reference sequence for *A. fumigatus*; however, no mutations related to environmentally acquired resistance in *A. fumigatus* were identified. The single point mutation G138C, previously identified to confer multi- and pan-azole resistance in *A. fumigatus* clinical isolates associated with therapeutic exposure to azoles, was found in two environmental *A. felis* isolates (from woodland soil in Australia). *cyp51A* homology modeling of mutations found only in isolates with high azole MICs compared to the sequences of isolates with low azole MICs did not show a significant change in the overall protein structure or direct interference of the mutation with azole drug binding to confer resistance. Analysis of mutations in the *cyp51A* gene provides good phylogenetic information on the AVSC, but these mutations are unlikely to be a cause of azole resistance in isolates with high MICs of one or more azole drugs (95% of AVSC isolates tested). The mechanisms of innate resistance and other causes of acquired azole resistance should be further investigated for the AVSC. The growth of all AVSC isolates was inhibited by luliconazole and olorofim (F901318) on CLSI antifungal susceptibility testing, indicating that they show promise as potential therapeutic options.

## MATERIALS AND METHODS

**Isolates.** Fifty-six archived AVSC isolates were retrieved from the Fungal Culture Biobank (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands ( $n = 55$ ), and from the National Mycology Reference Centre at SA Pathology, Adelaide, South Australia ( $n = 1$ ). This included 6 human isolates, 20 animal isolates, 25 environmental isolates, and 5 isolates of unknown origin (see Table S1 in the supplemental material).

**Antifungal susceptibility testing.** Antifungal susceptibility testing was performed using the guidelines in the M38-A3 document of CLSI for the broth microdilution antifungal susceptibility method ( $n = 37$ ) and MIC determination (67). Nineteen isolates failed to sporulate well enough to meet standardized conidial densities for inoculation; however, the *cyp51A* sequences were still determined for these isolates. Further, luliconazole (Nihon Nohyaku Co., Osaka, Japan) and olorofim (F2G, Manchester, UK) were dispensed into 96-well microtiter plates at a final concentration of 0.001 to 1  $\mu\text{g}/\text{ml}$ . The final concentration of dimethyl sulfoxide in the test wells was  $\leq 1\%$ . As clinical breakpoints have not yet been assigned to AVSC species, interpretation of MICs did not include designation of isolates as susceptible or resistant. Instead, MICs were interpreted as high or low (17), informed by established epidemiological cutoff values (ECVs) for *Aspergillus fumigatus* (ECVs of itraconazole, voriconazole, and isavuconazole, 1  $\mu\text{g}/\text{ml}$ ; ECV of posaconazole, 0.5  $\mu\text{g}/\text{ml}$ ) (68, 69). Luliconazole and olorofim (F901318) MIC values were not assigned as susceptible or resistant, as neither clinical breakpoints nor ECVs have been established for these compounds against *Aspergillus* species, so instead, only MICs are reported.

Geometric mean, range, MIC<sub>50</sub>, and MIC<sub>90</sub> values were calculated in Microsoft Office Excel (2013) software. Standard deviations and *t* test scores were calculated to determine if the triazole MICs for clinical isolates were higher than those for environmental isolates, with significance, determined using a two-tailed test, set at a *P* value of  $< 0.05$  (data not shown).

***cyp51A* gene amplification and sequencing.** For all isolates, DNA extraction was performed using a MoBio DNA isolation kit (Sanbio B.V., Uden, the Netherlands). Multiple primer pairs were designed to amplify the *cyp51A* gene.

Primer design was based on 3 primer pairs previously used for amplification of the *cyp51A* gene in *A. fumigatus* by Chen et al. (2005): CYP 1-L, CYP 1-R, CYP 2-L, CYP 2-R, CYP 3-L, and CYP 3-R (84). From these, degenerate primers were designed using MEGA, version 6 (MEGA6), software (70). The *cyp51A* gene was located in the whole-genome sequence of AVSC member *A. udagawae* IFM 46973 (71), available in GenBank (National Center for Biotechnology Information, Bethesda, MD, USA [<http://blast>



**TABLE 4** Primers used to target the *cyp51A* gene in AVSC members in this study

Primer sets for <i>cyp51A</i>	Primer pair	Sequence (5' to 3')
<i>Aspergillus udagawae</i>		
Forward 1	CYP0-F <sub>uda</sub> <sup>a</sup>	GACTTTCATATCTTGCTCAGC
Reverse 1	CYP1-R <sub>uda</sub> <sup>a</sup>	AGCCTTGAAAGTTCGGCGAG
Forward 2	CYP2-L <sup>b</sup>	CATGTGCCACTTATTGAGAAGG
Reverse 2	CYP2-R <sub>uda</sub> <sup>a</sup>	CCTTGCGCATGAGCGAGTGA
Forward 3	CYP3-L <sub>uda</sub> <sup>a</sup>	TTCCTCCGCTCCAGTACGAG
Reverse 3	CYP3-R <sub>uda</sub> <sup>a</sup>	CCTTTGATGTCCTCGATGAAA
Forward 4	CYP3-L <sub>uda</sub> <sup>a</sup>	TTCCTCCGCTCCAGTACGAG
Reverse 4	CYP4-R <sub>uda</sub> <sup>a</sup>	GATCGCACCGTGTCTTTG
Degenerate primers		
Forward 5	CYP0-F <sub>deg</sub> <sup>c</sup>	GRCKTTCAWATSTTGCTCAGC
Reverse 5	CYP1-R <sub>deg</sub> <sup>c</sup>	AGCCTTGAAARTTCGGYGAR
Forward 6	CYP2-L <sub>deg</sub> <sup>c</sup>	CATGTGCCACTYATYGAGAAGG
Reverse 6	CYP2-R <sub>deg</sub> <sup>c</sup>	CCTTGCGCATGAKMGAGTGA
Forward 7	CYP0-F <sub>deg</sub> <sup>c</sup>	GRCKTTCAWATSTTGCTCAGC
Reverse 7	CYP2-R <sub>deg</sub> <sup>c</sup>	CCTTGCGCATGAKMGAGTGA
Forward 8	CYP3-L <sup>b</sup>	TTCCTCCGCTCCAGTACAAG
Reverse 8	CYP4-R <sub>deg</sub> <sup>c</sup>	GATCRCACCRWRTCTTTG

<sup>a</sup>Primers identified from contig Aud0040 of *A. udagawae* (IFM 46973) (71) corresponding to the primers developed by Chen et al. (84).

<sup>b</sup>Primers used by Chen et al. (84).

<sup>c</sup>Degenerate primers developed for this study.

[ncbi.nlm.nih.gov/Blast.cgi](http://ncbi.nlm.nih.gov/Blast.cgi)) (accession numbers [BBXM01000001](#) to [BBXM01001029](#) and [BBXM01000040](#), region 670000-675000, contig Aud0040), and using MEGA6 software (70), a total of eight primer pairs were developed to amplify the *cyp51A* gene in AVSC members (Table 4). These were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Amplification was performed using conventional PCR in a Life Technologies Applied Biosystems thermal cycler (model 2720; Life Technologies Europe BV, Bleiswijk, the Netherlands) for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C; and then 5 min at 72°C and a hold at 10°C. In-house PCR product purification was performed in a Sensoquest lab cycler (Bioké, Leiden, the Netherlands), and Sanger sequencing was performed on a Hitachi/Applied Biosystems 3730xL genetic analyzer (Life Technologies [Thermo Fisher Scientific], USA).

The sequences were analyzed using the Seqman Pro tool in the DNASTAR program (version 12.1.0; Lasergene Molecular Biology and Lasergene Genomics, USA). Sequences were aligned in MEGA6 software using the MUSCLE alignment (70). Phylogenetic trees were constructed in MEGA6 software using the Kimura 2+ gamma distribution, the maximum likelihood discrete data method (tree searching method of 1,000 replicate trees), and bootstrapping (70).

Data were analyzed for mutations using the Fungal Resistance Database (FunResDB; The German National Reference Center for Invasive Fungal Infections, Jena, Germany), comparing the AVSC sequences with the sequence of the *A. fumigatus* reference strain with GenBank accession no. [AF338659](#) (12). Mutations present in isolates with demonstrated azole resistance were compared to those present in other isolates of the same species, as well as those present in all isolates in the complex.

***cyp51A* homology modeling.** The *A. fumigatus cyp51A* sequence (GenBank accession no. [AF338659](#)) was retrieved from Universal Protein Resources (<http://www.uniprot.org>), and a NCBI BLAST (blastp) search was performed to identify a suitable template. The crystal structure of *Saccharomyces cerevisiae cyp51A* (PDB accession number [5EQB](#)) was selected as the template for homology modeling, as this was the *cyp51A* protein that returned the highest sequence identity in the blastp search (resolution, 2.19 Å; maximum and total score, 506; query cover, 94%; identity, 51%). Five homology models were produced based on the template using Schrödinger's Prime program (72, 73), and the best model (model B) was selected after testing the quality of the models with the Verify 3D program (84.66% of the residues scored a three dimensional-one dimensional score of  $\geq 0.2$ ; Table S4) (74, 75). Homology models of *A. felis* (model F), *A. viridinutans* (model G), and *A. aureolus* (model H) were built on the basis of model B using the *cyp51A* amino acid sequences of isolates DTO 131-G1, DTO 050-F1, and DTO 278-B7, respectively (GenBank accession numbers are provided below, and the results obtained with the Verify 3D program are shown in Table S4). The models were verified with the Verify 3D program. Additionally, model F, the low-azole-MIC *A. felis* azole homology model, was subjected to mutations present in three *A. felis* isolates demonstrating high azole MICs (mutations T215S/G138C [model I] and S197C/Q340R [model J]; see Table 2 for isolate details). Finally, for models containing mutations at positions previously associated with azole resistance in *A. fumigatus* isolates (models H [V101] and I [G138]), mutations were added to *A. fumigatus* model B, producing model K (mutations from model I, G138C/T215S) and model L (mutations from model H, V101L/A103T/A234V/I360V/V428I/G505R/Q423D/F478V).

**Molecular docking and molecular mechanics-generalized Born surface area.** Models F to L were prepared for molecular docking using the Protein Preparation Wizard program (76). Two-dimensional (2D) structures of itraconazole, posaconazole, and voriconazole were first drawn using a 2D sketcher and

then prepared with the LigPrep program (Schrödinger release 2017-3). Docking studies for the three triazoles with these models were carried out via the Glide program with standard precision (SP) (77–79) to determine the binding affinity between the ligand and the receptor. For each model, conformers of the triazoles that interact with the protein with the expected binding mode in the SP study were chosen to carry on to the next step. They were subjected to minimization via the MacroModel program (Schrödinger release 2017-3), followed by the ConfGen program (80), to generate alternative low-energy conformations. A docking study for these conformers was then carried out via the Glide program with extra precision (XP) (77–79). Only the conformers with the best docking score in the XP analysis were taken into consideration. The docking scores obtained from the Glide XP analysis of each azole-resistant receptor-ligand interaction were compared to those for their azole-susceptible counterpart. Since models G (based on *Aspergillus viridinutans*) and H (based on *Aspergillus aureolus*) do not have a corresponding low-azole-MIC model, they were also compared with model F. The results were interpreted to indicate that the more negative that the docking score value was, the better that the binding affinity was. The MMGBSA [ $\Delta G(\text{binding})$ ], ligand strain energy, and receptor strain energy of the ligand-receptor calculations were also determined using the Prime program (81), based on XP docking studies; residues within 20 Å from the ligand were set flexible. Default settings were used for all calculations.  $\Delta G(\text{binding})$  indicated how strong the binding was between the ligand and receptor, and the ligand and receptor strain energy indicated how much energy that the ligand and protein required to distort themselves into the appropriate binding pose.

**Root mean square deviation scores.** The differences between model F and azole-resistant models G to J were measured via superimposition and determination of RMSD scores for comparison of structural similarity.

**Extrolite profiling.** Extrolite extraction was performed on all isolates after growth on Czapek yeast agar and yeast extract sucrose agar at 25°C and 37°C for 7 days. Three agar plugs were extracted according to the agar plug extraction method of Smedsgaard (82). Extracts were analyzed using ultrahigh-performance liquid chromatography (UHPLC) with a diode array detector (Dionex Ultramate 3000 UHPLC), and the compounds were identified against an internal database of UV spectra and information in the literature (83). The extrolite standards reported by Nielsen et al. (83) were used.

**Data availability.** All sequences were submitted to GenBank and may be found under accession numbers MF178270 to MF178326 (<https://www.ncbi.nlm.nih.gov/popset/?term=1388592591>).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00632-19>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

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