The Aspergillus fumigatus Spindle Assembly Checkpoint components, sldA and sldB, play roles in maintenance of triazole susceptibility

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INTRODUCTION

The rise of triazole resistance in *Aspergillus fumigatus* is of increasing concern as infection with resistant isolates is associated with increased treatment failure. Much remains unknown concerning adaptation to antifungal stress and development of antifungal resistance, threatening the future of triazoles for medical use. Reversible protein phosphorylation through protein kinase activity is a ubiquitous post-translational modification that mediates many cellular processes in eukaryotic organisms, including fungi. *A. fumigatus* is predicted to encode 147 protein kinases, the majority of which are uncharacterized, and the influence of these kinases on triazole susceptibility and adaptation to triazole drugs remains largely unknown. Here, we sought to reveal the impact of each of the predicted protein kinases on susceptibility to medical, mold-active triazoles.

METHODS

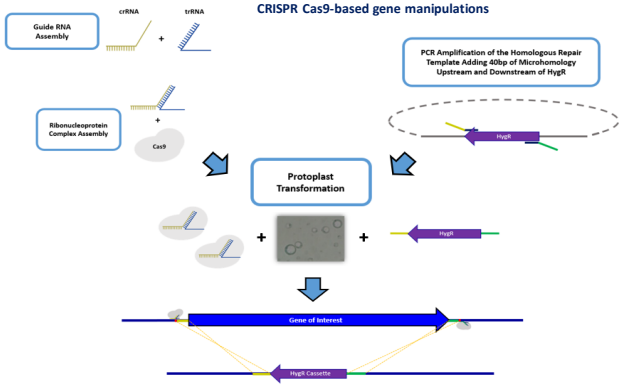
CRISPR/Cas9 gene editing was used to generate a library of 118 protein kinase disruption mutants in the wild type strain. Voriconazole minimum inhibitory concentrations (MIC) for the disruption strains were determined using CLSI broth microdilution assays and E-test. Screening revealed that disruption of the uncharacterized gene encoding the putative *sldA* kinase resulted in reduced susceptibility to the triazole antifungal voriconazole. This kinase is a direct ortholog of proteins involved in the cell cycle spindle assembly checkpoint of other eukaryotic organisms.[1] We sought to generate deletion strains lacking the entire gene coding sequence for *sldA* or that of a putative non-kinase binding partner, *sldB*. Targeted gene deletions and reconstitutions were performed by CRISPR/Cas9-mediated gene editing approaches. These mutants were re-examined for changes in growth or germination and for altered susceptibilities to a panel of mold-active triazoles, as well as the spindle poison benomyl. Culture for growth and stress analyses were carried out in standard *Aspergillus* minimal media.

Disruption of putative kinase genes impacts voriconazole susceptibility

MIC Assay. The minimum inhibitory concentration (MIC) to Voriconazole was determined for each strain by broth microdilution assays with liquid *Aspergillus* minimal media. MIC was considered the minimum concentration of azole that inhibited 100% of growth. MICs were determined in RPMI after 48h at 35°C, according to CLSI M38 document.[2] Representative results from multiple experiments are shown. Multiple putative kinase gene disruption strains exhibited altered susceptibility. Two mutants exhibited 4-fold increase in MIC over wild type; disruption of ssn3, which has been characterized previously, and the mutant *sldA*-1 which possesses disruption of a previously uncharacterized *A. fumigatus* gene which is predicted to encode a kinase ortholog of the *A. nidulans* SldA.

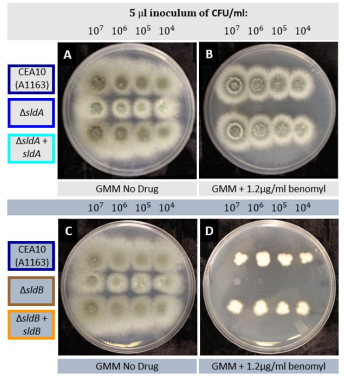
Gene ID	Kinase class	Predicted function	Voriconazole MIC
Wild Type CEA10	Na.	Na.	0.25ug/mL
npkA	CMGC/CDK	Cell Cycle, DNA Damage Response	0.25
chkA	CAMK	DNA Damage Response	0.25
chkB	CAMK	DNA Damage Response	0.25
tel1 / atmA	PIKK	DNA Damage Response	0.50
chkC	CAMK	DNA Damage Response	0.50
uvsB	PIKK	DNA Damage Response	0.50
ssn3	CDK	RNA Pol II Mediator	1.00
AFUB_074100	Other, S/T Kinase	(Cell cycle, Spindle Assembly Checkpoint, Putative)	1.00-2.00

CRISPR Cas9-based gene manipulations



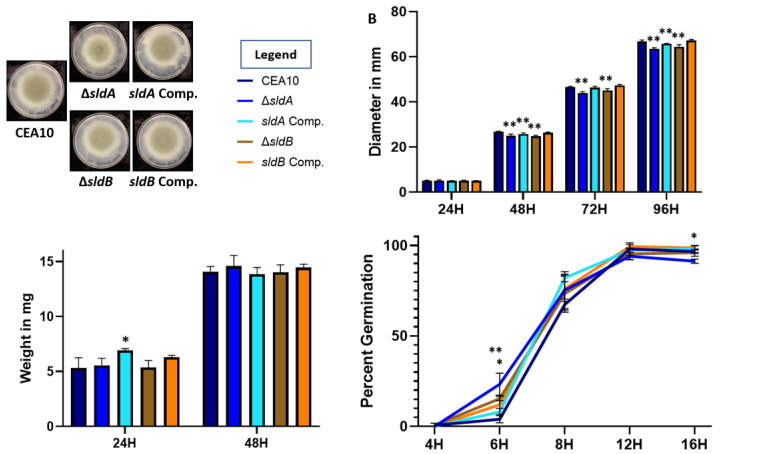
Construction of gene deletion mutants. The entire gene coding sequence for *sldA* or *sldB* was replaced by a homologous repair template providing hygromycin resistance. For each genetic manipulation, we designed two CRISPR RNAs (crRNA) one to target upstream of the Gene of Interest and one downstream. Guide RNA (gRNA) complexes were constructed in vitro by mixing equal amounts of one crRNA with transactivating CRISPR RNA (tracrRNA) in duplexing buffer and boiling the mixture at 95°C for 5 minutes. Ribonucleoprotein Complexes (RNP) were constructed by combining one gRNA complex with purified Cas9 enzyme and Cas9 duplexing buffer for 15 minutes at room temperature. Homology repair templates were constructed via PCR using primers which amplify the Hygromycin Resistance Cassette while adding 40bp homology upstream and downstream of the chosen locations for Cas9-generated DSB within the genome. For transformation, the CAS9-RNP complexes are both combined with repair template, CEA10 fungal protoplasts, and 60% PEG-CaCl2 and incubate for 50 minutes on ice. Transformation mix is plated on SMM overnight before addition of 150 µg/ml hygromycin to select for correct mutants.

Deletion of *sldA* or *sldB* results in increased susceptibility to benomyl



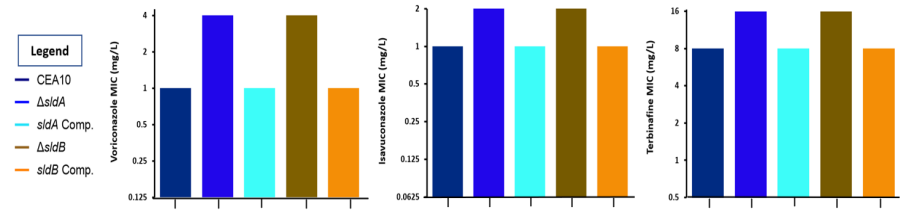
Spot Dilution Assay. *Aspergillus* minimal medium ± 1.2µg/ml benomyl. Strains and inoculums included are labeled. All plates were incubated for 72 hours at 37°C. (A) Representative image of strains wild type CEA10, deletion of sldA, and complementation of sldA at its native locus growing on solid minimal media without added drug. (B) Representative image of identical arrangement and inoculum of strains as in A, but with benomyl added to a final concentration of 1.2µg/mL. (C) Representative image of strains wild type CEA10, deletion of sldB, and complementation of sldB at its native locus growing on solid minimal media without added drug. (D) Representative image of identical arrangement and inoculum of strains as in C, but with benomyl added to a final concentration of 1.2µg/mL.

Loss of *sldA* or *sldB* does not result in vegetative growth defects or germination defects in *A. fumigatus*



Phenotypic characterization of *sldA* and *sldB* gene deletion mutants. (A) Comparison of growth of CEA10, Δ*sldA* and Δ*sldB* strains at 96H of growth on *Aspergillus* minimal medium. (B) Assay to measure radial growth in mm of colonies on solid *Aspergillus* minimal media over time. (C) Measure of total biomass accumulation in liquid *Aspergillus* minimal media over 24 and 48 hours. (D) Germination assay in liquid *Aspergillus* minimal media to monitor germination of strain conidia over time. For B-D, all results are presented as mean from three independent tests per strain. Error bars represent SD. One Way ANOVA with Tukey post hoc test. * = Significant at .05 ** = Significant at .01 *** = Significant at .001

Deletion of *sldA* or *sldB* results in reduced susceptibility to mold active antifungals



MIC Assay. *Aspergillus* minimal medium + Mold Active Antifungals. The minimum inhibitory concentration (MIC) to Voriconazole, Itraconazole and Terbinafine was determined for each strain by broth microdilution assays with liquid *Aspergillus* minimal media. MIC was considered the minimum concentration of azole that inhibited 100% of growth. MICs were determined in RPMI after 48h at 35°C, according to CLSI M38 document.[2] Representative results from multiple experiments are shown. Δ*sldA* and Δ*sldB* both reproduced the voriconazole susceptibility phenotype originally observed in the *sldA* disruption strain. Both strains also exhibited reduced susceptibility to itraconazole and terbinafine. The complemented strains however exhibit wild type susceptibility to each of these compounds.

CONCLUSIONS

- Loss of *A. fumigatus* genes *sldA* and *sldB* does not result in defective hyphal growth or conidial germination
- Loss of *A. fumigatus* genes *sldA* and *sldB* generates increased susceptibility to the spindle poison benomyl
- Loss of *A. fumigatus* genes *sldA* and *sldB* generates reduced susceptibility to triazole antifungals
- The previously uncharacterized *A. fumigatus* SldA kinase and its binding partner SldB likely play conserved roles in regulation of the SAC

1 Basenko, E. Y., J. A. Pulman, A. Shanmugasundram, O. S. Harb, K. Crouch, D. Starns, S. Warrenfeltz, C. Aurrecoechea, C. J. Stoeckert, Jr., J. C. Kissinger, D. S. Roos, and C. Hertz-Fowler. 2018. 'FungiDB: An Integrated Bioinformatic Resource for Fungi and Oomycetes', J Fungi (Basel), 4.

2 Clinical and Laboratory Standards Institute (2017). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved standard - Third edition. Document M38.