GAG, a polysaccharide cytotoxin?

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Background

Aspergillus fumigatus is a ubiquitous filamentous mold that causes necrotizing pneumonia in immunosuppressed individuals. A key virulence determinant of A. fumigatus is the synthesis of the adhesive, polycationic exopolysaccharide GAG, which is mediated by the products of a five gene cluster. One of these genes, *ega3,* encodes a cell membrane-anchored glycosyl hydrolase that is specific for deacetylated GAG.

Hypothesis

Cationic GAG is toxic to cell membranes and Ega3 protects fungal cells from the toxic effects of deacetylated GAG.

Methods

Genetic manipulations and transformation: All constructs were introduced by chemical transformation of *A. fumigatus* protoplasts. Deletion and complementation of *ega3* were done by CRISPR/Cas9. For deletion, two guide RNAs specific for *ega3* were used to direct cleavage by Cas9 in order to remove a segment of the gene. The hygromycin resistance cassette was used as a repair template. Complementation of *ega3* was done similarly using an *ega3* allele with a silent mutation and the phleomycin resistance cassette as a repair template. The tetracycline inducible *agd3* construct was produced by cloning the *agd3* locus into a plasmid containing the TetO7 operator. The *Δega3* mutant was then transformed with this plasmid.

RT-qPCR: RNA was extracted from indicated strains after 18 hours of growth and converted to cDNA using reverse transcriptase. Releative quantification of gene expression was measured using qPCR with *tef1* as a reference gene.

Western blot: Protein was extracted from indicated strains and loaded onto an SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and then detected using an anti-*ega3* primary antibody and HRP-conjugated secondary antibody.

Biofilm assay: Indicated strains were grown for 20 hours, washed, and adherent biomass was stained with crystal violet.

Deacetylated GAG detection: Culture supernatants of indicated strains were incubated in a high binding plate for 1 hour. After washing, adherent GAG was detected with biotinylated SBA-avidin HRP using TMB substrate for detection.

Methods

Growth rate determination: Conidia were inoculated in YPD medium with 0.1% Tween 80 on a 96 well plate. The plate was loaded into a Tecan plate reader incubated at 37ºC for 48 hours, measuring the optical density at 406nm was measured every hour.

ATP quantification in culture supernatants: Culture supernatants of the indicated strains were used as a substrate in the Invitrogen™ ATP Determination Kit as directed.

Chromium release assay: A549 cells were loaded with ^{51}Cr and incubated with the indicated culture supernatants for 20 hours. Cell damage was measured by ^{51}Cr release.

BMDM cell death quantification: Macrophages were differentiated from wild type mouse bone marrow then seeded on a 96 well plate. After allowing adherence overnight, culture supernatants of the indicated fungal strains plus propidium iodide were incubated with the cells for up to 24 hours. Propidium iodide fluorescence was measured at the indicated time points.

Results

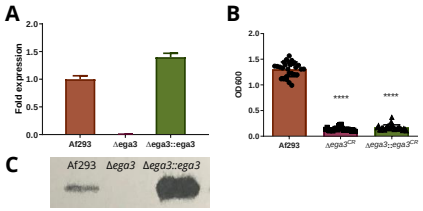

Figure 1: Deletion of Ega3 results in impaired biofilm formation that was not restored by complementation with a wild-type allele of *ega3*. A) Expression of *ega3* in the null mutant and complemented strain compared to Af293 wild type **B)** Biofilm formation of the null mutant and complemented strain **C)** Ega3 protein detection in the null mutant and complemented strain.

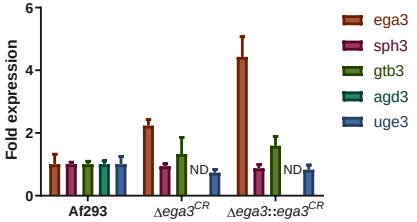

Figure 2: Expression of GAG cluster genes. The GAG deacetylase gene *agd3* is not expressed in *Δega3.*

Results

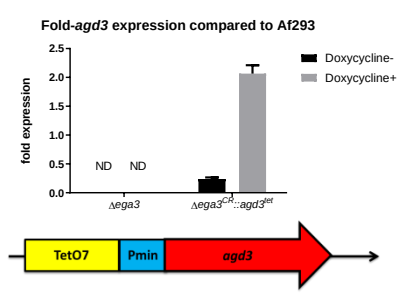

Figure 3: Expression of tetracycline-inducible *agd3* in *Δega3*. A) Expression of *agd3* with and without doxycycline **B)** Graphical representation of the construct used to transform *Δega3.*

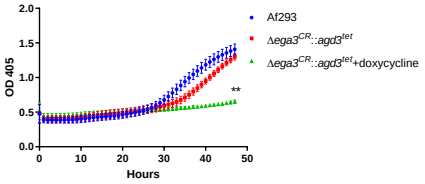

Figure 4: Expression of *agd3* in *Δega3* results in growth inhibition. The growth rates of the indicated strains were compared using a plate-reader based assay over the course of 48 hours. Error bars represent standard deviation. ** indicates a significant difference (p<.01) as determined by one-way ANOVA with Dunnett's multiple comparison test. N=3.

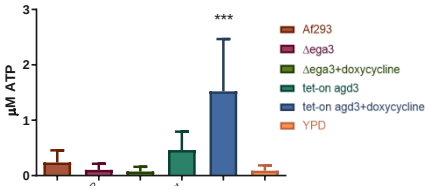

Figure 5: Expression of *agd3* in *Δega3* induces efflux of cytosolic contents. Detection of ATP in the culture supernatants of the indicated strains was used as a measure of cytosolic efflux.

Results

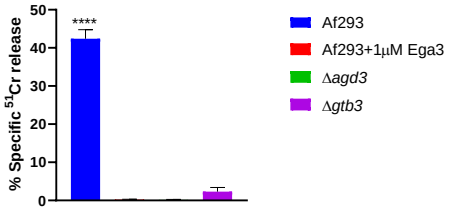

Figure 7: Deacetylated GAG damages A549 pulmonary epithelial cells. ^{51}Cr release was used as a measure of cell damage. Addition of 1μM recombinant Ega3 hydrolase domain prevented cell damage. Each bar represents the mean of at three independent experiments performed in triplicate with error bars indicating SEM. **** indicates p<0.0001.

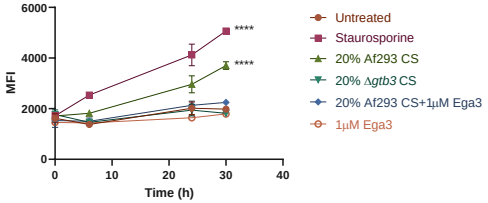

Figure 8: Deacetylated GAG causes BMDM cell death. Cell death was prevented by addition of 1μM recombinant Ega3 hydrolase domain. Means and standard deviations of measurements from 3 independent experiments were compared in a two way ANOVA using Tukey's multiple comparisons test. **** indicates a significant difference (p< 0.0001) compared to untreated. n=3.

Conclusions

- Deacetylated GAG is toxic to fungal and host cell membranes
- Ega3 acts as an antitoxin to protect *A. fumigatus* from GAG-induced injury
- GAG may be the first described member of a class of microbial polysaccharide toxins

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