

1 **An Alanine Aminotransferase is Required for Polysaccharide Regulation and Resistance**  
2 **of *Aspergillus fumigatus* Biofilms to Echinocandin Treatment**

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19 metabolism, echinocandins, antifungal drugs

20 **Abstract**

21 Alanine metabolism has been suggested as an adaptation strategy to oxygen limitation in  
22 organisms ranging from plants to mammals. Within the pulmonary infection microenvironment *A.*  
23 *fumigatus* forms biofilms with steep oxygen gradients defined by regions of oxygen limitation. A  
24 significant increase in alanine levels was observed in *A. fumigatus* cultured under oxygen limiting  
25 conditions. An alanine aminotransferase, AlaA, was observed to function in alanine catabolism  
26 and is required for several aspects of *A. fumigatus* biofilm physiology. Loss of *alaA*, or its catalytic  
27 activity, results in decreased adherence of biofilms through a defect in the maturation of the  
28 extracellular matrix polysaccharide galactosaminogalactan (GAG). Additionally, exposure of cell  
29 wall polysaccharides is also impacted by loss of *alaA* and loss of AlaA catalytic activity confers  
30 increased biofilm susceptibility to echinocandin treatment which is correlated with enhanced  
31 fungicidal activity. The increase in echinocandin susceptibility is specific to biofilms and chemical  
32 inhibition of *alaA* by the alanine aminotransferase inhibitor  $\beta$ -chloro-L-alanine is sufficient to  
33 sensitize *A. fumigatus* biofilms to echinocandin treatment. Finally, loss of *alaA* increases  
34 susceptibility of *A. fumigatus* to *in vivo* echinocandin treatment in a murine model of invasive  
35 pulmonary aspergillosis. Our results provide insight into the interplay of metabolism, biofilm  
36 formation, and antifungal drug resistance in *A. fumigatus* and describes a mechanism of  
37 increasing susceptibility of *A. fumigatus* biofilms to the echinocandin class of antifungal drugs.

38 **eLife Digest**

39 *Aspergillus fumigatus* is a ubiquitous filamentous fungus that causes an array of diseases  
40 depending on the immune status of an individual, collectively termed aspergillosis. Antifungal  
41 therapy for invasive pulmonary aspergillosis (IPA) or chronic pulmonary aspergillosis (CPA) is  
42 limited and too often ineffective. This is in part due to *A. fumigatus* biofilm formation within the  
43 infection environment and the resulting emergent properties, particularly increased antifungal  
44 resistance. Thus, insights into biofilm formation and mechanisms driving increased antifungal  
45 drug resistance are critical for improving existing therapeutic strategies and development of  
46 novel antifungals. In this work, we describe an unexpected observation where alanine  
47 metabolism, via the alanine aminotransferase AlaA, is required for several aspects of *A.*  
48 *fumigatus* biofilm physiology including resistance of *A. fumigatus* biofilms to the echinocandin  
49 class of antifungal drugs. Importantly, we observed that chemical inhibition of alanine  
50 aminotransferases is sufficient to increase echinocandin susceptibility and that loss of *alaA*  
51 increases susceptibility to echinocandin treatment in a murine model of IPA.

## 52 **Introduction**

53 *Aspergillus fumigatus* is a ubiquitous filamentous fungus with a prominent ecological role  
54 in the decomposition of organic carbon, that is easily isolated from compost piles and similar  
55 environments (Gugnani, 2003). Within compost piles a complex set of microenvironments can  
56 emerge along temperature and nutrient gradients that naturally form as saprophytes become  
57 metabolically active (Di Piazza et al., 2020; Sánchez et al., 2017). Thus, *A. fumigatus* has evolved  
58 a significant degree of metabolic flexibility and thermotolerance (Bhabhra & Askew, 2005; Ries et  
59 al., 2018). However, these saprophytic fitness traits also increase the fungus' pathogenic potential  
60 leading to *A. fumigatus* being the causative agent of a variety of immune-status dependent human  
61 diseases (Casadevall, 2017; Kanj et al., 2018; Robert & Casadevall, 2009), with the most lethal  
62 disease manifestation being Invasive Pulmonary Aspergillosis (IPA).

63 IPA occurs primarily in individuals with a suppressed innate immune system, such as  
64 individuals undergoing solid-organ transplantation or chemotherapy (Kanj et al., 2018; Kousha et  
65 al., 2011) Tragically, antifungal therapy options for IPA remain limited and are often ineffective,  
66 with recent clinical trials reporting 12-week mortality rates of 28-45% depending on therapeutic  
67 regimen and host immune status (Herbrecht et al., 2015; Maertens et al., 2016, 2021; Marr et al.,  
68 2015). One class of antifungal drugs, the echinocandins, inhibits synthesis of cell wall  $\beta$ -glucans  
69 and are better tolerated by patients than drugs belonging to the azole or polyene classes of  
70 antifungals. In many pathogenic yeasts, such as *Candida albicans*, echinocandin treatment has  
71 fungicidal activity and is utilized as a first-line treatment. While echinocandins yield some level of  
72 cell lysis when applied to *A. fumigatus* hyphae, these drugs are primarily fungistatic against *A.*  
73 *fumigatus* as it is intrinsically tolerant to echinocandins and will exhibit residual growth even at  
74 high concentrations of drug (Moreno-Velásquez et al., 2017). In many cases this tolerance results  
75 in a paradoxical phenomenon where the fungus will recover growth as the concentration of drug  
76 increases beyond a minimal effective concentration (MEC) (Aruanno et al., 2019; Moreno-

77 Velásquez et al., 2017; Wagener & Loiko, 2018). Thus, echinocandins have primarily been utilized  
78 as a salvage therapy for IPA and strategies to increase their efficacy in treatment of IPA are  
79 potentially of great clinical significance.

80 Recent studies have shown that *A. fumigatus* forms robust biofilms within the infection  
81 environment (Kowalski et al., 2019; Loussert et al., 2010). The hyphae within the *A. fumigatus*  
82 biofilm are coated with the extracellular matrix polysaccharide galactosaminogalactan (GAG),  
83 which is composed of a heterogenous mixture of galactose and N-acetylgalactosamine. GAG  
84 functions as a primary adherence factor for *A. fumigatus* biofilms, as well as an  
85 immunomodulatory compound (Fontaine et al., 2011; Gravelat et al., 2013; Lee et al., 2015; Speth  
86 et al., 2019). After synthesis GAG requires partial deacetylation via the Agd3 deacetylase to  
87 function in both capacities and strains lacking the ability to either produce GAG or deacetylate  
88 GAG are unable to adhere to surfaces (Bamford et al., 2020; Gressler et al., 2019; Lee et al.,  
89 2016). While some transcriptional regulatory machinery surrounding GAG biosynthesis and  
90 maturation has been described, mechanisms underlying biofilm formation and ECM regulation  
91 remain to be fully defined (Chen et al., 2020; Gravelat et al., 2013).

92 Insights into *A. fumigatus* biofilm formation are of great importance as these biofilms have  
93 been shown to display clinically relevant emergent properties, including increased resistance to  
94 antifungal drugs (Kowalski et al., 2020; Mowat et al., 2008; Seidler et al., 2008). A major factor  
95 contributing to increased drug resistance is the formation of oxygen limited, hypoxic,  
96 microenvironments within the biofilm (Kowalski et al., 2020, 2021). These same hypoxic  
97 microenvironments have been observed to exist in the infection environment and the ability to  
98 adapt to oxygen limitation is essential for disease progression and full virulence (Grahl et al.,  
99 2011; Willger et al., 2008). While some transcriptional regulators of *A. fumigatus* oxygen  
100 adaptation have been identified (Chung et al., 2014; Hagiwara et al., 2017; Willger et al., 2008),  
101 how the fungus metabolically adapts to low oxygen and how these metabolic pathways go on to

102 impact broader *A. fumigatus* physiology remain to be fully appreciated. In order to examine  
103 metabolic pathways that are potentially important for low oxygen adaptation we conducted a  
104 metabolomics experiment looking at the impact of acute exposure to oxygen limitation.

105 Analysis of metabolomics data described in this work, combined with published  
106 transcriptomics data, suggests a role for alanine metabolism in low oxygen adaptation (Barker et  
107 al., 2012; Chung et al., 2014; Hillmann et al., 2014; Losada et al., 2014). Alanine metabolism has  
108 been associated with adaptation to oxygen limitation in numerous organisms ranging from plant  
109 roots adapting to waterlogging (Lothier et al., 2020; Rocha et al., 2010) to exercise induced  
110 oxygen deprivation in muscle cells (Felig, 1973). Importantly, alanine is also one of the handful of  
111 amino acids detectable in human bronchoalveolar lavage (BAL) fluid and bronchial wash (BW)  
112 fluid, indicating it is readily available in the airway environment (Surowiec et al., 2016) and may  
113 serve as a potential carbon or nitrogen source for *A. fumigatus*. Here, we explore the role of  
114 fungal alanine metabolism via the alanine aminotransferase AlaA in *A. fumigatus*. Alanine  
115 aminotransferases catalyze the interconversion of pyruvate and alanine utilizing glutamate as an  
116 amino-group donor and thus participates in both carbon and nitrogen metabolism. While we  
117 observe that AlaA-mediated metabolic reactions are not essential for low oxygen growth, AlaA  
118 catalytic activity is critical for normal biofilm physiology where oxygen gradients naturally form.  
119 Moreover, AlaA is critical for growth and full fitness when alanine is the sole carbon or nitrogen  
120 source. Unexpectedly, *alaA* is required for maturation of the ECM polysaccharide GAG and  
121 exposure of cell wall polysaccharides. Furthermore, deletion or inhibition of AlaA results in a  
122 striking reduction of echinocandin resistance in *A. fumigatus* biofilms both *in vitro* and *in vivo*.

## 123 **Results**

### 124 **Acute exposure to low oxygen leads to the accumulation of carbohydrate metabolites and amino** 125 **acids.**

126 To examine the metabolic impact of acute exposure to oxygen limitation, fungal batch cultures  
127 were grown for 24 hours in ambient oxygen followed by either continued incubation at ambient O<sub>2</sub> or a 2-  
128 hour shift to an atmosphere of 0.2% O<sub>2</sub>. Intracellular metabolites were then extracted from biomass and  
129 relative quantities determined by LC-MS/MS. Acute exposure of fungal cultures to an oxygen limiting  
130 environment led to significant alterations in the relative abundance of 167 of 438 detected metabolites  
131 (38%) (Figure 1-figure supplement 1B, Supplemental File 1). Among these altered metabolites, an  
132 accumulation of several TCA cycle intermediates, lactate, and 4-amino-butyric acid (GABA) was  
133 observed. These metabolites are expected to increase during a rapid shift from a primarily oxidative  
134 metabolic state to a more fermentative metabolism (Figure 1A, Figure 1-figure supplement 1C). Other  
135 metabolites associated with fermentation such as acetate and ethanol were not detected via the method  
136 utilized, however other reports from similar culture conditions have observed ethanol fermentation as a  
137 major metabolic product during *A. fumigatus* growth in low oxygen conditions (Grahl et al., 2011).  
138 Additionally, decreased levels of glycolytic intermediates, in combination with increased intracellular  
139 trehalose content suggests a divergence of available carbon away from energy generation, in favor of  
140 carbon storage in the form of readily mobilized compounds, like trehalose (Figure 1-figure supplement  
141 1C).

142 Intriguingly, many metabolites related to nitrogen metabolism were increased. Specifically,  
143 alanine, GABA, glutamine, and several urea cycle intermediates (Figure 1A, Figure 1-figure supplement  
144 1D). This accumulation of nitrogen metabolites could be indicative of either nitrogen storage in the form  
145 of more favorable nitrogen sources, as is seen with carbon metabolism described above, and/or  
146 potentially indicative of nitrate fermentation as a strategy to recycle reducing potentials and allow  
147 glycolysis to continue. The accumulation of alanine was of particular interest due to an association of  
148 alanine and low oxygen adaptation in a wide-range of organisms including plants (Lothier et al., 2020;

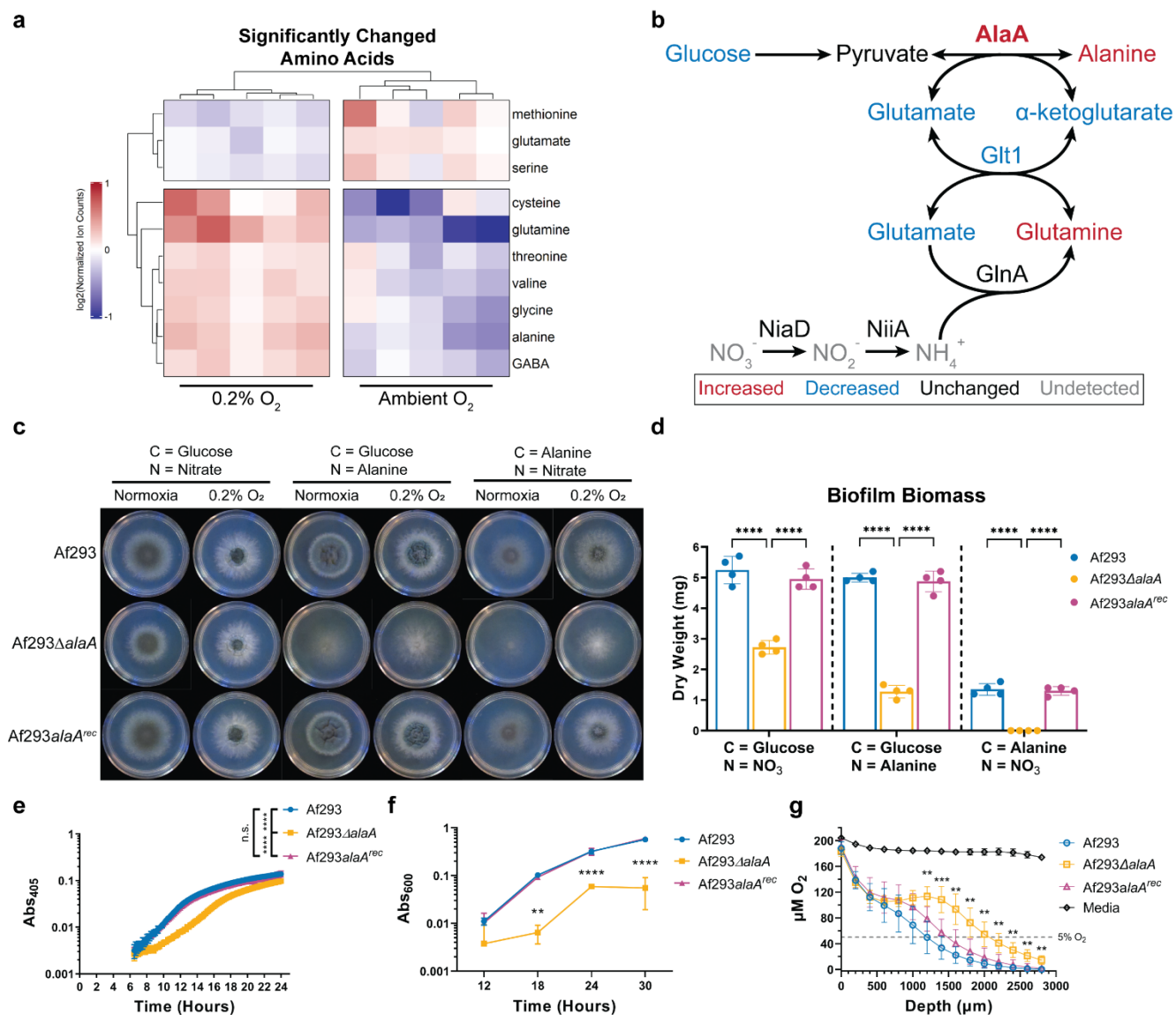
149 Rocha et al., 2010), crustaceans (Harrison, 2015), flies (Feala et al., 2007), and mammals (Felig et al.,  
150 1970). Additionally, several transcriptomic studies involving *A. fumigatus* and oxygen limitation have  
151 found that an alanine aminotransferase (Afu6g07770/AFUB\_073730) is highly increased in mRNA  
152 abundance upon exposure to low oxygen conditions (Figure 1B) (Barker et al., 2012; Chung et al., 2014;  
153 Hillmann et al., 2014; Losada et al., 2014). Thus, we further investigated the role of this alanine  
154 aminotransferase, herein named *alaA*, in *A. fumigatus* physiology.

155

### 156 **The alanine aminotransferase, *alaA*, is required for efficient catabolism of L-alanine.**

157 To assess the role of *alaA* in *A. fumigatus* metabolism and stress resistance, strains lacking the  
158 *alaA* gene were generated in the Af293 (Af293 $\Delta$ *alaA*) and CEA10 (CEA10 $\Delta$ *alaA*) backgrounds along with  
159 respective reconstituted strains in which *alaA* was ectopically re-introduced into the Af293 $\Delta$ *alaA* and  
160 CEA10 $\Delta$ *alaA* genomes under control of its native promoter (Af293*alaA*<sup>rec</sup> and CEA10*alaA*<sup>rec</sup>). Both  
161 Af293 $\Delta$ *alaA* and CEA10 $\Delta$ *alaA* colony biofilms grew on glucose minimal media (GMM), where glucose is  
162 the sole carbon source and nitrate is the sole nitrogen source, indicating that sufficient alanine was  
163 generated for colony biofilm growth independent of *alaA* under these *in vitro* conditions (Figure 1C, Figure  
164 1-figure supplement 2A). However, radial growth of the *alaA* null strains on solid GMM was approximately  
165 10% less than that of their respective WT and reconstituted strains at both ambient O<sub>2</sub> and 0.2% O<sub>2</sub>  
166 indicating a role for this protein in fungal metabolism in the presence of its preferred carbon source when  
167 growing as a colony biofilm (Figure 1C, Figure 1-figure supplement 2A). When the *alaA* null strains were  
168 grown with L-alanine as the sole carbon or sole nitrogen source the strains displayed severe colony  
169 biofilm growth defects (Figure 1C, Figure 1-figure supplement 2A). Surprisingly, both the wildtype (WT)  
170 and the *alaA* null strains grew more robustly at 0.2% O<sub>2</sub> than ambient O<sub>2</sub> when alanine was the sole  
171 carbon or nitrogen source, despite alanine being a non-fermentable carbon source (Figure 1C). Thus,  
172 *alaA* plays an important role in *A. fumigatus* metabolism in multiple carbon, nitrogen, and oxygen  
173 environments.





**Figure 1: An alanine aminotransferase is required for alanine catabolism and normal biofilm physiology.** A) Significantly changed amino acids upon acute exposure to a 0.2% oxygen environment. Ion counts were normalized to the mean ion count for each metabolite across all samples and  $\log_2$  transformed. Each column is a replicate ( $n = 5$  per condition). B) Reaction catalyzed by AlaA and its position in central carbon and nitrogen metabolism. Each metabolite and gene are color-coded according to their relative abundance upon exposure to a 0.2% oxygen environment. Metabolite data was obtained from the experiment in (A), and RNA-sequencing data was obtained from Chung, et al. 2014. C) Growth of *Af293 $\Delta$ alaA* on minimal media containing the indicated sole carbon and nitrogen sources in ambient oxygen (normoxia) and 0.2% oxygen environments. Images are representative of four replicate cultures. D) Dry biomass of biofilms grown in minimal media containing the indicated sole carbon and nitrogen sources for 24 hours ( $n = 4$ ). Each replicate is shown along with the mean  $\pm$  SD. E) Representative static growth assay of *Af293 $\Delta$ alaA* over 24 hours of biofilm growth ( $n = 6$  technical replicates). Experiment was repeated at least three times with similar results. F) Crystal violet adherence assay of biofilms grown for 12, 18, 24, and 30 hours ( $n = 3$ ). G) Oxygen concentration as a function of distance from the air-liquid interface in 24-hour biofilms ( $n \geq 7$ ). Culture volumes are approximately 3000 $\mu m$  in depth. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , n.s. = not significant by either Two-Way ANOVA with a Tukey's multiple comparison test (D, F, and G) or One-Way ANOVA with a Tukey's multiple comparison test (E). All graphs show the mean  $\pm$  SD unless otherwise stated.

175 **Robust adherence and growth of *A. fumigatus* biofilms is dependent on *alaA*.**

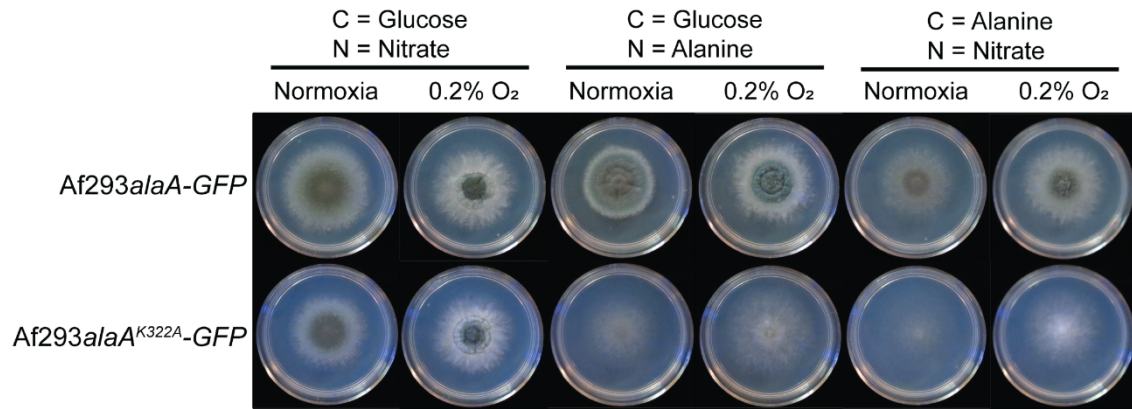
176 *A. fumigatus* submerged and colony biofilms naturally become increasingly oxygen deprived as  
177 they mature, albeit to different degrees where submerged biofilms form steeper oxygen gradients than  
178 colony biofilms (Kowalski et al., 2020, 2021). We next investigated the role of *alaA* in *A. fumigatus*  
179 submerged biofilms. To assess biofilm formation on GMM, and further quantify the role of AlaA in alanine  
180 metabolism, we quantified the dry biomass of submerged biofilms grown for 24 hours in GMM and with  
181 alanine as a sole carbon or nitrogen source. Loss of *alaA* resulted in a 40-50% decrease in submerged  
182 biofilm biomass in GMM. This growth defect was exacerbated when alanine was the sole carbon or  
183 nitrogen source, with no biomass recovered when alanine was the sole nitrogen source (Figure 1D).  
184 Additionally, we utilized a static growth assay to assess biofilm growth kinetics, which revealed that *alaA*  
185 null strains had a longer lag phase than their respective WT or reconstituted strains, indicative of a delay  
186 in conidial germination (Figure 1E, Figure 1-figure supplement 2B). To further determine if *alaA* had broad  
187 physiological impacts on *A. fumigatus* submerged biofilm formation, a crystal violet adherence assay was  
188 utilized to quantify adherence of the *alaA* null biofilms to abiotic surfaces. To account for any impacts of  
189 the germination delay on biofilm formation, the adherence of Af293Δ*alaA* was measured over a time  
190 course from an immature biofilm at 12 hours to a highly mature biofilm at 30 hours. At all timepoints after  
191 12 hours Af293Δ*alaA* had a severe defect in adherence compared to the WT and reconstituted strains  
192 (Figure 1F). CEA10Δ*alaA* was also tested for adherence and showed a similar inability to strongly adhere  
193 to surfaces (Figure 1-figure supplement 2C). Finally, we quantified oxygen levels within 24-hour biofilm  
194 cultures of Af293Δ*alaA*. The *alaA* null strain cultures were significantly more oxygenated than the WT  
195 and reconstituted strains' biofilms (Figure 1G). However, the portion of the culture containing the bulk of  
196 the biofilm's biomass, depth ~2000μm - 3000μm based on previous microscopy studies (Kowalski et al.,  
197 2020), was still below 5% O<sub>2</sub> and thereby experiencing hypoxia. Therefore, while the loss of *alaA* has an  
198 impact on colony biofilm growth, *alaA* appears to play a greater role in *A. fumigatus* submerged biofilm  
199 physiology where steep oxygen gradients naturally occur (Kowalski et al., 2020).

200 **Catalytic activity of AlaA is required for adherence and alanine growth, but not mitochondrial**  
201 **localization.**

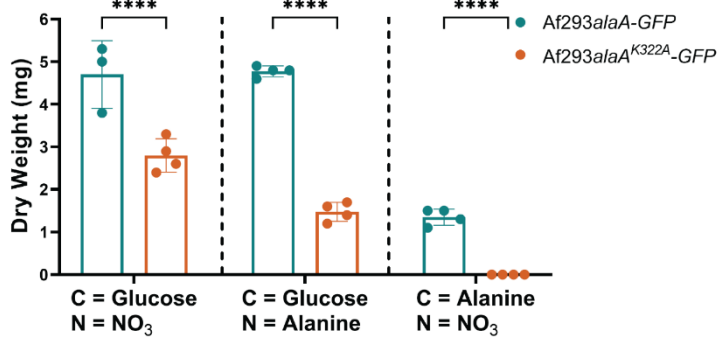
202 To begin determining how a gene involved in alanine metabolism is able to impact fungal  
203 metabolism and adherence, we generated a catalytically inactive allele of *alaA* to differentiate if the  
204 mechanism is through a moonlighting function of the protein or if it is through the catalyzed metabolic  
205 reaction. To generate a catalytically inactive allele, the conserved catalytic lysine residue at position 322  
206 (Figure 2-figure supplement 1) (Peña-Soler et al., 2014) was changed to an alanine and a C-terminal  
207 GFP tag was added. This construct was then transformed into the native locus of *alaA* in the Af293  
208 background (Af293*alaA*<sup>K322A</sup>-GFP). Additionally, the WT allele was modified with a C-terminal GFP tag  
209 and was transformed in the same manner (Af293*alaA*-GFP). Af293*alaA*-GFP and Af293*alaA*<sup>K322A</sup>-GFP  
210 grew on alanine as the sole carbon or sole nitrogen source in a manner similar to the WT and *alaA* null  
211 strains respectively, confirming that catalytic function was abolished and that the GFP tag did not interfere  
212 with protein function (Figure 2A, 2B). A crystal violet adherence assay revealed that Af293*alaA*<sup>K322A</sup>-GFP  
213 exhibited an adherence defect equivalent to the deletion of the entire *alaA* gene (Figure 2C). Confocal  
214 microscopy of these two strains in combination with Mitotracker™ Deep Red FM revealed that both the  
215 WT and catalytically inactive *alaA* alleles were stably expressed and localize to the mitochondria (Figure  
216 2D, Figure 2-figure supplement 2). Mitochondrial localization was surprising given that AlaA lacks a  
217 canonical mitochondrial localization signal and may suggest a role in mitochondrial function, as was found  
218 to be the case in tumor cells (Beuster et al., 2011). Therefore, AlaA catalytic activity is required for  
219 adherence and alanine catabolism, but not mitochondrial localization.

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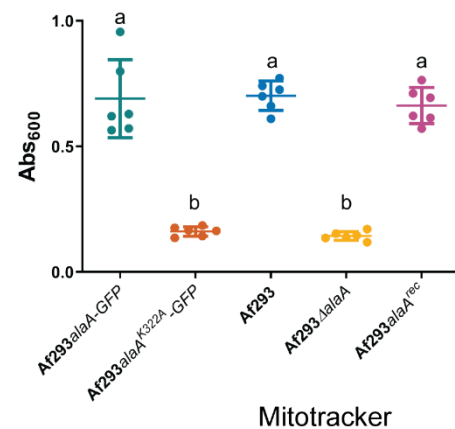
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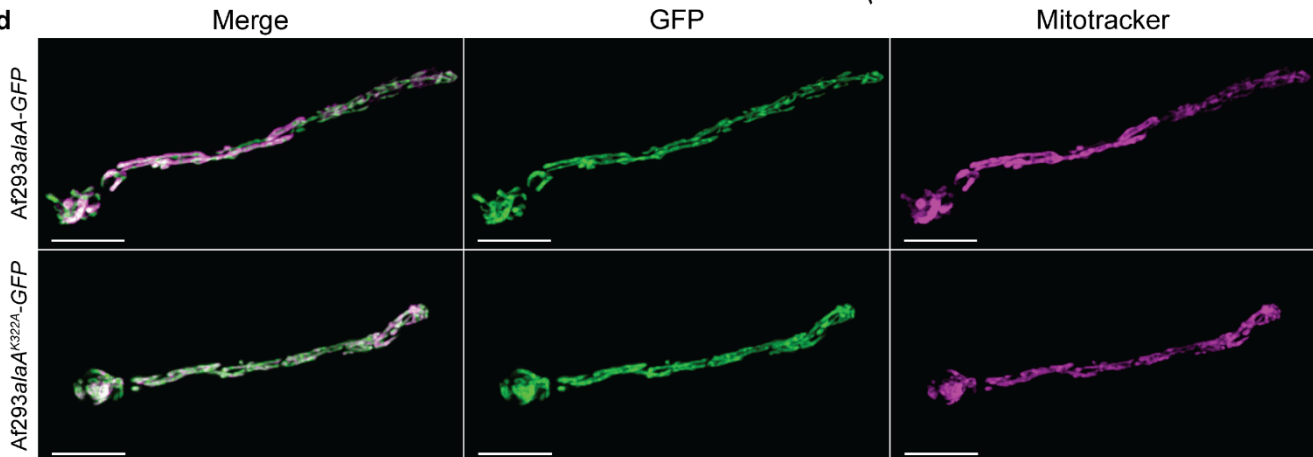
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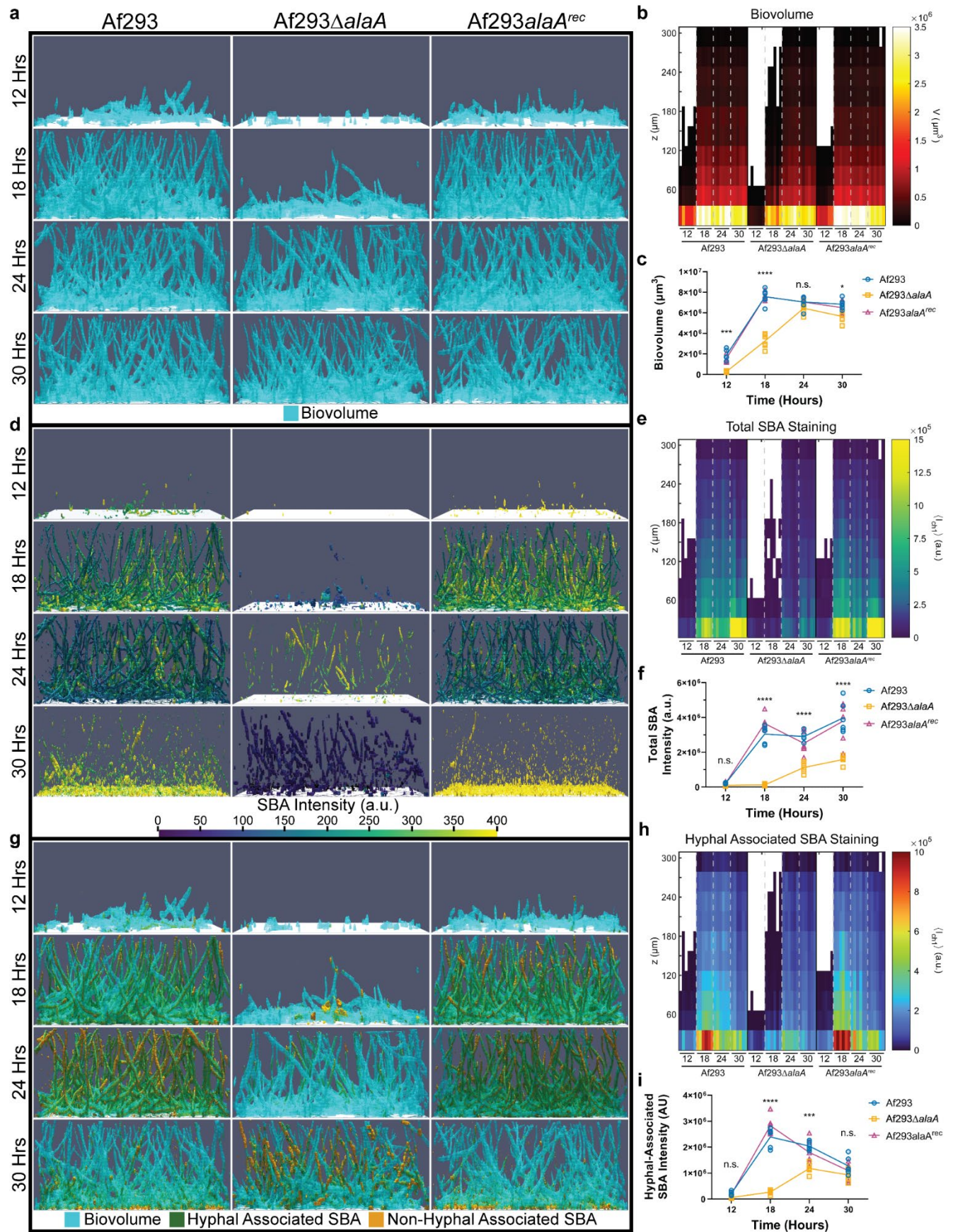
**Figure 2: Catalytic activity of AlaA is required for alanine catabolism and adherence of biofilms.** A) Growth of *Af293alaA<sup>K322A</sup>-GFP* on minimal media containing the indicated sole carbon and nitrogen sources in ambient oxygen (normoxia) and 0.2% oxygen environments. Images are representative of four replicate cultures. B) Dry biomass of biofilms grown in minimal media containing the indicated sole carbon and nitrogen sources ( $n \geq 3$ ). Each replicate along with the mean  $\pm$  SD are shown. \*\*\*\*  $p < 0.0001$  as determined by Two-Way ANOVA with a Tukey's multiple comparisons test. C) Crystal violet adherence assay of 24-hour biofilms ( $n = 6$ ). Each replicate along with the mean  $\pm$  SD are shown. a vs b  $p < 0.0001$  in all comparisons as determined by Two-Way ANOVA with a Tukey's multiple comparisons test. D) Representative micrographs of germlings containing C-terminal GFP tagged AlaA alleles (green) stained with Mitotracker™ Deep Red FM (magenta).

## 221 **Loss of *alaA* leads to alterations in the adherence mediating polysaccharide** 222 **galactosaminogalactan (GAG)**

223 The primary adherence factor for *A. fumigatus* submerged biofilms studied to date is the  
224 extracellular matrix polysaccharide galactosaminogalactan (GAG) (Gravelat et al., 2013; Lee et al., 2015,  
225 2016), and the lack of adherence observed in the *alaA* null strain suggests a role in GAG production or  
226 maturation. To examine GAG production, we first utilized a fluorescently labeled lectin specific to N-  
227 acetyl-D-galactosamine (GalNAc) residues found in the GAG polysaccharide, FITC-Soybean Agglutinin  
228 (SBA). Biofilms of Af293, Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> were stained with SBA to visualize GAG and  
229 calcofluor white, which binds chitin, to visualize biomass at 12, 18, 24, and 30 hours of growth (Figure  
230 3A, D, G). Spinning-disk confocal microscopy was utilized to image the first 300 $\mu$ m of the biofilms,  
231 followed by quantification using BiofilmQ (Hartmann et al., 2021). As seen in growth curve experiments  
232 (Figure 1F), Af293 $\Delta$ *alaA* had a lower biovolume at 12 and 18 hours (Figure 3A-C). Total SBA staining of  
233 the GAG polysaccharide was quantified as the sum intensity of the SBA stain in each image, revealing  
234 that Af293 $\Delta$ *alaA* biofilms had less total SBA staining than the WT and reconstituted strains starting at 18  
235 hours of growth (Figure 3D-F). While the SBA staining tightly associated with the cell wall at all timepoints  
236 in Af293 $\Delta$ *alaA*, at 30 hours in the WT and reconstituted strains the SBA staining pattern shifted from  
237 hyphal associated to primarily staining the extracellular milieu (Figure 3D, G). We quantified the hyphal  
238 associated SBA staining as the sum intensity of SBA stain that overlapped with the segmented calcofluor  
239 white stain, therefore showing GAG in relation only to hyphal biovolume. In the WT and reconstituted  
240 strain biofilms hyphal associated SBA peaked at 18 hours and decreased at 24 and 30 hours as matrix  
241 was shed from the hyphae into the extracellular milieu (Figure 3G-I). This was in contrast to total SBA  
242 staining, which remained relatively consistent from 18-30 hours of growth in the WT and reconstituted  
243 strains (Figure 3E).

244 To chemically define how GAG was being altered in the *alaA* null strain, monosaccharide analysis  
245 of ECM polysaccharides and an enzyme-linked lectin assay (ELLA) were conducted. Monosaccharide  
246 analysis revealed that the *alaA* null strain's ECM polysaccharide composition was similar to that of the

247 WT strain (Figure 4A). This finding suggests that the altered ECM is primarily due to a change in the  
248 maturation of the ECM polysaccharides rather than a difference in the base polysaccharides produced.  
249 After GAG has been synthesized, partial deacetylation by the Agd3 deacetylase is necessary for  
250 functional adherence (Bamford et al., 2020; Lee et al., 2016). To test if GAG maturation was altered, we  
251 utilized an ELLA in combination with treatment of the ECM by recombinant Agd3. In principle,  
252 deacetylated GAG in supernatants allows for adherence to the walls of a polystyrene plate, whereas fully  
253 acetylated GAG is unable to adhere and is easily removed by washing. Adherent, and therefore  
254 deacetylated, GAG can then be quantified by binding of a biotinylated SBA lectin coupled to a  
255 streptavidin-conjugated horseradish peroxidase. Additionally, the presence of fully acetylated GAG can  
256 be detected by pre-treating samples with recombinant Agd3, producing de-acetylated, adherent, GAG  
257 that can then be detected by SBA. A strain lacking the *agd3* gene, which only produces fully acetylated  
258 GAG (Lee et al., 2016), was utilized as a control. The *alaA* and *agd3* null strains both yielded low levels  
259 of adherent (deacetylated) GAG compared to the WT, and this was rescued by treatment of ECM with  
260 recombinant Agd3 protein (Figure 4B). Therefore, *alaA* is not required for GAG production, but rather is  
261 required for deacetylation and maturation of the GAG polysaccharide into its functional form. Finally,  
262 mRNA abundance of *uge3* and *agd3* was measured from RNA isolated from 24-hour biofilms of Af293,  
263 Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> to begin to distinguish if the observed differences in GAG are through a  
264 transcriptional or post-transcriptional mechanism of regulation. No differences in expression of *uge3* were  
265 observed (Figure 4C). While a statistically significant decrease of ~20% in *agd3* mRNA levels was  
266 observed (Figure 4D), it is unclear if that level of mRNA difference could cause the degree of altered  
267 GAG deacetylation observed. Thus, while loss of *alaA* has a modest impact on *agd3* at the transcriptional  
268 level, the impact of *alaA* on GAG maturation is likely to be primarily post-transcriptional.

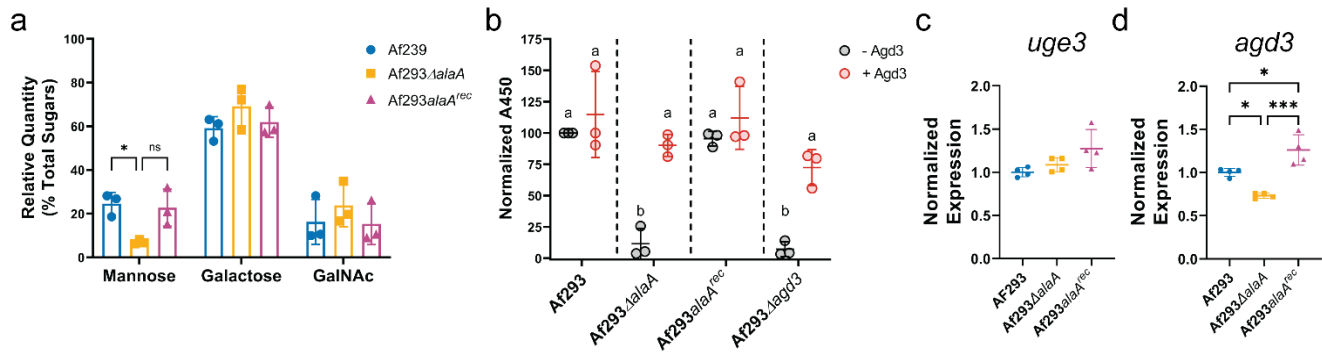


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**Figure 3: Loss of *alaA* alters extracellular matrix staining by the galactosaminogalactan binding lectin SBA.** A) Representative image renderings of biovolume in the first 300 $\mu$ m of biofilms grown for 12, 18, 24, and 30 hours. Biofilms were stained with calcofluor white and FITC-SBA followed by fixing with paraformaldehyde. Biovolume was determined by segmentation of the calcofluor white stain of each image. B) Heatmap of biovolume as a function of height from the base of the biofilm. C) Global segmented biovolume quantifications of each biofilm. D) Representative image renderings of FITC-SBA staining intensity corresponding to biomass images in (A). Renderings show FITC-SBA matrix intensity mapped onto the segmented FITC-SBA stain. E) Heatmap of FITC-SBA intensity as a function of height from the base of the biofilm. F) Sum intensity quantification of FITC-SBA staining for each biofilm. G) Representative merged image renderings of the segmented biovolume (calcofluor white), shown in blue, and segmented FITC-SBA stain shown in orange. Hyphal associated SBA staining will appear green as a result of the overlap between the two channels. SBA was considered hyphal-associated or non-hyphal associated based on overlap with the segmented biomass. H) Heatmap of hyphal associated FITC-SBA intensity as a function of height from the base of the biofilm. I) Sum intensity quantification of hyphal associated FITC-SBA staining for each biofilm. Each graph and heatmap shows the individual replicates for each timepoint (n = 6). For (C, F, and I), the line goes through the mean of each timepoint. \* p < 0.05, \*\*\* p < 0.001, \*\*\*\* p < 0.0001, n.s. = not significant as determined by Two-Way ANOVA with a Tukey's multiple comparison's test for (C, F, and I).



271



**Figure 4: *alaA* is required for proper deacetylation of galactosaminogalactan.** A) Monosaccharide analysis of extracellular matrix polysaccharides (n = 3). \* p < 0.05, n.s. = not significant by Two-Way ANOVA with a Tukey's multiple comparisons test. B) Enzyme linked lectin assay (ELLA) of biofilm extracellular matrix treated or untreated with recombinant Agd3. a vs b p < 0.05 for all comparisons as determined by Two-Way ANOVA with a Tukey's multiple comparisons test. C-D) Expression of *uge3* (C) and *agd3* (D) in 24-hour biofilm cultures as determined by RTqPCR. \* p < 0.05, \*\*\* p < 0.001 as determined by One-Way ANOVA with a Tukey's multiple comparisons test for C and D. For all graphs each replicate along with the mean +/- SD is shown.

272 **Deletion of *alaA* leads to cell wall changes and increased susceptibility of biofilms to**  
273 **echinocandins.**

274         Given that GAG maturation was substantially impacted by loss of *alaA* and that *alaA* plays a role  
275 in metabolism, we asked if loss of *alaA* impacts additional cell wall polysaccharides. To test this  
276 hypothesis, germlings of Af293, Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> were stained with calcofluor white (to  
277 measure total chitin), wheat-germ agglutinin (WGA) (to measure surface exposed chitin), and soluble  
278 Dectin-1 Fc (to measure surface exposed  $\beta$ -glucans). Curiously, Af293 $\Delta$ *alaA* germlings had lower WGA  
279 staining (exposed chitin), despite no difference in calcofluor white staining (total chitin) (Figure 5A-B).  
280 Loss of *alaA* also decreased exposure of the immunostimulatory  $\beta$ -glucan polysaccharide, as determined  
281 by Dectin-1 Fc staining (Figure 5C). These results were surprising, as it has been shown that GAG  
282 masks  $\beta$ -glucans, and perturbations to GAG synthesis (Gravelat et al., 2013) or maturation (Lee et al.,  
283 2016) normally result in higher levels of Dectin-1 Fc staining. Together this suggests Af293 $\Delta$ *alaA* has  
284 lower levels of total cell wall  $\beta$ -glucans and that *alaA* is required for WT cell wall organization.

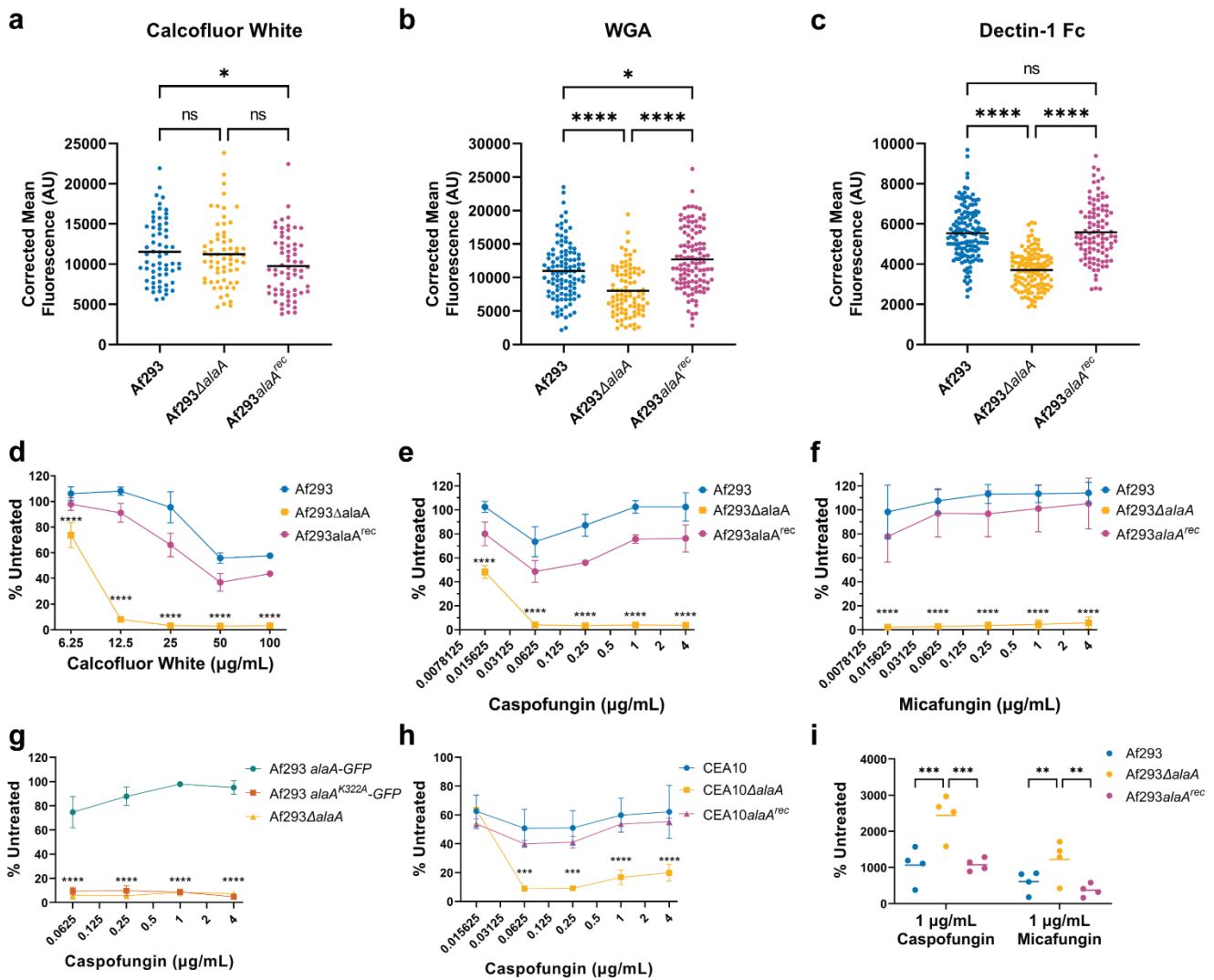
285         To determine if these cell wall changes translated to functional phenotypes, biofilms of Af293,  
286 Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> were tested for sensitivity to the cell wall perturbing agents calcofluor white  
287 and the echinocandin class of antifungal drugs. Biofilms were grown to maturity (24 hours) prior to  
288 application of cell wall stress for 3 hours. Viability was then compared to untreated biofilms via reduction  
289 of the metabolic dye XTT. Af293 $\Delta$ *alaA* biofilms were significantly more susceptible to damage by  
290 calcofluor white at all concentrations tested, with greater than 90% inhibition beginning at 12.5  $\mu$ g/mL  
291 (Figure 5D). In contrast, the WT and reconstituted strains maintained at least 30% viability at even the  
292 highest concentration tested (100  $\mu$ g/mL) (Figure 5D). We next tested the strains for susceptibility to the  
293 echinocandin caspofungin. The WT and reconstituted biofilms displayed minimal damage regardless of  
294 concentration. Quantification also reveals signs of the paradoxical effect in this system, where the fungus  
295 will recover growth as the concentration of drug increases beyond a MEC (Figure 5E). Thus, similar to  
296 MEC and agar colony biofilm plate assays that begin with conidia, mature WT *A. fumigatus* biofilms are  
297 caspofungin tolerant. However, *alaA* null biofilms were highly susceptible to caspofungin and reached

298 >90% inhibition at a concentration of 0.0625 µg/mL. Unlike WT biofilms, the *alaA* null biofilms did not  
299 display evidence of a paradoxical effect, with increasing concentrations of caspofungin yielding  
300 equivalent or greater damage (Figure 5E).

301 To test if this increased susceptibility to caspofungin extended to other echinocandins, these  
302 experiments were validated with another echinocandin, micafungin. Similar to caspofungin treatment, the  
303 biofilms of WT and reconstituted strains were highly resistant to treatment with micafungin, whereas  
304 Af293Δ*alaA* was inhibited >90% at even the lowest concentration of drug tested, 0.015625 µg/mL (Figure  
305 5F). The catalytically inactive strain (Af293*alaA*<sup>K322A</sup>) was also tested for caspofungin sensitivity and  
306 displayed the same phenotype as Af293Δ*alaA* (Figure 5G). Finally, to ensure this phenotype was not  
307 specific to the Af293 reference strain, these phenotypes were validated in another reference background,  
308 CEA10. CEA10, CEA10Δ*alaA*, and CEA10*alaA*<sup>rec</sup> biofilms were tested for susceptibility to caspofungin  
309 and again the loss of *alaA* resulted in increased echinocandin susceptibility (Figure 5H). Moreover, the  
310 increased susceptibility of the *alaA* null mutant was confirmed by measuring adenylylase kinase release  
311 (Didone et al., 2011). In brief, adenylylase kinase is normally present in very low quantities extracellularly  
312 and increased release of adenylylase kinase is indicative of cell lysis. Treatment of Af293Δ*alaA* biofilms  
313 with caspofungin or micafungin resulted in a greater release of adenylylase kinase into the supernatant,  
314 indicating that loss of *alaA* potentiates the fungicidal effects of echinocandins (Figure 5I, FIGURE 5-  
315 FIGURE SUPPLEMENT 1). Thus, the presence and catalytic activity of AlaA are required for the high  
316 level of echinocandin resistance observed in phylogenetically diverse *A. fumigatus* biofilms.

317 We next tested if the increased susceptibility of Af293Δ*alaA* to echinocandins was biofilm specific  
318 or if it extended to more traditional measures of drug resistance and tolerance that begin with exposing  
319 dormant conidia to the drug. Tolerance, or the ability to grow in the presence of a fixed concentration of  
320 drug, to caspofungin was measured using radial growth of conidia on agar plates at three concentrations  
321 of caspofungin (0.25 µg/mL, 1 µg/mL, and 4 µg/mL). No significant differences in colony biofilm growth  
322 were observed between the strains at any concentration tested on agar surfaces (Figure 5-figure  
323 supplement 2A-C). Additionally, the paradoxical effect was observed in all three strains tested, with

324 increased growth as the concentration of caspofungin increased. Intriguingly, no difference in resistance  
325 to caspofungin was observed when a minimal effective concentration (MEC) assay was utilized with  
326 conidia of Af293, Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> (Figure 5-figure supplement 2D). Therefore, the  
327 increased susceptibility of Af293 $\Delta$ *alaA* to echinocandins is a biofilm specific phenomenon, as no  
328 difference in resistance or tolerance to caspofungin is observed when the drug is applied to dormant  
329 conidia.



330

**Figure 5: Loss of *alaA* leads to cell wall changes and increased susceptibility of biofilms to echinocandins.**

Germlings were stained with calcofluor white to quantify total chitin content (A), FITC-wheat germ agglutinin (WGA) to quantify surface exposed chitin (B), and Dectin-1 Fc to quantify surface exposed  $\beta$ -glucans (C). Each data-point represents an individual germling across three independent cultures per strain for each cell wall stain and the lines correspond to the mean. \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ , n.s. = not significant as determined by One-Way ANOVA with a Tukey's multiple comparisons test. D-F) 24-hour biofilms were established in the absence of drug and treated with calcofluor white (D), caspofungin (E), or micafungin (F) at the indicated concentrations for 3 hours and viability was determined by XTT assay. Mean  $\pm$  SD are shown for  $n \geq 3$  for each experiment. \*\*\*\*  $p < 0.0001$  as determined by Two-Way ANOVA with a Tukey's multiple comparison test. The highest p-value for *Af293* $\Delta*alaA* compared to both *Af293* and *Af293**alaA*<sup>rec</sup> is shown. G) *Af293**alaA*<sup>K322A</sup>-GFP biofilms were grown for 24-hours, treated with caspofungin at the indicated concentrations for 3 hours, and viability was determined by XTT assay. Mean  $\pm$  SD are shown for  $n = 3$  replicates. ****  $p < 0.0001$  as determined by Two-Way ANOVA with a Tukey's multiple comparison test. The highest p-values for *Af293**alaA*<sup>K322A</sup>-GFP and *Af293* $\Delta$ *alaA* in comparison to *Af293**alaA*-GFP are shown. No significant difference was observed between *Af293**alaA*<sup>K322A</sup>-GFP and *Af293* $\Delta$ *alaA*. H) *CEA10* $\Delta$ *alaA* biofilms were grown for 24-hours, treated with caspofungin at the indicated concentrations for 3 hours, and viability was determined by XTT assay. Mean  $\pm$  SD are shown for  $n = 3$  replicates. ***  $p < 0.001$ , ****  $p < 0.0001$  as determined by Two-Way ANOVA with a Tukey's multiple comparison test. The highest p-values for *CEA10* $\Delta$ *alaA* compared to both *CEA10* and *CEA10**alaA*<sup>rec</sup> are shown. I) Adenylate kinase release assay as a quantification of cell lysis. 24-hour biofilms were treated with 1  $\mu$ g/mL of caspofungin (left) or micafungin (right) for 3-hours and supernatant adenylate kinase activity was quantified. Each replicate and the mean are shown ( $n = 4$ ). **  $p < 0.01$ , ***  $p < 0.001$  as determined by Two-Way ANOVA with a Tukey's post-test.$

331 **Chemical Inhibition of AlaA by  $\beta$ -chloro-L-alanine decreases adherence and increases**  
332 **susceptibility of *A. fumigatus* biofilms to caspofungin.**

333 Given the potential clinical significance of increasing *A. fumigatus* biofilm susceptibility to  
334 echinocandins, we next tested whether chemical inhibition of AlaA was sufficient to confer similar  
335 phenotypes observed in the null or catalytically inactive mutant strains. The chemical  $\beta$ -chloro-L-alanine  
336 has previously been shown to inhibit mammalian alanine aminotransferases (Beuster et al., 2011;  
337 Golichowski & Jenkins, 1978; Morino et al., 1979), and thus we tested if  $\beta$ -chloro-L-alanine treatment  
338 could recapitulate the adherence and caspofungin phenotypes observed in the *alaA* null strain. Af293,  
339 Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> were incubated with 10-fold increasing concentrations of  $\beta$ -chloro-L-  
340 alanine from 0.1 $\mu$ M to 1000 $\mu$ M and tested for adherence via crystal violet adherence assay. Increasing  
341 concentrations of  $\beta$ -chloro-L-alanine resulted in decreased adherence for Af293 and Af293*alaA*<sup>rec</sup>, with  
342 EC<sub>50</sub> values of 10.49  $\mu$ M and 15.90  $\mu$ M respectively (Figure 6A). At 100 $\mu$ M of  $\beta$ -chloro-L-alanine,  
343 adherence of the WT and reconstituted strains was inhibited to slightly above that of the *alaA* null strain.  
344 Importantly, adherence of the Af293 $\Delta$ *alaA* was unaltered by any concentration of  $\beta$ -chloro-L-alanine  
345 tested indicating some level of chemical specificity for AlaA (Figure 6A). Additionally, treatment of the  
346 GFP-tagged AlaA and catalytically inactive strains with  $\beta$ -chloro-L-alanine yielded similar results to the  
347 WT and *alaA* null strains, respectively, indicating that the compound is acting through the catalytic activity  
348 of the AlaA enzyme (Figure 6B).

349 To test if  $\beta$ -chloro-L-alanine treatment increases susceptibility of biofilms to echinocandins,  
350 biofilms were grown in the presence of 10 $\mu$ M and 100 $\mu$ M  $\beta$ -chloro-L-alanine to represent a range of  
351 values that encompass both the EC<sub>50</sub> value determined by the adherence assay results (10 $\mu$ M) and the  
352 concentration that yielded an *alaA* deletion-like phenotype (100 $\mu$ M) (Figure 6A). The  $\beta$ -chloro-L-alanine  
353 treated biofilms of Af293, Af293 $\Delta$ *alaA*, and Af293*alaA*<sup>rec</sup> were tested for sensitivity to caspofungin  
354 treatment. In the WT and reconstituted strains efficacy of caspofungin increased as the concentration of  
355  $\beta$ -chloro-L-alanine increased (Figure 6C). Curiously, even in the *alaA* null strain basal XTT reduction  
356 decreased as the concentration of  $\beta$ -chloro-L-alanine increased. However, the *alaA* null strain remained

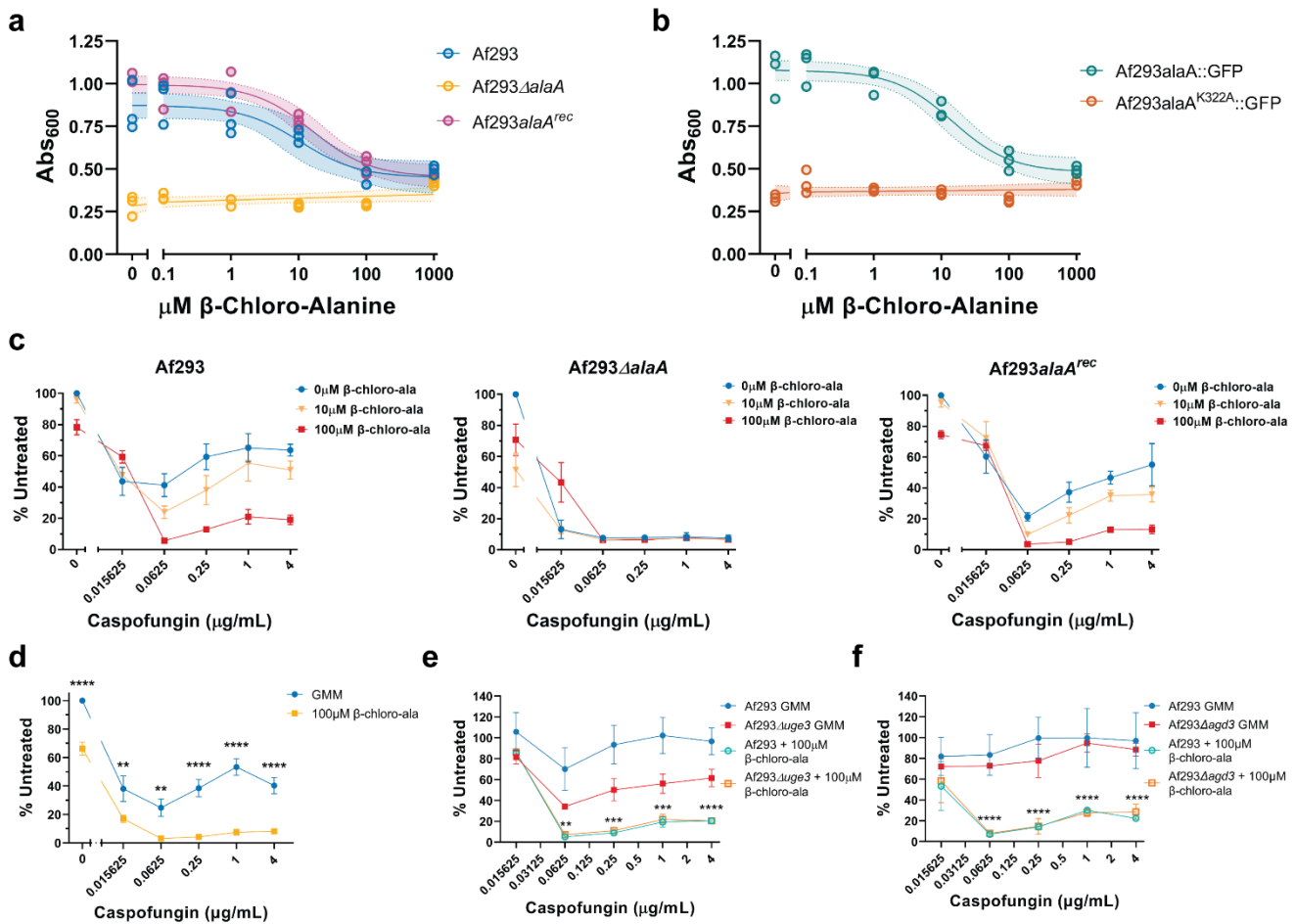
357 highly susceptible to caspofungin regardless of the concentration of  $\beta$ -chloro-L-alanine (Figure 6C).  
358 Additionally, treatment of CEA10 with  $\beta$ -chloro-L-alanine increased caspofungin susceptibility of biofilms  
359 validating that this is not specific to the Af293 strain background (Figure 6D). Together these data  
360 establish the proof of concept that chemical inhibition of AlaA is a possible strategy for increasing  
361 susceptibility of *A. fumigatus* biofilms to echinocandins.

362

### 363 **Altered extracellular matrix is not the primary factor impacting caspofungin susceptibility.**

364 In other fungi, the biofilm extracellular matrix has been shown to be a major factor in reducing  
365 antibiotic efficacy against biofilms (Taff et al., 2013). Therefore, we wanted to test if the increased  
366 susceptibility to caspofungin could be attributed to the altered GAG composition of the *alaA* null strain.  
367 To do this we utilized a strain lacking the UDP-glucose-4-epimerase required to produce GAG  
368 (Af293 $\Delta$ *uge3*) and a strain lacking the deacetylase required for the maturation of GAG (Af293 $\Delta$ *agd3*) in  
369 combination with  $\beta$ -chloro-L-alanine treatment (Gravelat et al., 2013; Lee et al., 2016). In an attempt to  
370 overcome the inability of Af293 $\Delta$ *uge3* and Af293 $\Delta$ *agd3* to adhere to abiotic surfaces (Gravelat et al.,  
371 2013; Lee et al., 2016), we performed experiments with these strains on collagen-coated tissue culture  
372 plates. Collagen is a mammalian extracellular matrix component abundant in the lung, and we observed  
373 that this treatment was sufficient to partially restore adherence of both strains, indicating the existence of  
374 GAG-independent mechanisms of adherence to alternative substrates found in mammalian lungs (Figure  
375 6-figure supplement 1). Af293, Af293 $\Delta$ *uge3*, and Af293 $\Delta$ *agd3* biofilms were established on collagen-  
376 coated plates with or without 100 $\mu$ M  $\beta$ -chloro-L-alanine and then subsequently treated with caspofungin.  
377 All three strains were highly susceptible to caspofungin when AlaA was inhibited by  $\beta$ -chloro-L-alanine  
378 (Figure 6E, 6F). Untreated Af293 $\Delta$ *uge3* was inhibited by caspofungin treatment to a greater extent than  
379 the WT strain. However, this increased susceptibility was far less severe than observed in  $\beta$ -chloro-L-  
380 alanine treated biofilms and was not observed in the deacetylase deficient strain (Af293 $\Delta$ *agd3*) (Figure  
381 6E). Therefore, GAG contributes to caspofungin resistance to some degree, but it is not the primary factor  
382 responsible for the increased susceptibility when AlaA is chemically inhibited or genetically altered.

383



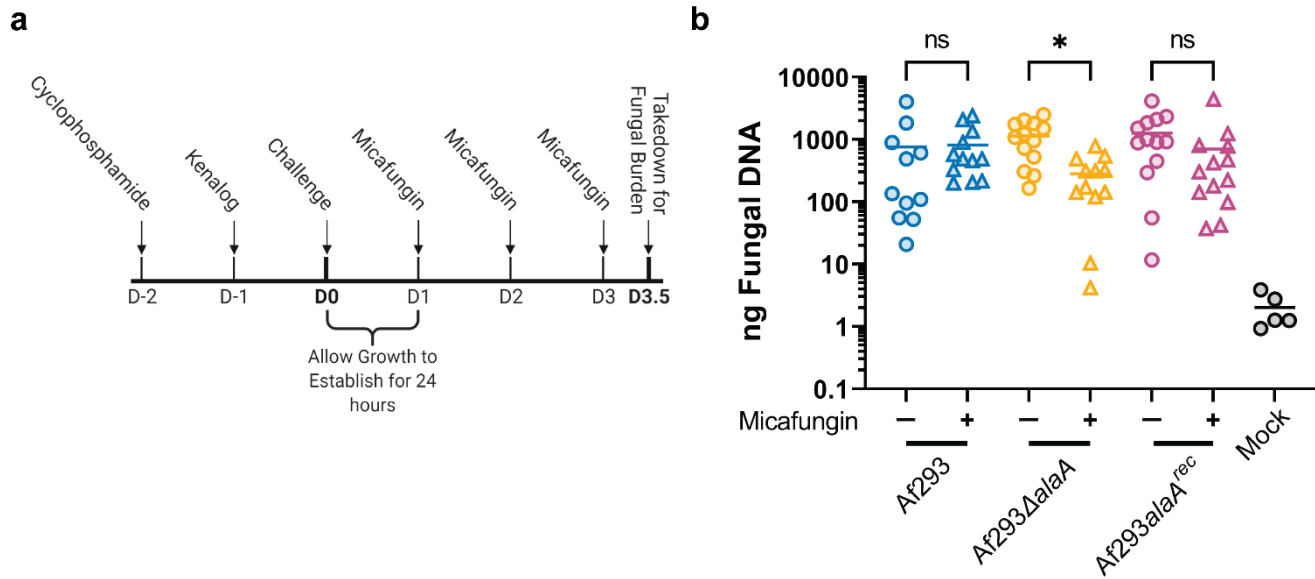
**Figure 6: Chemical inhibition of AlaA by β-chloro-L-alanine is sufficient to decrease adherence and increase susceptibility of biofilms to caspofungin.** A-B) Crystal violet adherence assay of 24-hour biofilms grown in the presence of increasing concentrations of β-chloro-L-alanine. Each replicate is shown (n = 3) along with a non-linear regression using a dose-response model (line) +/- 95% confidence interval (shaded area). C) Susceptibility of 24-hour biofilms of *Af293* (left), *Af293ΔalaA* (middle), and *Af293alaA<sup>rec</sup>* (right) established in the presence of 0, 10, or 100 μM β-chloro-L-alanine. Biofilms were treated with the indicated concentrations of caspofungin for 3-hours and viability was assessed by XTT assay. Mean +/- SD are shown (n = 3). D) Susceptibility of 24-hour CEA10 biofilms established in the presence or absence of 100 μM β-chloro-L-alanine to caspofungin treatment. Biofilms were treated with the indicated concentrations of caspofungin for 3-hours and viability was assessed by XTT assay. Mean +/- SD are shown (n = 3). \*\* p < 0.01, \*\*\*\* p < 0.0001 as determined by Two-Way ANOVA with Tukey's multiple comparisons test. E-F) Susceptibility of 24-hour *Af293Δuge3* (E) and *Af293Δagd3* (F) biofilms established in the presence or absence of 100 μM β-chloro-L-alanine to caspofungin treatment. Biofilms were treated with the indicated concentrations of caspofungin for 3-hours and viability was assessed by XTT assay. Mean +/- SD are shown (n = 3). \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001 as determined by Two-Way ANOVA with a Tukey's multiple comparisons test. The highest p-values for β-chloro-L-alanine treated groups vs their respective untreated groups are shown.



384 ***alaA* is required for echinocandin resistance *in vivo*.**

385 Finally, we sought to determine if *alaA* plays a role in echinocandin resistance *in vivo* within lung  
386 infection microenvironments. To address this question, we utilized a chemotherapy murine model of  
387 invasive pulmonary aspergillosis (IPA). Outbred CD1 mice were immunosuppressed with  
388 cyclophosphamide and triamcinolone then challenged with conidia of Af293, Af293 $\Delta$ *alaA*, or  
389 Af293*alaA*<sup>rec</sup>. The infection was allowed to establish for 24 hours followed by three treatments with either  
390 0.9% NaCl or 1mg/kg micafungin every 24 hours (Figure 7A). 12 hours after the final micafungin treatment  
391 relative fungal burden was determined by qPCR quantification of *A. fumigatus* 18S rDNA. The dosage of  
392 micafungin treatment used had no significant impact on fungal burden in mice inoculated with the WT or  
393 reconstituted strains. Moreover, loss of *alaA* at the time point examined did not significantly impact fungal  
394 burden levels in the untreated groups (Figure 7B). In contrast, there was a 4-fold reduction in fungal  
395 burden in mice inoculated with Af293 $\Delta$ *alaA* and treated with micafungin compared to untreated mice  
396 (Figure 7B). Thus, loss of *alaA in vivo* significantly increases the susceptibility of *A. fumigatus* to sub-  
397 effective concentrations of the echinocandin micafungin.

398



**Figure 7: *alaA* is required for echinocandin resistance *in vivo*.** A) Experimental outline for determining *in vivo* echinocandin resistance using a chemotherapy model of invasive aspergillosis. Outbred CD1 mice were immunosuppressed with 150mg/kg cyclophosphamide 48 prior to fungal challenge and 40mg/kg triamcinolone 24 hours prior to fungal challenge. Mice were challenged with *A. fumigatus* or PBS (mock) at D0, and infection was allowed to establish for 24 hours. Mice were treated with either 1mg/kg micafungin or 0.9% NaCl every 24 hours from D1 to D3. 12 hours after the final micafungin treatment mice were sacrificed for fungal burden determination by qPCR. B) qPCR quantification of total ng fungal DNA in lungs of mice challenged with the indicated *A. fumigatus* strains and treated or untreated with 1mg/kg micafungin according to design in (A). Each datapoint and the mean are shown (n ≥ 12 for each experimental group and n = 5 for mock infected mice across two independent experiments). \* p < 0.05, n.s. = not significant as determined by Kruskal-Wallis with a Dunn's multiple comparisons test.

## 399 **Discussion**

400 The vast majority of antifungal drug studies and discoveries are based on treatment of the conidial  
401 state of *A. fumigatus* despite the fact that clinical treatment is largely in the context of an already  
402 established infection where *A. fumigatus* exists as a biofilm. Here we discovered a gene involved in the  
403 metabolism of alanine that confers biofilm specific echinocandin resistance to *A. fumigatus*. Given the  
404 results presented in this work, as well as others, we have shown that antifungal treatment of biofilms is  
405 far less effective than treatment of conidia (Kowalski et al., 2020; Seidler et al., 2008). Mechanisms to  
406 increase efficacy of antifungal drugs in the context of *A. fumigatus* biofilms are of great clinical need and  
407 may even bridge the discrepancies between relatively low, but rising, rates of antifungal resistance *in*  
408 *vitro* and the relatively high rates of clinical treatment failure (Herbrecht et al., 2015; Maertens et al., 2016,  
409 2021; Marr et al., 2015). Both genetic and chemical disruption of AlaA results in increased susceptibility  
410 of biofilms to echinocandin treatment, suggesting inhibition of this enzyme is a potential treatment option  
411 for clinically enhancing the efficacy of this class of antifungals. While alanine aminotransferase inhibitors  
412 have already been developed (Beuster et al., 2011; Golichowski & Jenkins, 1978; Morino et al., 1979),  
413 these molecules were discovered using mammalian alanine aminotransferases (AlaA has 45.77% and  
414 45.19% amino acid sequence identity with human GPT1 and GTP2, respectively (Figure 2-figure  
415 supplement 1B-C)). Thus, the chemical inhibition performed in this study serves as a proof of principle  
416 for targeting AlaA activity in a combination therapy approach. However, these molecules could provide  
417 chemical building blocks for development of more fungal specific and/or potent alanine aminotransferase  
418 inhibitors.  $\beta$ -chloro-L-alanine has been utilized in a murine cancer model where inhibition of murine  
419 alanine aminotransferase was found to decrease the Warburg effect and increase mitochondrial activity  
420 of tumor cells (Beuster et al., 2011). While this is promising for future studies *in vivo*, further safety and  
421 pharmacological studies are needed.

422 We originally found *alaA* through investigation of datasets associated with low oxygen adaptation.  
423 AlaA catalyzes interconversion of pyruvate and alanine without direct involvement of reducing potentials  
424 or any high energy molecules, such as ATP. However, the reduction of nitrate to alanine would consume

425 five reducing potentials and this pathway is suggested to be important for a variety of systems in the  
426 adaptation to low oxygen (Feala et al., 2007; Felig et al., 1970; Harrison, 2015; Lothier et al., 2020; Rocha  
427 et al., 2010). It is possible that in these systems alanine serves as a nitrogen sink to prevent toxic  
428 ammonium accumulation during the conversion of nitrate to ammonium. Therefore, we had originally  
429 hypothesized that AlaA function was a critical means of recycling reducing potentials during low oxygen  
430 growth and were surprised to find that AlaA plays a significant role in polysaccharide regulation and  
431 biofilm formation despite the minimal impact on growth in a low oxygen environment. This result could be  
432 due to a high redundancy in the number of mechanisms encoded by the fungus to balance reducing  
433 potentials, or it could suggest that alanine metabolism has a more specific role in adaptation to natural  
434 oxygen gradients formed by respiration and/or adaptation to stochastic fluctuations in environmental  
435 oxygen that naturally occur during filamentous fungal biofilm growth.

436 The involvement of an alanine aminotransferase in polysaccharide regulation is even more  
437 perplexing when one considers that none of the components of the reaction (alanine, pyruvate,  
438 glutamate, and  $\alpha$ -ketoglutarate) have any obvious or direct role in any known biochemical pathways to  
439 generate *A. fumigatus* cell wall or extracellular matrix components. AlaA also does not appear to be  
440 essential for maintaining basal levels of alanine for protein production, as genetic disruption of *alaA* did  
441 not yield alanine auxotrophy. Here we describe that the catalytic activity of the AlaA protein is essential  
442 for polysaccharide regulation. Additionally, transcriptional data corresponding to the *uge3* and *agd3*  
443 genes essential for synthesis and maturation of GAG, respectively, suggests that the mechanism of  
444 regulation is predominantly post-transcriptional and potentially indirect from alterations in fungal  
445 metabolism (Figure 4C-D). However, the exact mechanism through which this reaction regulates  
446 polysaccharide biosynthesis and maturation remains to be further studied. Further investigation into this  
447 mechanism could yield significant insight into the interplay between metabolism, biofilm formation, and  
448 antifungal drug resistance to help inform development of novel biofilm targeted antifungal drugs.

449

## 450 **Materials and Methods**

### 451 **Strains and growth conditions**

452 Mutant strains were made in the *A. fumigatus* Af293 and CEA10 strains, and therefore Af293 and  
453 CEA10 were used as the wildtype (WT) strains as appropriate for each experiment. Strains were stored  
454 as conidia in 25% glycerol at -80°C and maintained on 1% glucose minimal media (GMM; 6g/L NaNO<sub>3</sub>,  
455 0.52g/L KCL, 0.52g/L MgSO<sub>4</sub>•7H<sub>2</sub>O, 1.52g/L KH<sub>2</sub>PO<sub>4</sub> monobasic, 2.2mg/L ZnSO<sub>4</sub>•7H<sub>2</sub>O, 1.1mg/L  
456 H<sub>3</sub>BO<sub>3</sub>, 0.5mg/L MnCl<sub>2</sub>•4H<sub>2</sub>O, 0.5mg/L FeSO<sub>4</sub>•7H<sub>2</sub>O, 0.16mg/L CoCl<sub>2</sub>•5H<sub>2</sub>O, 0.16mg/L  
457 CuSO<sub>4</sub>•5H<sub>2</sub>O, 0.11mg/L (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>•4H<sub>2</sub>O, 5mg/L Na<sub>4</sub>EDTA, 1% glucose; pH 6.5). Solid media  
458 was prepared by addition of 1.5% agar. All experiments were performed with GMM unless explicitly stated  
459 otherwise. For experiments where alanine was the sole carbon or nitrogen source, glucose or NaNO<sub>3</sub>  
460 were replaced with alanine at an equimolar quantity of carbon or nitrogen atoms, respectively. For all  
461 experiments, *A. fumigatus* was grown on solid GMM at 37°C 5% CO<sub>2</sub> for 3 days to produce conidia.  
462 Conidia were collected using 0.01% Tween-80, counted using a hemacytometer, and diluted in either  
463 0.01% Tween-80 or media to the final concentration used in each assay.

### 464 **Strain construction**

465 *alaA* null mutants were generated by replacing the *alaA* open-reading frame  
466 (AFUB\_073730/Afu6g07770) with the dominant selection marker *ptrA* in both the Af293 and CEA10  
467 backgrounds. The replacement construct was generated using overlap PCR to fuse ~1kb upstream and  
468 ~1kb downstream of the open reading frame of *alaA* to the *ptrA* marker. The resulting construct was  
469 transformed into protoplasts of each strain and mutants were selected for on osmotically stabilized  
470 minimal media (GMM plus 1.2M sorbitol) containing 100 µg/L pyrithiamine hydrobromide (Sigma).  
471 Reconstitution of the *alaA* gene was performed by PCR amplification of the *alaA* locus for each strain  
472 from ~1.1kb upstream of the start codon to ~700bp downstream of the stop codon using primers  
473 containing PacI and AsclI digestion sites. The resulting PCR products were digested with PacI and AsclI  
474 restriction enzymes and individually ligated into a plasmid containing the hygromycin resistance marker

475 *hygR*. The resulting plasmids were ectopically transformed into protoplasts derived from the *alaA* null  
476 strain for each plasmid's respective background. Reconstituted mutants were selected for on osmotically  
477 stabilized minimal media containing both 175µg/mL hygromycin B (VWR) and 100µg/L pyrithiamine to  
478 ensure the mutated locus remained intact. GFP tagged alleles of WT *alaA* and catalytically inactive  
479 *alaA*<sup>K322A</sup> were generated at the *alaA* native locus in the Af293 background using the *ptrA* marker. The  
480 WT allele was generated using overlap PCR to fuse ~1kb upstream of the stop codon of *alaA*, excluding  
481 the stop codon, to a fragment containing an in-frame *gfp* linked to a *trpC* terminator from *A. nidulans* and  
482 the *ptrA* marker, along with the same ~1kb downstream of the stop codon that was used in the deletion  
483 construct. The catalytically inactive mutation was generated using nested PCR from the mutation site to  
484 immediately before the stop codon in order to modify the AAG lysine codon to a GCC alanine codon.  
485 This fragment was then fused with 500bp upstream of the point mutation, along with the in frame *gfp*-  
486 *trpC*<sub>terminator</sub> *ptrA* fragment, and ~1kb downstream of the *alaA* stop codon. The two alleles were  
487 transformed into Af293 protoplasts and mutants were selected using pyrithiamine. Sanger sequencing  
488 was used to confirm each allele.

489 Protoplasts were generated using lysing enzyme from *Trichoderma harzianum* (Sigma) and  
490 transformed as previously described (Willger et al., 2008). Protoplasts were plated on sorbitol stabilized  
491 minimal media (GMM + 1.2M sorbitol) containing pyrithiamine. For hygromycin selection protoplasts were  
492 allowed to recover without hygromycin selection until germtubes were visible by inverted microscope  
493 (overnight at 37°C). At which point 0.6% agar media containing hygromycin was added to a final  
494 concentration of 175µg/mL. All strains were single spored and checked for correct integration, or  
495 presence of construct in the case of the ectopic reconstituted strains, via PCR and southern blot.  
496 Additionally, the basal expression of *alaA* was checked by RT-qPCR on RNA extracted from 24-hour  
497 biofilms for the reconstituted strains using the *alaA* null mutants as negative controls (Figure 1-figure  
498 supplement 3).

## 499 **Metabolomics**

500 Cultures for metabolomics were performed with 100mL of  $10^6$  conidia/mL of CEA10. Shaking  
501 liquid cultures were performed in baffled flasks with a foam stopper to allow rapid environmental  
502 acclimation to changes in oxygen tension. Cultures were grown for 24 hours at 37°C 200RPM in ambient  
503 oxygen followed by either continued incubation at ambient oxygen or a shift to 0.2% O<sub>2</sub> for two hours.  
504 Biomass was harvested by filtering through Miracloth, washed thoroughly with water, and flash frozen in  
505 liquid nitrogen. The biomass was then lyophilized and 100mg dry weight was submitted to Metabolon for  
506 LC-MS/MS analysis, metabolite identification, and relative quantification. Data processing and figure  
507 generation was performed in R using the ComplexHeatmap (Gu et al., 2016) and Pathview (Luo &  
508 Brouwer, 2013) packages based on the relative ion counts and statistical measures given by Metabolon.

### 509 **Growth Assays**

510 For assays of growth on alanine as a carbon or nitrogen source, an equal molarity of carbon or  
511 nitrogen atoms were added for each indicated molecule to our base minimal media lacking NaNO<sub>3</sub> and  
512 glucose. Agar plates were inoculated with  $10^3$  conidia and incubated for 72 hours at 37°C 5% CO<sub>2</sub> in  
513 either ambient oxygen or in a chamber that maintained oxygen at a concentration of 0.2% (InvivoO<sub>2</sub> 400  
514 Workstation, Ruskinn Baker). Biofilm biomass cultures were inoculated with 20mL of  $10^5$  conidia/mL in  
515 GMM and grown in petri plates for 24 hours at 37°C 5% CO<sub>2</sub>. Supernatants and air-liquid interface growth  
516 were removed, and biofilms were harvested using a cell scraper. Biomass was washed 2X with ddH<sub>2</sub>O  
517 with centrifuging at 5000RPM for 10 minutes to spin down biomass, frozen at -80°C, lyophilized, and dry  
518 weight was measured. For liquid kinetic growth assays of biofilms, 200μL of  $10^5$  conidia/mL in GMM  
519 was inoculated in six technical replicates per strain in a 96 well plate. Plates were incubated statically in  
520 a plate reader at 37°C with Abs<sub>405</sub> readings every 15 minutes over the first 24 hours of growth.

### 521 **Crystal Violet Adherence Assay**

522 U-bottomed 96-well plates were inoculated with  $10^5$  conidia/mL in GMM and incubated statically  
523 for the indicated time at 37°C 5% CO<sub>2</sub> to allow biofilms to form. To remove non-adherent cells, media  
524 was removed, and biofilms were washed twice with water via immersion followed by banging plate onto

525 a stack of paper towels. Adherent biomass was stained with 0.1% (w/v) crystal violet for 10 minutes and  
526 biofilms were washed twice with water to remove excess crystal violet. Crystal violet was then dissolved  
527 in 100% ethanol, supernatants were transferred to a flat-bottomed plate, and absorbance at 600nm was  
528 quantified. Dose-response assays testing the impact of  $\beta$ -chloro-L-alanine on adherence were fit with a  
529 non-linear regression based on a dose-response model (GraphPad Prism 9) to calculate EC<sub>50</sub> values.  
530 For assays testing the impact of collagen coating, 50 $\mu$ L of Collagen Coating Solution (Sigma) was applied  
531 to half the wells of a 96-well U-bottom plate overnight at room temperature. The solution was removed,  
532 and wells were washed one time with PBS prior to inoculation. All crystal violet adherence assays were  
533 performed with 3-6 technical replicates and data presented represent at least three biological replicates.

### 534 **Oxygen Quantification**

535 Oxygen was quantified as previously described (Kowalski et al., 2020) using a Unixense Oxygen  
536 Measuring System 1-CH (Unisense OXY METER) equipped with a micromanipulator (Unisense  
537 MM33), motorized micromanipulator stage (Unisense MMS), motor controller (Unisense MC-232), and  
538 a 25 $\mu$ m Clark-type/amperometric oxygen sensor (Unisense OX-25). The SensorTrace Suite Software  
539 v3.1.151 (Unisense) was utilized to obtain and analyze the data. Falcon 35mm petri dishes (Fisher)  
540 were coated with 2mL of 0.6% agar GMM to protect the microelectrode from breaking when performing  
541 deep profiling into the biofilms. 3mL of 10<sup>5</sup> conidia/mL in GMM was inoculated into the plates and  
542 incubated for 24 hours at 37°C 5% CO<sub>2</sub>. The meniscus of the culture was ~3mm above the surface of  
543 the agar pad, and thus oxygen was measured at the center of each culture in 200 $\mu$ m steps, with  
544 technical duplicates at each step, from the air-liquid interface to 2800 $\mu$ m into the culture. Oxygen  
545 quantification was performed immediately upon removal of the culture from the incubator. At least  
546 seven independent biofilms were measured for each strain across two experiments along with three  
547 media only cultures that lacked fungus.

### 548 **Fluorescent Microscopy**



549 Fluorescent confocal microscopy was performed on an Andor W1 Spinning Disk Confocal with a Nikon  
550 Eclipse Ti inverted microscope stand.

### 551 *AlaA Localization Studies*

552 Af293*alaA-GFP*, Af293*alaA<sup>K322A</sup>-GFP*, and Af293 were cultured in GMM on MatTek® dishes at  
553 37°C in GMM until germlings were visible on an inverted light microscope, ~9 hours for Af293*alaA-GFP*  
554 and Af293, and ~10 hours for Af293*alaA<sup>K322A</sup>-GFP*. Media was removed and replaced with fresh GMM  
555 containing 100nm MitoTracker™ Deep Red FM (ThermoFisher). Cultures were incubated for 30 minutes  
556 at 37°C to allow mitochondrial staining. Images were acquired with a 60X oil-immersion objective at  
557 488nm (GFP) and 637nm (MitoTracker) on the Andor W1 Spinning Disk Confocal. Images were  
558 deconvolved and max intensity z-projections were generated using the Nikon NIS-Elements AR software.  
559 Experiment was also performed with WT Af293 as a negative control for autofluorescence (Figure 2-  
560 figure supplement 2). At least ten images for each strain were taken across four replicate cultures.

### 561 *Fungal biofilm imaging and quantification*

562 Biofilms were grown in 2mL of GMM at 10<sup>5</sup> conidia/mL in 35mm glass bottom MatTek® dishes  
563 for the indicated duration of time at 37°C 5% CO<sub>2</sub>. At the indicated time, 1mL of media was removed and  
564 500uL of 30µg/mL FITC-SBA (Vector Laboratories) was added to each culture. Cultures were incubated  
565 at room temperature for 1 hour to allow staining. Biofilms were then fixed via addition of 500uL 4%  
566 paraformaldehyde in PBS and counterstained with 200uL of 275µg/mL calcofluor white (Fluorescent  
567 Brightener-28 (Sigma)). Images of the first ~300µm of the biofilms were acquired on the Andor W1  
568 Spinning Disk Confocal with a 20X multi-immersion objective (Nikon) at 405nm (calcofluor white) and  
569 488nm (FITC-SBA).

570 For quantification and analysis of biofilms the BiofilmQ framework was used. A detailed  
571 explanation of BiofilmQ can be found in previous publications (Hartmann et al., 2021). Briefly, both  
572 biomass (calcofluor white) and matrix (FITC-SBA) were thresholded and then segmented into discrete  
573 objects with 20 voxel cubes for further analysis. Total biovolume measures were achieved by taking the

574 summed volume of the segmented calcofluor white signal for each biofilm. Total matrix intensity was  
575 determined by summing the intensity signal of FITC-SBA in each cube of segmented matrix signal  
576 across the entire image. We measured hyphal associated matrix by taking the sum of FITC-SBA  
577 intensity that was overlapping in each cube of segmented biomass. Representative images were  
578 rendered using the VTK output feature of BiofilmQ. These files could then be rendered in ParaView  
579 (Ayachit, 2015) using Ospray ray tracing. Matrix intensity per individual cube as determined in BiofilmQ  
580 was then mapped onto the segmented matrix images.

### 581 Cell wall staining and quantification

582 Strains were grown in GMM in the center of MatTek® dishes until germlings were visible by an  
583 inverted light microscope, ~9 hours for Af293 and Af293 $\Delta$ ala<sup>rec</sup> and ~10 hours for Af293 $\Delta$ alaA.  
584 Supernatants were removed and cells were washed with PBS. For calcofluor white staining, germlings  
585 were fixed with 4% paraformaldehyde for 15 minutes, washed with PBS and stained with 25 $\mu$ g/mL  
586 calcofluor white (Fluorescent Brightener 28, Sigma) in PBS for 15 minutes. Calcofluor white was  
587 removed, germlings were washed with PBS, and maintained in 2mL PBS at room-temperature until  
588 imaging. For FITC-WGA staining, germlings were stained with 5 $\mu$ g/mL FITC-WGA in GMM for 30 minutes  
589 at room temperature. Germlings were washed with PBS and fixed with 4% paraformaldehyde for 15  
590 minutes. WGA stained germlings were then washed with PBS and maintained in 2mL PBS at room  
591 temperature until imaging. Finally, for Dectin-1 staining germlings were fixed with 4% paraformaldehyde  
592 for 15 minutes and washed with PBS. Blocking solution (RPMI + 10% FCS + 0.025% Tween-20) was  
593 applied for 1 hour at room temperature. Blocking solution was removed and 5 $\mu$ g/mL of Dectin-1-Fc in  
594 blocking solution was applied for 1 hour at room temperature. Germlings were washed with PBS and the  
595 secondary antibody AlexaFluor 488 anti-human IgG (ThermoFisher) was added at a 1/300 dilution in  
596 PBS for 1 hour at room temperature. Germlings were washed one final time with PBS and cells were  
597 maintained in 2mL PBS until imaging. All staining took place in the dark and extreme care was taken to  
598 not disrupt the Af293 $\Delta$ alaA germlings.

599 All germlings were imaged on the Andor W1 Spinning Disk Confocal with a 60X oil-immersion  
600 objective using 405nm for calcofluor white and 488nm for FITC-WGA and Dectin-1-Fc. Cell wall staining  
601 was quantified using Fiji (ImageJ). Z-stacks were assembled using a sum-intensity Z-projection. Regions  
602 of interest (ROI's) were drawn around each individual germling within a given image, along with a region  
603 lacking any germlings to account for background fluorescence. Within each ROI the area, sum intensity,  
604 and mean intensity were quantified. To obtain corrected mean intensity measurements, the mean  
605 background intensity was multiplied by the area of the ROI to calculate total background contribution.  
606 The total background contribution was subtracted from the ROI's sum intensity and this value was divided  
607 by the area of the ROI yielding the final corrected mean fluorescence intensity. Each cell wall stain was  
608 performed in triplicate cultures and at least three fields of view were obtained for each culture. For FITC-  
609 WGA the staining pattern was almost entirely absent from the germ-tube and enough natural size  
610 heterogeneity was found both within and between cultures to act as a confounding variable. Thus, for  
611 FITC-WGA ROIs were drawn around each conidial body, where the staining was present, rather than the  
612 entire germling.

### 613 **Extracellular matrix monosaccharide analysis and ELLA**

#### 614 Enzyme Linked Lectin Assay (ELLA)

615 100 $\mu$ L of 10<sup>5</sup> conidia per mL in GMM were inoculated into wells of 96 well plate and incubated  
616 for 24h. Culture supernatants were then transferred to a 384 well plate Immulon 4HBX with or without  
617 500pM of recombinant Agd3. After a 1-hour incubation period, wells were washed three times with 1X  
618 TBS – 0.05% Tween20. A preincubated solution of 30nM soybean agglutinin lectin coupled to biotin and  
619 1/700 avidin-HRP in TBS-T was added to the wells and incubated for 1 hour. After 3 TBS-T washes,  
620 detection was performed using Ultrasensitive TMB read at 450nm. Normalization of the values were  
621 performed reporting the absorbance reads to the absorbance of Af293.

#### 622 Extracellular Matrix Monosaccharide Composition by Gas Chromatography Coupled to Mass 623 Spectrometry

624 100ml of GMM was inoculated with  $10^4$  conidia per mL and incubated for 3 days at 37°C at  
625 200rpm. Culture supernatants were filtered by Miracloth prior to being dialyzed for 3 days against Milli-  
626 Q® water and lyophilized. About 0.5mg of dried material was then derivatized into TriMethylSilyl  
627 derivatives. Samples were hydrolyzed with either 2 M trifluoroacetic acid for 2 hours at 110°C or 6 M  
628 hydrochloric acid (HCl) for 4 hours at 100°C. Monosaccharides were then converted in methyl glycosides  
629 by heating in 1 M methanol-HCl (Sigma-Aldrich) for 16 hours at 80°C. Samples were dried and washed  
630 twice with methanol prior to re-N-acetylating hexosamine residues. Re-N-acetylation was performed by  
631 incubation with a mix of methanol, pyridine, acetic anhydride (10:2:3) for 1 hour at room temperature.  
632 Samples were then treated with hexamethyldisilazane-trimethylchlorosilane-pyridine solution (3:1:9;  
633 ThermoFisher) for 20 min at 110°C. The resulting TMS methyl glycosides were dried, resuspended in 1  
634 ml of cyclohexane, and injected in the Agilent 7890B GC - 5977A MSD. Identification and quantification  
635 of the monosaccharides was performed using a mix of monosaccharide calibrants injected at different  
636 concentrations as a reference. Quantification was finally normalized to an equivalent of 1mg of material  
637 before comparison between groups.

### 638 **RNA Extraction and RTqPCR**

639 RNA was extracted from 24-hour biofilm cultures in a 6-well plate. Supernatant was removed  
640 and 500µL of TRIsure™ (Bioline Reagents) was immediately applied to the biofilms. Biofilm  
641 suspensions were centrifuged, and supernatant was removed. Biomass was resuspended in 200µL  
642 TRIsure™ flash frozen in liquid nitrogen, and bead beat with 2.3mm beads. Homogenate was brought  
643 to a final volume of 1mL with TRIsure™, bead beaten a second time, and RNA was extracted following  
644 the manufacturer's protocol. 5µg of RNA was DNase treated with TURBO DNA-free™ kit (Invitrogen)  
645 according to manufacturer's protocol. 500ng of DNase-treated RNA was run on an agarose gel to  
646 ensure RNA integrity. 500ng of DNase-treated RNA was used for cDNA synthesis as previously  
647 described (Beattie et al., 2017). The RTqPCR data were collected on a CFX Connect Real-Time PCR  
648 Detection System (Bio-Rad) with CFX Maestro Software (Bio-Rad). Gene expression was normalized  
649 to *tefA* expression for all experiments. Primers used for RTqPCR are listed in Table S2.

## 650 **Antifungal drug susceptibility**

### 651 Biofilm Assays

652 To test susceptibility of biofilms to inhibition by calcofluor white and caspofungin, 500 $\mu$ L of 10<sup>5</sup>  
653 conidia per mL in GMM were inoculated into wells of 24 well plates and biofilms were grown statically for  
654 24 hours at 37°C 5% CO<sub>2</sub>. Any air-liquid interface growth was removed using a sterile pipette tip, the  
655 supernatant was removed from each well, and fresh media containing the indicated concentration of  
656 calcofluor white or caspofungin was added to the biofilm. Biofilms were incubated for a further 3 hours at  
657 37°C 5% CO<sub>2</sub>, washed with PBS, and 300 $\mu$ L of XTT solution was added to each well (0.5mg/mL XTT  
658 [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (VWR) with 25 $\mu$ M menadione  
659 in PBS). XTT solution was incubated for 1 hour at 37°C to allow reduction of the dye. 150 $\mu$ L of  
660 supernatants were transferred to a flat-bottomed 96 well plate and the absorbance at 450nm was read  
661 on a plate reader. Abs<sub>450nm</sub> values of the treated samples were compared to untreated biofilms to calculate  
662 the relative metabolic activity as the percent of untreated control for each strain. All XTT assays were  
663 performed on at least three biological replicates.

664 For the adenylate kinase release assay biofilms were grown and treated in the same manner as  
665 above. After three hours of echinocandin treatment supernatants were collected and allowed to cool to  
666 room temperature. XTT assay was performed on the biofilms according to the protocol above for matched  
667 XTT data. Relative adenylate kinase levels were measured on 40 $\mu$ L of supernatants via the ToxiLight™  
668 Non-Destructive Cytotoxicity BioAssay Kit (Lonza) according to the manufacturer's instructions.  
669 Chemiluminescence was measured on a Synergy Neo2 multi-mode plate reader (BioTek). Experiment  
670 was performed on four independent biological replicates.

### 671 Conidia Assays

672 Minimal effective concentration (MEC) assays were performed by inoculating 100 $\mu$ L of 2\*10<sup>5</sup>  
673 conidia/mL in GMM into 96 well flat-bottomed plates containing 100 $\mu$ L serial 2-fold dilutions of  
674 caspofungin in GMM from 8 $\mu$ g/mL to 0.015625 $\mu$ g/mL along with a no drug control. Cultures were grown

675 statically for 24 hours and viewed under an inverted light microscope for the concentration at which gross  
676 morphological changes characteristic of caspofungin treatment became visible. This concentration was  
677 deemed the MEC. Radial growth assays were performed by inoculating GMM agar plates containing the  
678 indicated quantity of caspofungin with  $10^3$  conidia in 2 $\mu$ L 0.01% Tween-80 and incubating at 37°C 5%  
679 CO<sub>2</sub> for 72 hours. Images are representative of four biological replicates.

## 680 **Murine Fungal Burden Assay**

681 All mice were housed in autoclaved cages at 3-4 mice per cage and provided food and autoclaved  
682 water *ad libitum*. Female outbred CD-1 (Charles River Laboratory), 20-24 grams, were immune-  
683 suppressed with 150mg/kg cyclophosphamide (Ingenus Pharmaceuticals, LLC) interperitoneally 48  
684 hours prior to inoculation and 40mg/kg triamcinolone acetonide (Kenalog-10, Bristol-Myers Squibb)  
685 subcutaneously 24 hours prior to fungal challenge. Mice were administered  $10^6$  conidia in 30 $\mu$ L PBS  
686 intranasally under isoflurane anesthesia. Mock mice were administered 30 $\mu$ L sterile PBS. Micafungin  
687 treated mice were administered 1mg/kg micafungin (Mycamine<sup>®</sup>, Astellas Pharma) interperitoneally at  
688 24, 48, and 72 hours post-fungal inoculation. Untreated mice were administered 100 $\mu$ L 0.9% saline  
689 (vehicle control) interperitoneally, at 24, 48, and 72 hours post-fungal inoculation. Mice were sacrificed  
690 at 84 hours post-fungal inoculation and lungs were harvested for fungal burden.

691 Lungs were divided between two 2mL screw cap tubes and physically chopped using a dissecting  
692 scissors, flash frozen in liquid nitrogen, and lyophilized for 48 hours. The freeze-dried lungs were then  
693 bead beaten with 2.3mm Zirconia beads and DNA was extracted using the E.Z.N.A.<sup>®</sup> Fungal DNA Mini  
694 Kit (Omega Bio-tek) with the following modifications. Bead beaten lungs were resuspended in 600 $\mu$ L FG1  
695 buffer, bead beaten a second time and incubated at 65°C for 1 hour. Samples were centrifuged and  
696 supernatants from the split lung samples were combined in a new tube. The protocol was continued with  
697 200 $\mu$ L of the combined supernatant according to the manufacturer's instructions with two elution steps  
698 using 100 $\mu$ L molecular grade water heated to 65°C. qPCR quantification of fungal DNA was performed  
699 as previously described (Li et al., 2011). The fungal burden experiment was performed two times with  $n$   
700  $\geq 6$  in each experimental group per experiment and  $n = 5$  mock across the two experiments. Four mice

701 across the two experiments, including one in the Af293 $\Delta$ alaA treated group, were censored for either  
702 unsuccessful infection or fungal DNA extraction based on the criteria of having less fungal DNA than the  
703 highest mock control value.

## 704 **Statistics and Reproducibility**

705 All statistical analyses were performed in GraphPad Prism 9 with the exception of metabolomics  
706 statistics which were performed by Metabolon. Unless otherwise noted all experiments were performed  
707 with a minimum of three biologically independent samples.

708

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716

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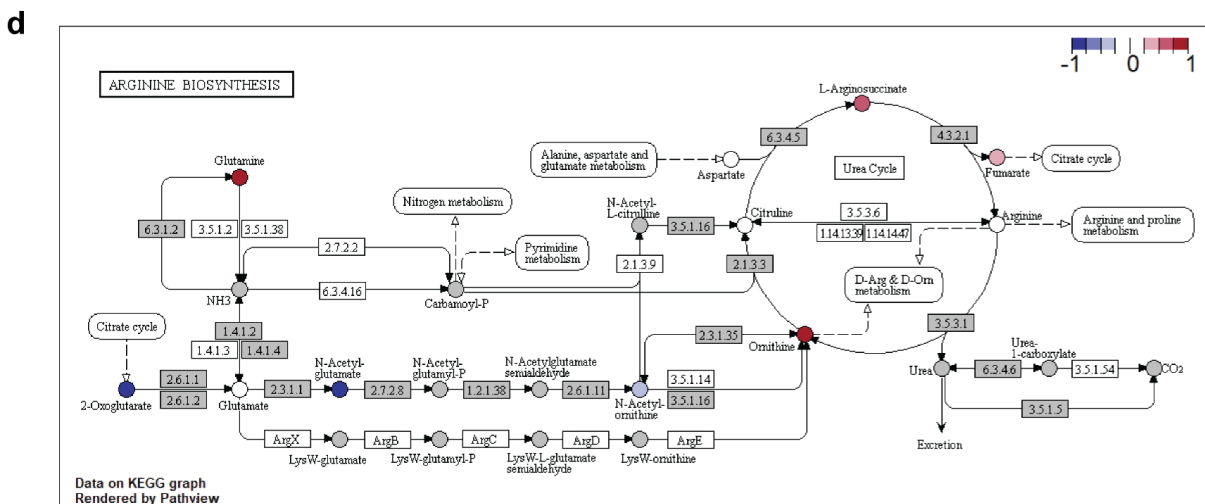
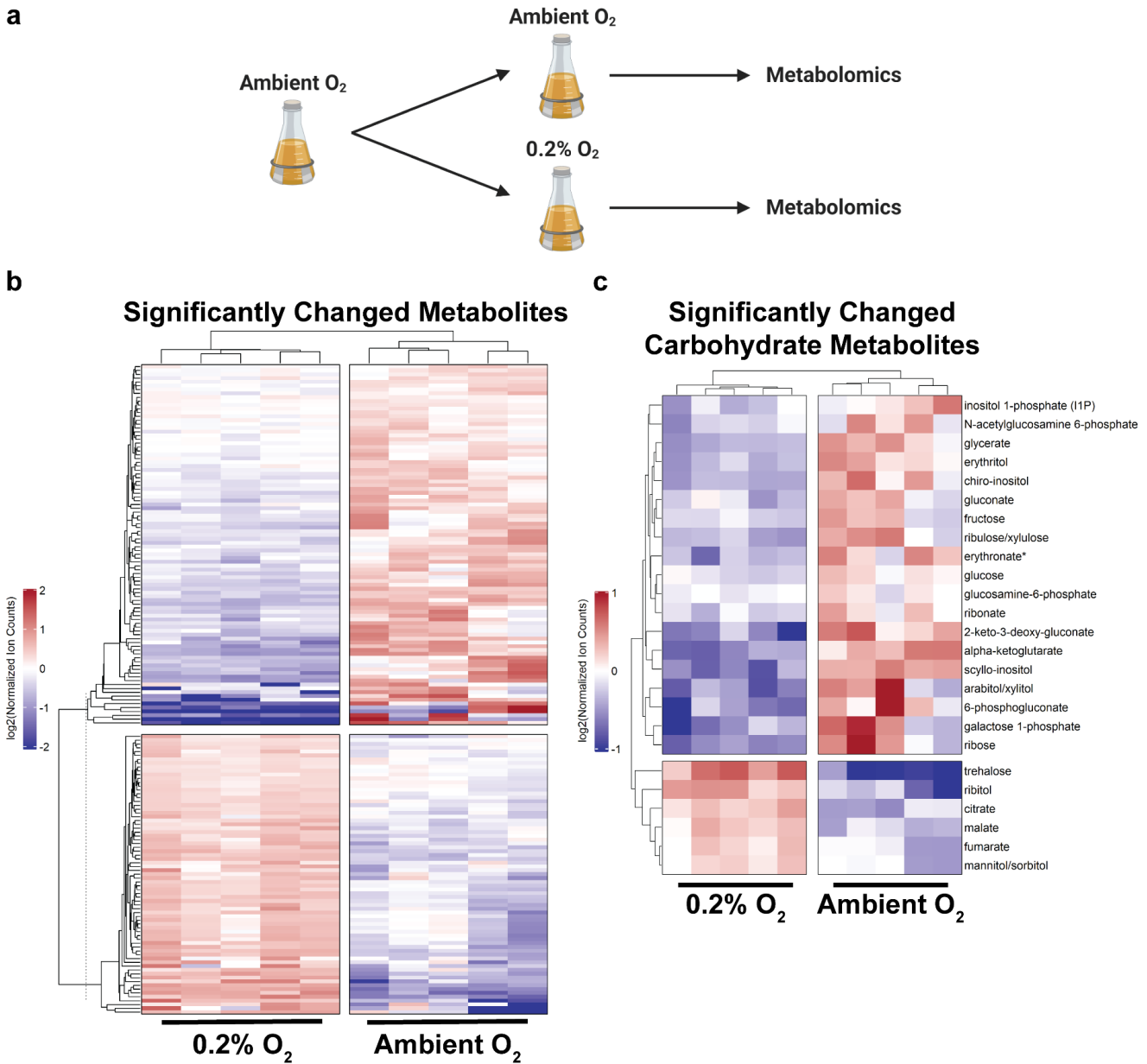
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931 **Supplemental Figures and Tables**

932



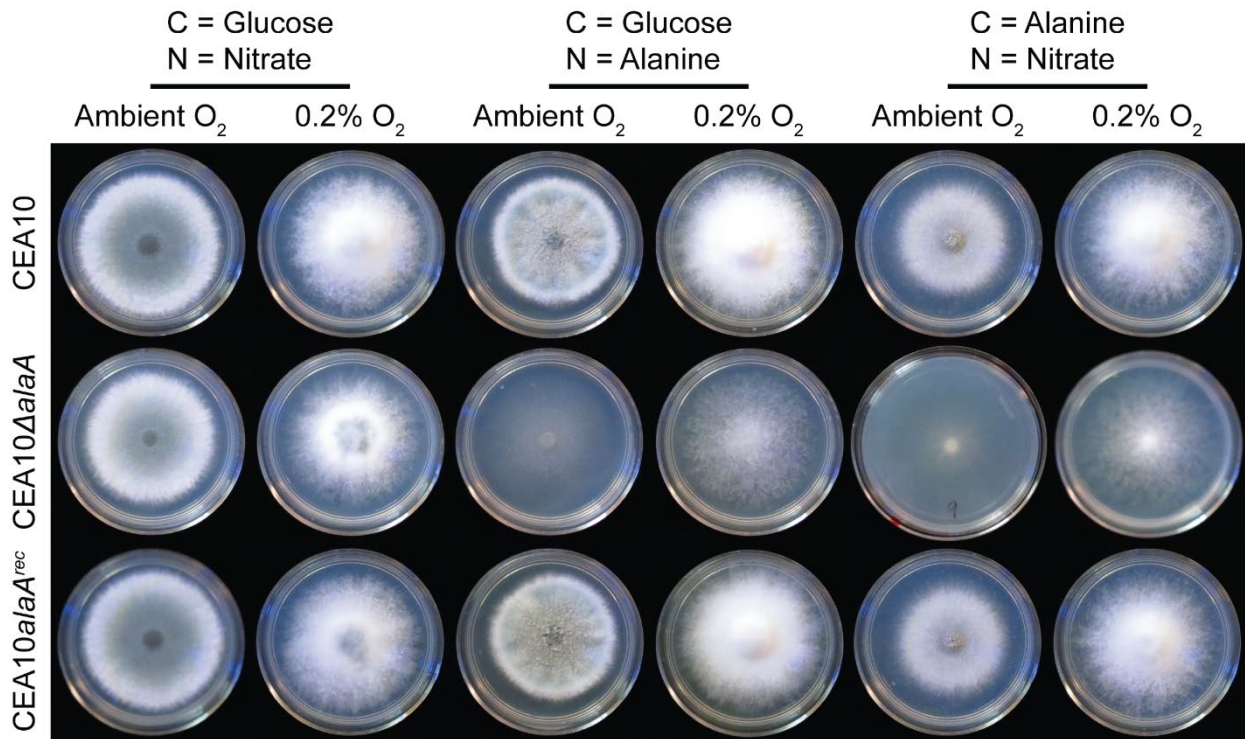
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**Figure 1-figure supplement 1: Acute exposure to a low oxygen environment significantly changes *A. fumigatus* metabolism.** A) Outline of the metabolomics experiment. Shaking culture were grown for 24 hours in liquid GMM followed by either a shift to a 0.2% oxygen environment or continued incubation in ambient oxygen for two hours. Biomass was then harvested, flash frozen to quench metabolic reactions, lyophilized, and submitted for metabolomics. B) Overview of all significantly altered metabolites detected in experiment. C) Significantly changed carbohydrate related metabolites in the two conditions. For C-D ion counts were normalized to the average ion count for the respective metabolite across all samples and  $\log_2$  transformed. Each column corresponds to an individual sample (n = 5 per condition). D) Average relative abundance of metabolites in 0.2% vs ambient oxygen ( $\log_2$  transformed) mapped onto the Arginine Biosynthesis KEGG map using the Pathview R package. Red indicates greater abundance in 0.2% oxygen, blue indicates greater abundance in ambient oxygen, white indicates no difference between conditions, and metabolites not detected are in grey.

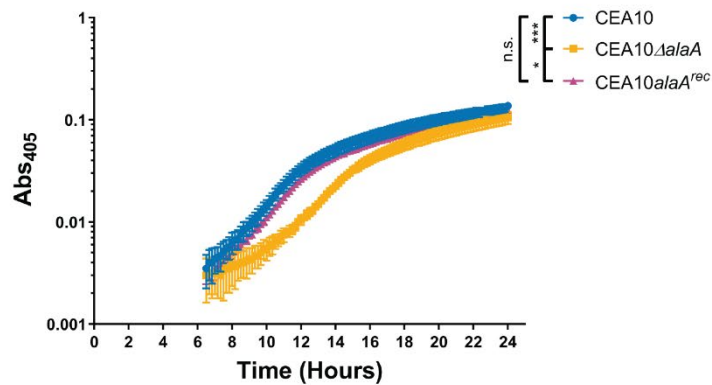


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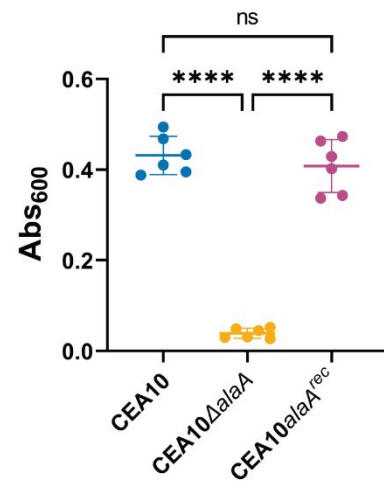
a



b

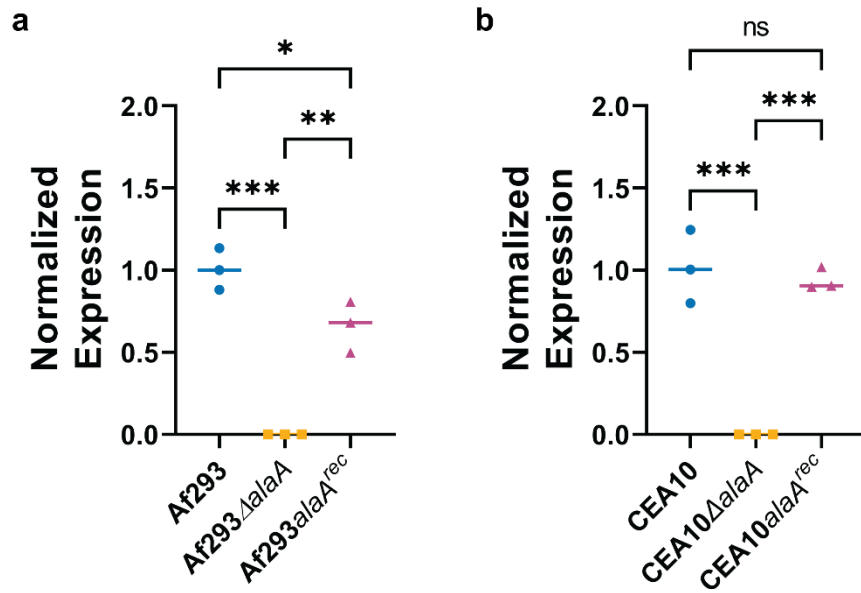


c



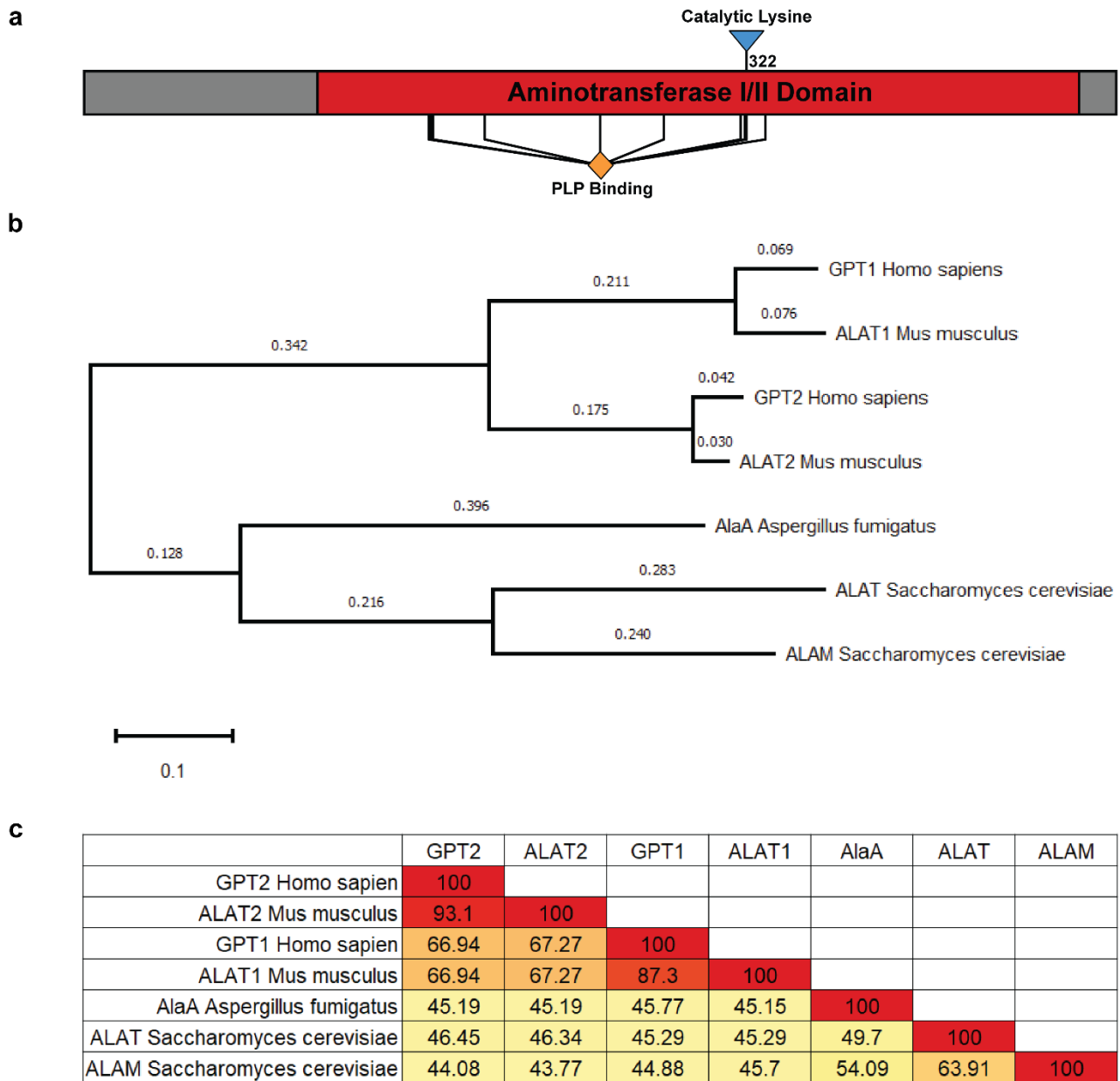
**Figure 1-figure supplement 2: *alaA* is required for alanine catabolism and biofilm physiology in the CEA10 strain background.** A) Growth of CEA10Δ*alaA* on minimal media containing the indicated sole carbon and nitrogen sources in ambient oxygen and 0.2% oxygen environments. B) Static growth assay of CEA10Δ*alaA* over the first 24 hours of biofilm growth. Mean +/- SD of 6 technical replicates is shown. Experiment was repeated a minimum of 3 times with similar results. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  by One-Way ANOVA with a Tukey's multiple comparisons test C) Crystal violet adherence assay of 24-hour biofilms ( $n = 6$ ). \*\*\*\*  $p < 0.0001$ , n.s. = not significant as determined by One-Way ANOVA with a Tukey's multiple comparisons test.

935



**Figure 1-figure supplement 3: RTqPCR of *alaA* expression in *alaA* deletion and reconstituted strains.** A-B) RNA was harvested from 24-hour biofilms and *alaA* expression was determined by RTqPCR (n = 3). Each replicate along with the median are shown. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, n.s. = not significant as determined by One-Way ANOVA with a Tukey's multiple comparisons test.

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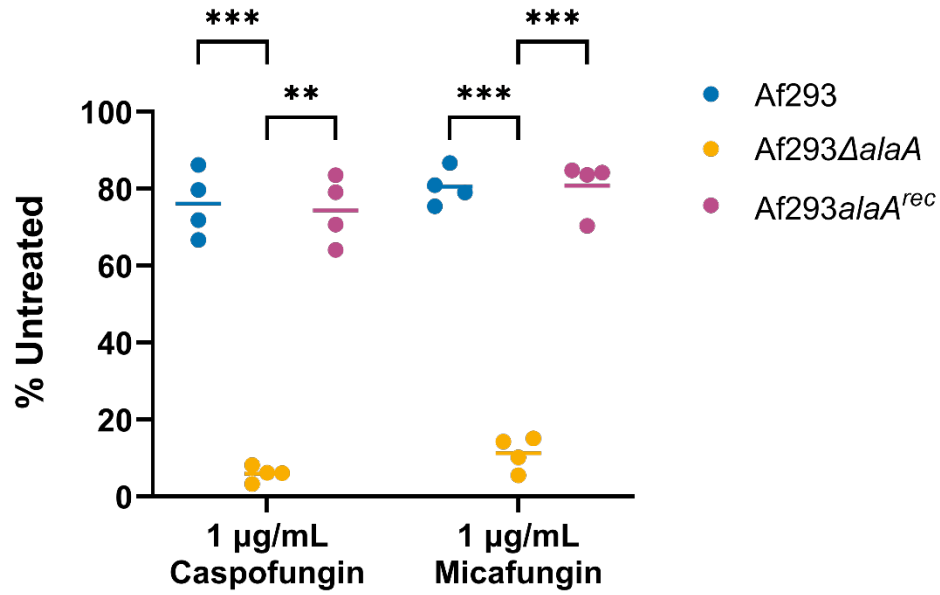
**Figure 2-figure supplement 1: AlaA protein domain architecture and degree of similarity to alanine aminotransferases of several model systems.** A) AlaA linear protein structure highlighting the aminotransferase class I/II domain, predicted PLP-binding residues, and the catalytic lysine residue mutated in the catalytic null strain (*Af293alaA<sup>K322A</sup>-GFP*). B) Phylogeny of AlaA relative to human, murine, and *Saccharomyces cerevisiae* alanine aminotransferases. All species except *A. fumigatus* encode two alanine aminotransferases in their genomes. Proteins were aligned in MEGA X using MUSCLE and a maximum-likelihood tree was generated. Scale bar and branch lengths refer to substitutions per site. C) Percent identity matrix of the alanine aminotransferases shown in (B).

937



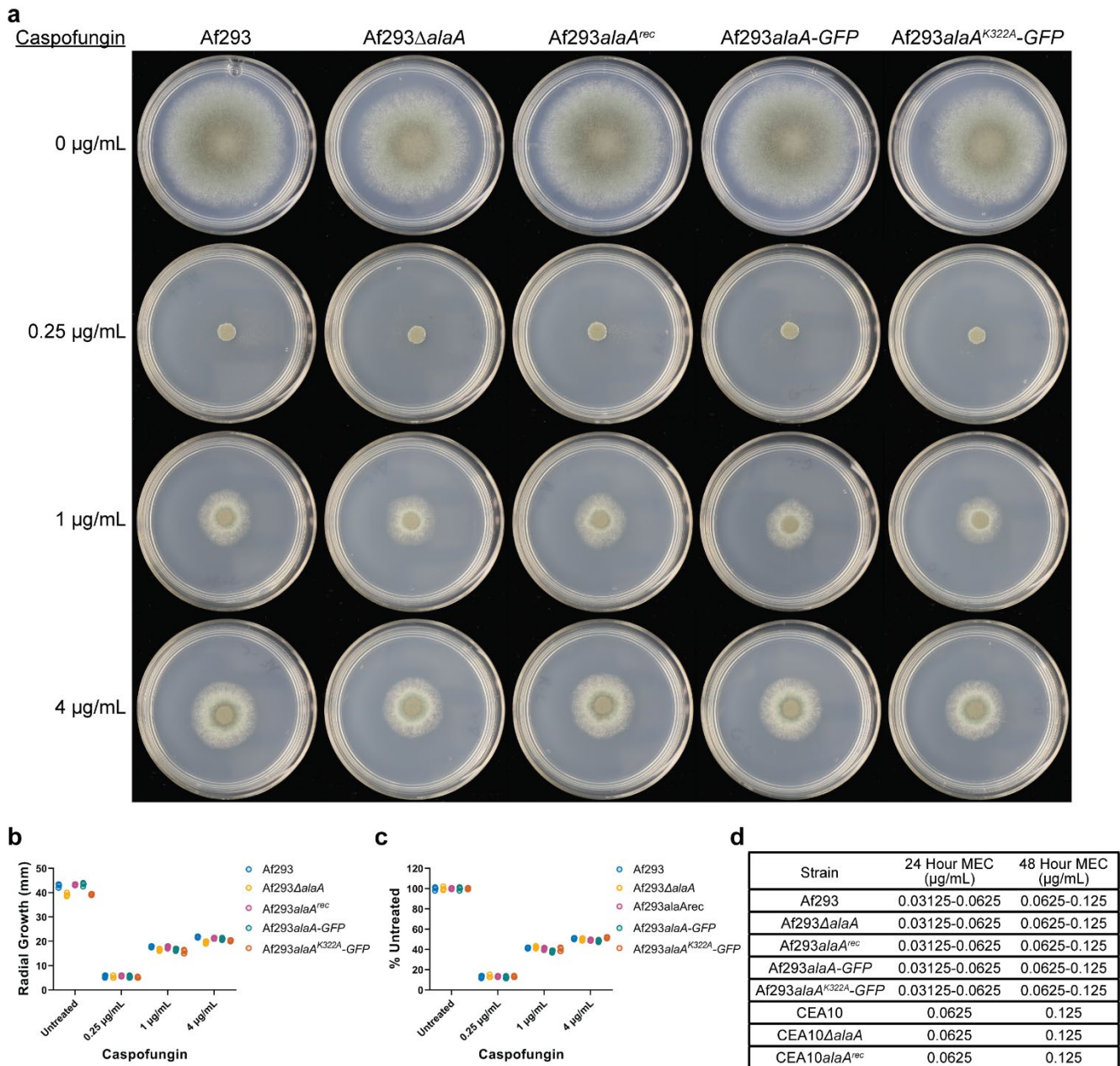
**Figure 2-figure supplement 2: Af293 wildtype control for AlaA localization experiments.** Representative micrographs of wildtype Af293 germlings stained with Mitotracker™ Deep Red FM (magenta). No fluorescence was observed in the GFP channel.

938



**Figure 5-figure supplement 1: XTT assay corresponding to the cultures used in the adenylate kinase release assay.** Biofilms were grown for 24 hours and treated with 1 μg/mL caspofungin (left) or micafungin (right) for 3 hours. Supernatants were used to quantify adenylate kinase activity (Figure 5I) and an XTT assay was performed to measure viability of biofilm biomass. Each replicate and mean are shown (n = 4). \*\* p < 0.01, \*\*\* p < 0.001 as determined by Two-Way ANOVA with a Tukey's multiple comparisons test.

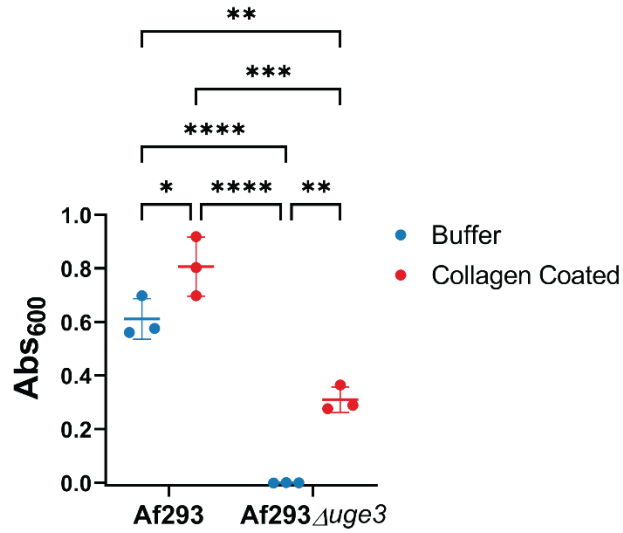
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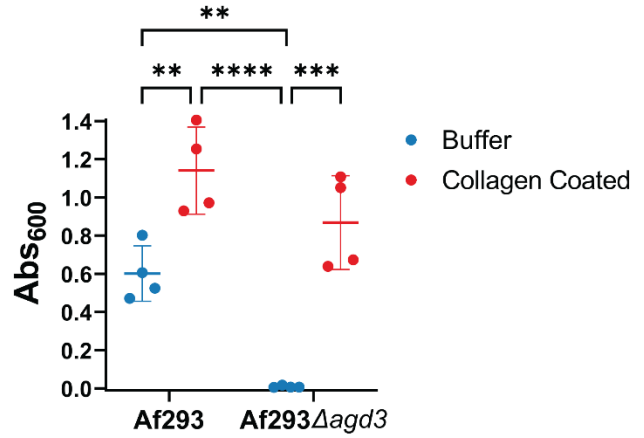
**Figure 5-figure supplement 2: Increased susceptibility of *alaA* null strains to caspofungin is biofilm specific.** A) Conidial radial growth assays of the indicated strains grown on the indicated concentrations of caspofungin in GMM for 72 hours. Images are representative of four replicate cultures. B) Quantification of radial growth as the mm diameter for each colony (n = 4). C) Radial growth normalized to the untreated control for each strain. Individual replicates and mean are shown (n = 4) for (B-C). D) Minimum effective concentration (MEC) of caspofungin for the indicated strains at 24 and 48 hours of incubation in GMM containing increasing concentrations of caspofungin (n = 3).

940

a



b



**Figure 6-figure supplement 1: Collagen coating partially rescues adherence of Af293 $\Delta$ uge3 and Af293 $\Delta$ agd3.** A-B) Crystal violet adherence assay of Af293 $\Delta$ uge3 (A) and Af293 $\Delta$ agd3 (B) in wells of a 96-well plate coated with collagen or PBS (buffer). Each replicate along with the mean +/- SD are shown (n  $\geq$  3). \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001 as determined by Two-Way ANOVA with a Tukey's multiple comparisons test.

941 **Table S1: Fungal strains used in this study.**

Strain	Background Strain	Genotype	Origin
Af293	Reference Strain	N/A	Nierman et al., 2005
Af293 $\Delta$ <i>alaA</i>	Af293	$\Delta$ <i>alaA</i> ; <i>ptrA</i> <sup>+</sup>	This Study
Af293 <i>alaA</i> <sup>rec</sup>	Af293 $\Delta$ <i>alaA</i>	<i>alaA</i> <sup>+</sup> ; <i>ptrA</i> <sup>+</sup> ; <i>hygR</i> <sup>+</sup>	This Study
CEA10	Reference Strain	N/A	Girardin et al., 1993
CEA10 $\Delta$ <i>alaA</i>	CEA10	$\Delta$ <i>alaA</i> ; <i>ptrA</i> <sup>+</sup>	This Study
CEA10 <i>alaA</i> <sup>rec</sup>	CEA10 $\Delta$ <i>alaA</i>	<i>alaA</i> <sup>+</sup> ; <i>ptrA</i> <sup>+</sup> ; <i>hygR</i> <sup>+</sup>	This Study
Af293 <i>alaA</i> -GFP	Af293	<i>alaA</i> -GFP; <i>ptrA</i> <sup>+</sup>	This Study
Af293 <i>alaA</i> <sup>K322A</sup> -GFP	Af293	<i>alaA</i> <sup>K322A</sup> -GFP; <i>ptrA</i> <sup>+</sup>	This Study
Af293 $\Delta$ <i>agd3</i>	Af293	$\Delta$ <i>agd3</i> ; <i>hygR</i> <sup>+</sup>	Lee et al., 2016
Af293 $\Delta$ <i>uge3</i>	Af293	$\Delta$ <i>uge3</i> ; <i>hygR</i> <sup>+</sup>	Gravelat et al., 2013

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943 **Table S2: Primers used for RTqPCR.**

Target Gene	Primer Sequence
<i>tefA</i> (Afu1g06390)	GTGACTCCAAGAACGATCCC
<i>tefA</i> (Afu1g06390)	AGAACTTGCAAGCAATGTGG
<i>uge3</i> (Afu3g07910)	CGACCCAGAATGGACTAT
<i>uge3</i> (Afu3g07910)	ACGACGACAGGAAGTAAG
<i>agd3</i> (Afu3g07870)	GTGGGTTGAGACGATTG
<i>agd3</i> (Afu3g07870)	AAGGAAGTTCTCGGACAT
<i>alaA</i> (Afu6g07770)	GGTGATCGGTCAGTGCCTGG
<i>alaA</i> (Afu6g07770)	GGCTTCGTACAGGGCGAGG

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