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Quantitative monitoring of mycelial growth of *Aspergillus fumigatus* in liquid culture by optical density

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June 10, 2021

Dr. Yoshitsugu Miyazaki
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Re: Spectrum00063-21 (Quantitative monitoring of mycelial growth of *Aspergillus fumigatus* with modified surface polysaccharides in liquid culture)

Dear Dr. Yoshitsugu Miyazaki:

Thank you for submitting your manuscript to Microbiology Spectrum. As you will see the reviewers support publication of a revised paper. Please revise the paper along the lines suggested by the reviewers. Given that this manuscript was initially submitted to another ASM journal with a different focus, I encourage you to specifically address the outlined points to reflect the scope of Microbiology.
1. The authors provide a different strategy for rapid screening of various compounds that can alter growth or viability of A. fumigatus. The method relies on use of mutant strain with severely altered cell wall composition. Thus, it becomes unclear how well use of this strain will reflect the wild type phenotypes, especially given the heterogeneity within the species. Reviewer #2 specifically noted that the discussion is rather short and it is advisable to add such explanation.
2. I especially agree with Reviewer #1 comments 2 and 5. As this appears as a technique manuscript a clear and well detailed explanation of all experimental procedures, materials used, etc. is needed. This would ensure the reproducibility of the presented approach. Regarding comment 5 either a direct comparison between solid and liquid medium growth and justification for benefits (growth rate, ease of experimental setup, low costs...) have to be included. I strongly recommend such paired solid-liquid growth comparison between wt and mutant using a handful of conditions as the goal of the study is to demonstrate wide usability of the presented approach.
Of note, problems with the file containing comments made by reviewer 2 were noted. I have attached the correct file as an attachment.

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Slavena Vylkova

Editor, Microbiology Spectrum

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Reviewer comments:

Reviewer #1 (Comments for the Author):

In this manuscript, Ken and colleagues constructed a mutant strain of A. fumigatus that lacks the alpha-glucan and galactosaminogalactan in the cell wall. Based on this mutant strain, they developed a strategy to assess the cell growth by measuring optical density. As the authors proposed, this new strain may be applied for high throughput anti-fungal drug screening in shaken liquid growth condition. However, there are several concerns for the high throughput screening strategy. Substantially more experiment should be performed before it could be validated strategy
.
Major points:
1, Growth of unicellular organism and filamentous fungi (multicellular) are fundamentally different. Use of optical density for measuring the growth of unicellular organism is based on their uniform distribution. In contrast, filamentous fungi grow in a way to elongate the existing mycelia, as the authors show in Fig.2D. The conidia and mycelia of filamentous fungi tend to cluster together and form pellets in a shaken liquid culture. Even the Δags1Δgtb3 mutant strain, the mycelia were not totally separated from each other. They still form small but visible pellets in the shaken liquid medium, especially in the YG medium. If so, it is hard to agree with the concept that a simple measurement of optical density could faithfully represent the cell density.

2, Materials and Methods section. For the measurement of optical density, authors described that "100µL was mixed with 100 µL of 100 mM sodium phosphate buffer (pH 7.0) containing 4% paraformaldehyde in a 96-well plate". However, this statement was not clear. Especially when the pellets of the colonies become larger in late time points, the transfer of such colony suspension cannot be performed by simple pipetting (not sure if it is a problem for Δags1Δgtb3 mutant strain, but it must be a problem for a wildtype strain). Authors should add more details for these steps. Otherwise the result could be quite inconsistent due to different operations.

3, Regarding to the data consistency, optical density measurements were performed in different medium cultures, such as AMM, YG and RPMI. The time course of OD600 results were shown in Fig2C and FigS5B. Comparing these data, the growth rate was different in each medium and data reproducibility was also quite different. Especially in YG medium the data reproducibility was the worst. As Fig. S5A showed, Δags1Δgtb3 mutant strain formed more visible pellets in YG medium than that in RPMI and AMM. Authors should compare such data and draw a conclusion for the best growth condition (medium selection and time for growth) of the proposed strategy. Otherwise, such variations will greatly limit the use of this strategy.

4, The strategy that described in this manuscript based on a Δags1Δgtb3 mutant strain. As the authors and many other publications noted, such mutant strain has very different cell wall architecture, which means it responds differently as a wildtype strain. In addition, mutant A. fumigatus strains that lack alpha-glucan and GAG are both less virulent. Therefore, it would be hard to tell if the outcomes from this strategy could also be useful for the clinical important strains, which should not be alpha-glucan and GAG defective strains.

5 Other strategies have also be used for testing drug sensitivity of filamentous fungi, such as testing colony growth on drug containing solid medium, or testing the pellets diameter in a shaken liquid culture. To validate the application of the proposed strategy in this manuscript, authors should compare these different methods and discuss how the proposed method may overwhelm other methods.

Minor point:
1 The title of the manuscript was not straightforward to represent the work in this study.
2 line 208, gene names are not italic
3 there is a typo in line 322, "he" should be "the"
4 Did author test the GAG content in gtb3 single deletion strain? It would be very helpful to show that gtb3 directly regulated GAG formation in A. fumigatus.
5 In their previous work, authors have generated other alpha-glucan and GAG defective A nidulans and A oryzae strains. Authors may consider to test their strategy with more strains to validate this idea.

Reviewer #2 (Comments for the Author):

incorporate all the comments indicated in the manuscript.

Staff Comments:

Preparing Revision Guidelines

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Quantitative monitoring of mycelial growth of *Aspergillus fumigatus* with modified surface polysaccharides in liquid culture

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Keywords: filamentous fungi, hyphal aggregation, optical density, α-1,3-glucan, galactosaminogalactan

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Running title (< 54 characters)

Quantification of *A. fumigatus* by optical density

Abstract

Filamentous fungi form multicellular hyphae, which generally form pellets in liquid shake cultures, during the vegetative growth stage. Because of these characteristics, growth-monitoring methods commonly used in bacteria and yeast have not been applied to filamentous fungi. We have recently revealed that the cell wall polysaccharide α-1,3-glucan and extracellular polysaccharide galactosaminogalactan (GAG) contribute to hyphal aggregation in *Aspergillus oryzae.* Here, we tested whether *Aspergillus fumigatus* shows dispersed growth in liquid media that can be quantitatively monitored, similar to that of yeasts. We constructed a double disruptant mutant of both the primary α-1,3-glucan synthase gene *ags1* and the putative GAG synthase gene *gtb3* in *A. fumigatus* AfS35, and found that the hyphae of this mutant were fully dispersed. Although the mutant completely lost α-1,3-glucan and GAG, its growth and susceptibility to antifungal agents were not significantly different from those of the parental strain. Biomass of the mutant in shake-flask cultures was proportional to optical density for at least 18 h. We were also able to quantify the dose response of hyphal growth to antifungal agents by measuring optical density. Overall, here we established a convenient strategy to monitor *A. fumigatus* hyphal growth. Our method can be directly used for screening for novel antifungals against *Aspergillus* species.

Introduction

Growth of bacteria or yeast can be easily quantified and their cultures can be used for susceptibility testing. However, because filamentous fungi form mycelia, limited methods have been developed to monitor their growth. Conventionally, growth of filamentous fungi has been assessed by measuring dry or wet biomass (1). The former approach is very accurate but time consuming because it requires tens of milligrams of dried fungi (1). The latter approach is fast, but results in considerable variation for samples of the same dry weight (1). To precisely quantify fungal mass, several direct or indirect measurement methods have been developed. The mass of fungi grown in wood is quantified by chitin content (2). The amount of ergosterol, a unique component of fungal cells, is also useful (3, 4). Quantitative PCR can be used to quantify fungal cells in soil and infected hosts. Banerjee et al. have measured turbidity of ground hyphal cells (5). A method for measurement of fluorescence of formazan produced by living cells from 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-tetrazolium-5-carboxanilide (XTT) has been developed (6). Recently, several methods based on image analysis have been reported (7-10).

In filamentous fungi, the surface structure differs between conidia and hyphae. The outer layer of conidia, termed the rodlet layer, is composed of polymerized

55 hydrophobin (11, 12) and is underlaid by melanin (13). Below the melanin is the cell
56 wall composed mainly of polysaccharides, i.e. glucan, chitin, and mannan (13, 14).
57 When hyphae extend from conidia, the polysaccharide layer is exposed to the surface,
58 and the structure of the polysaccharide network is continuously modified (15). In
59 *Aspergillus* species, hyphae have α-glucan in the outermost layer, which covers the
60 β-1,3-glucan and chitin layers (16). In the hyphal growth stage, filamentous fungi
61 secrete extracellular matrix (ECM) composed mainly of polysaccharides, proteins, and
62 lipids (13, 17). In *Aspergillus* species, the main ECM polysaccharides are
63 galactosaminogalactan (GAG), α-glucan, and galactomannan (18).
64 In shake-flask cultures, extended hyphae of filamentous fungi sometimes form
65 pellets, which compromise the accuracy of growth monitoring. During hyphal growth,
66 specific interactions of conidial wall components have been thought to be the primary
67 cause of aggregation (19, 20), but the specific component has been revealed only
68 recently. Fontaine et al. have reported that α-1,3-glucan directly contributes to
69 aggregation of germinating conidia in *Aspergillus fumigatus* (21). We have reported that
70 α-1,3-glucan is an adhesive factor for hyphae in the model fungus *Aspergillus nidulans*
71 and the industrial fungus *Aspergillus oryzae* (22-24). He et al. have also reported
72 α-1,3-glucan as a hyphal adhesive factor in *A. nidulans* (25). We have revealed that

GAG contributes to hyphal aggregation in *A*. *oryzae*, and that the hyphae of a strain deficient in both α-1,3-glucan and GAG are dispersed in shake-flask cultures (26). In the present study, we studied aggregation of *A. fumigatus*, a conditional pathogen of medical importance. We constructed a double disruptant of *ags1* and *gtb3*, which encode putative primary α-1,3-glucan and GAG synthases, respectively, in *A*. *fumigatus*, and observed that the hyphae of the mutant were dispersed. We monitored the growth of the mutant by measuring optical density at 600 nm (OD_{600}) and assessed whether the value of OD_{600} is directly proportional to the biomass. We compared MICs determined by OD_{600} measurements and by the standard Clinical and Laboratory Standards Institute (CLSI) antifungal susceptibility testing.

Materials and Methods

Strains and growth media

Strains used in this study are listed in Table 1. The nonhomologous end-joining deficient strain of *A. fumigatus*, AfS35, was used for all genetic manipulations (27). *Aspergillus* minimal medium (AMM) (28) was used for harvesting conidia and liquid cultivation of *A. fumigatus* strains. Czapek–Dox (CD) medium (Becton Dickinson and Company, Sparks, NV, USA) was used for transformation of *A. fumigatus*. YG medium (29) was used for liquid cultivation in some experiments. RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS; 0.165 M; pH 7.0) was used for shake-flask and plate cultures to evaluate susceptibility to antifungal agents.

Construction of plasmids and strains

The sequences of all primers are listed in Table S1. Transformation of *A. fumigatus* was performed as described previously (30) with some modifications. Briefly, conidia were inoculated into YG medium (10 mL) and incubated at 37°C with shaking at 150 rpm. Conidial suspension was mixed with 10 mL of KCl–citric acid solution (1.1 M KCl, 100 mM potassium citrate buffer, pH 5.8) containing 0.4 g of VinoTaste Pro (Novozymes, Bagsværd, Denmark) and incubated for 1 h at 30°C with gentle shaking. Generated

protoplasts were transformed with 2–5 μg of DNA cassettes, plated onto CD medium supplemented with 1 M sucrose, and incubated for 15 h at 37°C. Then CD medium containing 0.35% Bacto Agar and 400 μg/mL hygromycin was overlaid onto the CD plate.

Construction of the Δ*ags1* strain: The first round of PCR amplified gene fragments containing the 5′ non-coding region (amplicon 1) and the coding region (amplicon 2) of *ags1* from AfS35 genomic DNA, and the *hph* gene (amplicon 3) from pSK397 (27) (Fig. S1A). Amplicon 1 was amplified with primers Afags1-LU and Afags1-LL, amplicon 2 with primers Afags1-RU and Afags1-RL, and amplicon 3 with primers 397-5 and 397-3. The three resulting PCR products were gel-purified and fused into a disruption cassette in the second round of PCR. The resulting PCR product was gel-purified and transformed into the AfS35 strain (Fig. S1B). Replacement of the *ags1* gene was confirmed by PCR (Fig. S1C).

Construction of the Δ*ags1* (loxP) strain: The *ags1* gene was disrupted by using the Cre/*loxP* marker recycling system (31). The plasmid pAH-Cre was first constructed (Fig. S2A) as follows. The *hph* marker (amplicon 1) was amplified from pSK397. A fragment

120 containing the *lox71*, *xynG2* promoter (P*xynG2*), and *Cre* (amplicon 2) was amplified
121 from the plasmid pAAG-Cre (31). A fragment containing *ampR* and ori (amplicon 3)
122 was amplified from pUC19. Amplicon 1 was amplified with primers IF1-lox66-hph-Fw
123 and 397-5, amplicon 2 with primers IF2-PxynG2-Fw and IF2-lox71-TagdA-Rv, and
124 amplicon 3 with primers IF3-lox71-pUC19 and IF3-lox66-pUC19. The three amplicons
125 were fused by using a NEBuilder HiFi DNA Assembly kit (New England Biolabs,
126 Ipswich, MA, USA). The pAH-Cre plasmid was used as a template for PCR with
127 primers M13-47 and RV-M, resulting in amplicon C. PCR was performed to amplify
128 gene fragments containing the 5′ non-coding region (amplicon L) and the coding region
129 (amplicon R) of *ags1* from AfS35 genomic DNA. Amplicon L was amplified with
130 primers Afags1-LU and Afags1-LL-M13, and amplicon R with primers
131 Afags1-RU-M13 and Afags1-RL. Amplicons C, L, and R were gel-purified and fused
132 into a disruption cassette by PCR (Fig. S2B). The resulting PCR product was
133 gel-purified and transformed into the AfS35 strain (Fig. S2C). Candidate strains were
134 selected on AMM medium containing 400 μg/mL hygromycin and then cultured on
135 AMM medium without hygromycin containing 1% xylose to induce *Cre* expression (Fig.
136 S2C). Strains sensitive to hygromycin were isolated by culture on AMM plates with or
137 without hygromycin. Replacement of the *ags1* gene was confirmed by PCR (Fig. S2D).

Disruption of the *gtb3* gene to generate single (Δ*gtb3*) and double (Δ*ags1*Δ*gtb3*) mutants: The first round of PCR amplified gene fragments containing the 5′ non-coding region (amplicon 1) and the coding region (amplicon 2) of *gtb3* from AfS35 genomic DNA, and the *hph* gene (amplicon 3) from pSK397 (Fig. S3A). Amplicon 1 was amplified with primers Afgtb3-LU and Afgtb3-LL, amplicon 2 with primers Afgtb3-RU and Afgtb3-RL, and amplicon 3 with primers 397-5 and 397-3. The three resulting PCR products were gel-purified and fused into a disruption cassette in the second round of PCR. The resulting PCR product was gel-purified and transformed into the AfS35 strain to generate Δ*gtb3* or into the Δ*ags1* (loxP) strain to generate Δ*ags1*Δ*gtb3* (Fig. S3B). Replacement of the *gtb3* gene was confirmed by PCR (Fig. S3C).

Biofilm visualization

Conidia (final concentration, 1×10^6/mL) of the AfS35, Δ*ags1*, Δ*gtb3*, and Δ*ags1*Δ*gtb3* strains were inoculated into 1 mL of AMM medium in a polystyrene 24-well plate and incubated for 24 h at 37°C. Culture medium was removed, the plate was washed twice with PBS, and 0.5 mL of 0.5% (w/v) crystal violet solution was added. The plate was incubated at room temperature for 5 min. Excess stain was removed, and the plate was

156 washed twice with water. The biofilm was imaged with a flatbed scanner (GT-X970;
157 Seiko Epson Corp., Nagano, Japan).
158
159 *Determination of cell wall components*
160 Mycelia were cultured for 24 h in AMM medium, collected by filtering through
161 Miracloth (Merck Millipore, Darmstadt, Germany), washed twice with water, and
162 freeze-dried. Cell wall components were fractionated as described previously with some
163 modifications (22). The mycelia were ground with a mortar and pestle, and the powder
164 (1 g) was suspended in 40 mL of 0.1 M sodium phosphate buffer (pH 7.0). The mycelial
165 suspension was autoclaved at 121°C for 60 min and centrifuged at 10,000 × *g* for 10
166 min. The supernatant was retained, and the pellet was resuspended in 40 mL of 0.1 M
167 phosphate buffer (pH 7.0), autoclaved, and centrifuged again at 10,000 × *g* for 10 min.
168 The supernatants from the first two centrifugations were combined, dialyzed against
169 water, and lyophilized, resulting in the hot-water soluble (HW) fraction. The pellet was
170 suspended in 50 mL of 0.1 M NaOH for 6 h at 4°C. The suspension was centrifuged at
171 10,000 × *g* for 10 min, and the pellet was suspended in 50 mL of 0.1 M NaOH and
172 centrifuged again at 10,000 × *g* for 10 min. The supernatant was designated as the
173 diluted-alkali-soluble fraction. The pellet was suspended in 50 mL of 2 M NaOH for 24

174 h at 4°C and centrifuged as above. The supernatant and the pellet were designated as the
175 alkali-soluble (AS) and alkali-insoluble (AI) fractions, respectively. These fractions
176 were neutralized with acetic acid, dialyzed against water, and the AS fraction was
177 centrifuged. The supernatant was designated as AS1, and the precipitate as AS2. All the
178 fractions were freeze-dried and weighed. Monosaccharide composition of the HW, AS2,
179 and AI fractions was quantified according to Yoshimi et al. (22).
180
181 *In vitro antifungal susceptibility testing*
182 MICs and Minimal effective concentrations (MECs) of AfS35 and Δ*ags1*Δ*gtb3* strains
183 against micafungin (MCFG), caspofungin (CPFG), amphotericin B (AMB), flucytosine
184 (5FC), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), and miconazole
185 (MCZ) were evaluated using Frozen Plate for Antifungal Susceptibility Testing of
186 Yeasts (Eiken Chemicals, Tokyo, Japan) according to the method for CLSI M38-A2
187 (32).
188
189 *Measurement of optical density*
190 To measure the turbidity of conidia and mycelia in shake-flask culture, conidia (final
191 concentration, 1.0×10^7/mL) of AfS35 or Δ*ags1*Δ*gtb3* strain were inoculated into 50

192 mL of AMM, YG, or RPMI medium in a 200-mL Erlenmeyer flask and rotated at 160
193 rpm at 37°C. The culture broth (2 mL) was withdrawn at each sampling point, and 100
194 μL was mixed with 100 μL of 100 mM sodium phosphate buffer (pH 7.0) containing
195 4% paraformaldehyde in a 96-well plate. OD_{600} was measured in a microplate reader
196 (Synergy LX, BioTek, Winooski, VT, USA). The morphology of conidia and mycelia
197 was observed under an IX81 inverted fluorescence microscope (Olympus, Tokyo,
198 Japan).

199 To measure the turbidity of the dilution series of mycelial suspensions, conidia
200 (final concentration, 1.0×10^7/mL) of the Δ*ags1*Δ*gtb3* strain were inoculated into 50
201 mL of AMM medium in a 200-mL Erlenmeyer flask and rotated at 160 rpm at 37°C for
202 18 h. The culture broth was filtered through Miracloth, and the mycelia were
203 resuspended in 10 mL of water; 5 mL of the suspension was mixed with 5 mL of water
204 (2-fold dilution). By repeating this procedure, the dilution series were prepared; 200 μL
205 of each dilution was dispensed into 5 wells of a 96-well plate, and OD_{600} was measured.
206 The remaining mycelial suspension (4 mL) was freeze-dried and weighed.

207

208 *Evaluation of antifungal susceptibility of the Δ*ags1*Δ*gtb3 *strain by optical density*
209 To evaluate the susceptibility of the Δ*ags1*Δ*gtb3* strain to MCFG, AMB, 5FC, ITC and

VRC, conidia (final concentration, 5.0×10^6/mL) were inoculated into 500 µL of RPMI medium containing an antifungal agent in a 48-well plate and rotated at 300 rpm using MicroMixer E-36 (Taitec, Koshigaya, Japan) at 35°C for 15 h, and OD_{600} was measured in triplicate. Each test was performed in triplicate, and standard deviations were determined. Relative growth rate was calculated as the percentage of OD_{600} at each drug concentration relative to the mean OD_{600} in the absence of the drug.

MCFG, AMB, 5FC, ITC and VRC were prepared according to CLSI M38-A2 (32). Briefly, water-insoluble AMB, VRC, and ITC were dissolved in DMSO, and then the dilution series were prepared by mixing with DMSO. Water-soluble MCFG and 5FC were dissolved in RPMI medium and the dilution series were prepared by mixing with RPMI.

Results

The double disruptant of ags1 and gtb3 of *A. fumigatus* growth in in shake-flask culture

Aspergillus fumigatus possesses three α-1,3-glucan synthase genes, *ags1–3*, and *ags1* has a primary role in α-1,3-glucan biosynthesis (33-35). Here, we constructed an *ags1* disrupted strain of *A. fumigatus* and examined its macromorphology in shake-flask culture. Hyphal pellets were smaller in the Δ*ags1* strain than in the AfS35 strain (Fig. 1A), suggesting that α-1,3-glucan contributes to hyphal aggregation in *A. fumigatus*; this result was consistent with the phenotypes of α-1,3-glucan-deficient strains of *A. oryzae*, *A. nidulans*, and *A. niger* (22, 23, 25, 36, 37). We also constructed a strain with disrupted *gtb3* gene, which encodes putative GAG synthase (38). Although the pellet morphology of the Δ*gtb3* strain was similar to that of AfS35 (Fig. 1A), it did not form a biofilm (Fig. S4A). To generate a double mutant, the *ags1* and *gtb3* genes were sequentially disrupted (Δ*ags1*Δ*gtb3*); the hyphae of the Δ*ags1*Δ*gtb3* strain were completely dispersed in shake-flask culture (Fig. 1A), consistent with the *A. oryzae* mutant deficient in α-1,3-glucan and GAG (26). All the mutants constructed here showed similar radial growth and conidiation on agar plates (Fig. S4B). Antifungal susceptibilities of Δ*ags1*Δ*gtb3* and AfS35 were similar (Table 2).

241 To validate that the hyphal dispersion of Δ*ags1*Δ*gtb3* was caused by a lack of
242 α-1,3-glucan and GAG, we analyzed the cell wall components. The AS2 fraction mainly
243 contains α-1,3-glucan with a small amount of galactomannan, and the AI fraction
244 contains β-1,3-glucan, chitin, and galactomannan (35, 39). The AS2 fraction from the
245 AfS35 strain contained 5.2% of glucose (AS2-Glc), and the AI fraction contained 1.7%
246 of glucosamine (AI-GlcN) and 7.5% of glucose (AI-Glc) (Fig. 1B). The minor
247 components (<1% each) were galactose (AS2-Gal and AI-Gal) and mannose (AI-Man)
248 (Fig. 1B). Galactosamine was scarcely detected in the AS2 and AI fractions from the
249 AfS35 strain. The Δ*ags1*Δ*gtb3* strain contained only 0.1% of AS2-Glc, which was
250 significantly less than in AfS35 ($P < 0.05$; Fig. 1B), suggesting that α-1,3-glucan was
251 almost abolished by disruption of the *ags1* gene. The HW fraction from AfS35
252 contained 0.019% of galactosamine, whereas galactosamine was scarcely detected in the
253 HW fraction from the Δ*ags1*Δ*gtb3* strain (Fig. 1C), suggesting that disruption of the
254 *gtb3* gene resulted in a complete loss of GAG. The contents of AI-GlcN, AI-Gal, AI-Glc,
255 and AI-Man were slightly higher in the Δ*ags1*Δ*gtb3* strain than in AfS35 (Fig. 1B),
256 which might be attributable to compensation of the defect in α-1,3-glucan and/or GAG
257 in the Δ*ags1*Δ*gtb3* strain.
258

Growth of the Δags1Δgtb3 strain can be monitored by optical density at 600 nm

Dispersed hyphal morphology of the Δ*ags1*Δ*gtb3* strain prompted us to try to monitor its growth by turbidity. Conidia of the AfS35 and Δ*ags1*Δ*gtb3* strains were inoculated into 50 mL of AMM liquid medium and cultured with shaking at 37°C for up to 24 h. At the indicated time points, the culture was dispensed into a 96-well plate and fixed, and OD_{600} was measured (Fig. 2A). Pellets became visible in AfS35 from 9 h and their size increased with time, whereas the Δ*ags1*Δ*gtb3* hyphae were continuously dispersed (Fig. 2B). The OD_{600} measurements of AfS35 showed that, for example, the first and third quartiles from 12 measurements were 0.894 and 0.337, respectively, at 15 h (Fig. 2C), suggesting no correlation between apparent AfS35 growth and OD_{600}. In the Δ*ags1*Δ*gtb3* strain, the first and third quartiles were 0.633 and 0.595 at 15 h (Fig. 2C), suggesting that measurement of OD_{600} is suitable for monitoring the growth of the mutant. The OD_{600} measurements seemed to be reproducible at least until 18 h in the Δ*ags1*Δ*gtb3* strain (Fig. 2C). Microscopic observations showed that AfS35 formed aggregates as early as during conidial swelling at 6 h, whereas pellet formation was scarce in the Δ*ags1*Δ*gtb3* strain up to 24 h (Fig. 2D). Although the growth rate differed in RPMI and YG media, the growth of the Δ*ags1*Δ*gtb3* strain could also be monitored by OD_{600} values (Fig. S5A, B).

To evaluate the linearity of OD_{600} values versus biomass, a dilution series of Δ*ags1*Δ*gtb3* culture was prepared, and OD_{600} was measured. A plot of biomass versus the reciprocal of dilution rate showed that the dilution was performed accurately (Fig. S6A). The relationship between OD_{600} values and the reciprocal of dilution rate was linear below OD_{600} = 0.75 (Fig. S6B). Using a standard curve, biomass could be determined from OD_{600} values (Fig. S6C). Overall, growth of the Δ*ags1*Δ*gtb3* strain in shake-flask culture could be easily and precisely monitored by measuring culture turbidity.

*Antifungal susceptibility of the Δ*ags1*Δ*gtb3 *strain evaluated from turbidity measurements*

As an application of the monitoring method, we evaluated the dose response of growth to the presence of antifungal agents: AMB, 5FC, ITC, VRC, and MCFG. The Δ*ags1*Δ*gtb3* strain was grown in RPMI medium containing an antifungal agent for 15 h in a 48-well plate, and then OD_{600} was measured. Growth was repressed in the presence of the ITC and VRC in a dose-dependent manner (Fig. 3). Relative growth rate was around 10% in spite of high drug concentrations because of the presence of conidia in each well. Conidia were swollen but did not germinate in the presence of high

295 concentrations of the drugs (data not shown). Drug concentrations that caused 50%
296 inhibition of growth were 0.125 μg/mL for VRC and 0.0313 μg/mL for ITC (Fig. 3).
297 Growth was completely inhibited at 2 μg/mL of VRC and 1 μg/mL of ITC (Fig. 3),
298 which was in agreement with MICs determined by CLSI M38-A2 (Table 2). The
299 concentrations that caused 50% growth inhibition were 0.0625 μg/mL for AMB and 4
300 μg/mL for 5FC (Fig. 3). Growth was completely inhibited at 0.5 μg/mL of AMB and
301 256 μg/mL of 5FC (Fig. 3). Growth inhibition by MCFG was hard to evaluate (Fig. 3)
302 because this drug had a limited effect in the early stage of germination. Overall, the
303 growth of the Δ*ags1*Δ*gtb3* strains could be quantified by turbidity in a 48-well plate in
304 the presence of the antifungal agents tested except for MCFG.
305
306

Discussion

In the present study, we constructed a double disruptant of the *ags1* and *gtb3* genes, which have roles in α-1,3-glucan and GAG biosynthesis, respectively, and observed that the hyphae of the mutant were fully dispersed in shake-flask culture (Fig. 1A). Using this mutant, we accurately monitored hyphal growth by measuring turbidity for the first time in filamentous fungi (Fig. 2) and evaluated dose-dependent growth in the presence of antifungal agents (Fig. 3).

Unlike in unicellular microorganisms including yeasts, the colony forming unit values in filamentous fungi cannot be determined from turbidity. Growth of filamentous fungi is conventionally monitored using methods that have limitations such as measurement of dry mass (1). The dry mass method is easy and precise, but it requires tens of milligrams of material and is time consuming (1). Because of the biomass requirement, precise quantification of cells during the early stage of germination is particularly difficult. Here, we used turbidity to quantify a limited amount of cells of a filamentous fungus. In AMM, biomass started increasing at 9–12 h and this increase flattened out at 18–24 h, and the increase per unit time (i.e., slope of the line connecting data points) was greatest at 12–15 h (Fig. 2C).

Biosynthesis of GAG is thought to be regulated by a cluster of five genes (*uge3*,

325 *sph3*, *ega3*, *agd3*, and *gtb3* in *A. fumigatus*) (40). Disruption of the *uge3* and *sph3* genes
326 results in the absence of GAG in *A. fumigatus* (41, 42). The *gtb3* disruptant constructed
327 here showed no biofilm formation (Fig. S4A). GalN was hardly detected in the *gtb3*
328 disruptant (Fig. 1C), suggesting that Gtb3 is essential for GAG biosynthesis. Although
329 Gtb3 seems to be involved in synthesizing polymers of galactose and
330 *N*-acetylgalactosamine from UDP-galactose and UDP-*N*-acetylgalactosamine (38, 43),
331 direct biochemical evidence has not been reported. To unveil the mechanism of GAG
332 biosynthesis, further biochemical analyses are needed.

333 Here, we quantified the growth of *A. fumigatus* in the presence of antifungal
334 agents by OD_{600} using the strain with dispersed hyphae. This strain could be used as a
335 model for the high-throughput screening of antifungal compounds. However, our study
336 has a limitation that has to use of a gene-deletion strain. The decrease in growth in the
337 presence of an antifungal agent depended on its type, but the mutant hyphae remained
338 dispersed in spite of the agent. These results suggest that the gene expression alteration
339 induced by antifungal agents should be more clearly observed in the mutant than in the
340 parental strain, which forms hyphal pellets. Genome-editing can accelerate generation
341 of a strain that deficient in α-1,3-glucan and GAG from clinically isolated strain. Cell
342 sorting might be used to isolate single germinating conidia of *A. fumigatus* that are

resistant to some antifungal agents. We believe that dispersion of cells could dramatically extend the applicability of the analytical methods for filamentous fungi.

Acknowledgements

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475 **Figure Legends**

476 **Figure 1. Characterization of the Δ*ags1*, Δ*gtb3*, and double-disruptant strains of *A.***

477 ***fumigatus*.** (**A**) Growth characteristics of each strain. Conidia (5.0×10^5/mL) of AfS35

478 (control) or mutant strains were inoculated into AMM liquid medium and rotated at 160

479 rpm at 37°C for 24 h. (**B**) Monosaccharide composition of cell wall AS2 and AI

480 fractions from the AfS35 and Δ*ags1*Δ*gtb3* strains. Error bars are standard deviation

481 from three replicates. **P* < 0.05 (Student's *t*-test). (**C**) Galactosamine content in the HW

482 fraction from the AfS35 and Δ*ags1*Δ*gtb3* strains.

483 **Figure 2. Evaluation of growth of the AfS35 and Δ*ags1*Δ*gtb3* strains by turbidity.**

484 (**A**) Scheme of the experiment. Conidia were inoculated into AMM liquid medium and

485 aliquots were withdrawn at the indicated time points. The culture broth was mixed with

486 4% paraformaldehyde (PFA) in a 96-well plate, and the OD_{600} values were measured

487 with a microplate reader. (**B**) Growth of the AfS35 and Δ*ags1*Δ*gtb3* strains. (**C**) Time

488 course of OD_{600}. The OD_{600} values were calculated from 12 measurements per time

489 point and are shown as box plots. Lines in boxes indicate medians, and crosses indicate

490 averages. Circles indicate outliers. (**D**) Time course of hyphal morphology of the AfS35

491 and Δ*ags1*Δ*gtb3* strains.

492 **Figure 3. Growth of the Δ*ags1*Δ*gtb3* strain in the presence of antifungal agents**

monitored by OD_{600}. Conidia (5.0×10^6/mL) were inoculated into 500 μL of RPMI liquid medium containing the indicated antifungal agent in a 48-well plate and rotated at 300 rpm at 35°C for 15 h. The OD_{600} values were measured with a microplate reader. Error bars are standard deviations from three biological replicates.

Responses to Reviewers

Reviewer #1

In this manuscript, Ken and colleagues constructed a mutant strain of A. fumigatus that lacks the alpha-glucan and galactosaminogalactan in the cell wall. Based on this mutant strain, they developed a strategy to assess the cell growth by measuring optical density. As the authors proposed, this new strain may be applied for high throughput anti-fungal drug screening in shaken liquid growth condition. However, there are several concerns for the high throughput screening strategy. Substantially more experiment should be performed before it could be validated strategy.

Major points:

1, Growth of unicellular organism and filamentous fungi (multicellular) are fundamentally different. Use of optical density for measuring the growth of unicellular organism is based on their uniform distribution. In contrast, filamentous fungi grow in a way to elongate the existing mycelia, as the authors show in Fig. 2D. The conidia and mycelia of filamentous fungi tend to cluster together and form pellets in a shaken liquid culture. Even the Δags1Δgtb3 mutant strain, the mycelia were not totally separated from each other. They still form small but visible pellets in the shaken liquid medium, especially in the YG medium. If so, it is hard to agree with the concept that a simple measurement of optical density could faithfully represent the cell density.

Our experiments suggest that AMM and RPMI medium are suitable for the evaluation of the growth of the *A. fumigatus*Δ*ags1*Δ*gtb3* strain by optical density. To gain insight into why small but visible pellets were formed in YG medium, we labelled the Δ*ags1*Δ*gtb3* mutant grown in YG medium for 24 h with alpha-1,3-glucanase

alpha-1,3-glucan–binding domain (AGBD) fused with GFP (AGBD-GFP), and observed clear labelling of the septa and the outline of the cell. As *A. fumigatus* has three alpha-1,3-glucan synthase genes (*ags1—3*), alpha-1,3-glucan in labeled hyphae of the Δ*ags1*Δ*gtb3* mutant was likely synthesized by Ags2 and/or Ags3. In *A. oryzae* Δ*agsA*Δ*agsB*Δ*agsC*Δ*sphZ*Δ*ugeZ* (AGΔ-GAGΔ) all the three genes encoding alpha-1,3-glucan synthases were disrupted, and this mutant had fully dispersed hyphae in all the media tested including YG medium. Although the data on *A. oryzae* AGΔ-GAGΔ are consistent with the above data on *A. fumigatus* Δ*ags1*Δ*gtb3*, further experiments are needed to prove the effect of alpha-1,3-glucan on pellet formation in the Δ*ags1*Δ*gtb3* mutant. We have revised the manuscript in lines 354—370.

2, Materials and Methods section. For the measurement of optical density, authors described that "100μL was mixed with 100 μL of 100 mM sodium phosphate buffer (pH 7.0) containing 4% paraformaldehyde in a 96-well plate". However, this statement was not clear. Especially when the pellets of the colonies become larger in late time points, the transfer of such colony suspension cannot be performed by simple pipetting (not sure if it is a problem for Δags1Δgtb3 mutant strain, but it must be a problem for a wildtype strain). Authors should add more details for these steps. Otherwise the result could be quite inconsistent due to different operations.

We have added more details as follows.

Line 196—199: The culture (2 mL) was withdrawn with wide-bore tips at each sampling point, and 100 μL of the culture was mixed by pipetting with wide-bore tips with 100 μL of 100 mM sodium phosphate buffer (pH 7.0) containing 4% paraformaldehyde in a 96-well plate.

3, Regarding to the data consistency, optical density measurements were performed in

different medium cultures, such as AMM, YG and RPMI. The time course of OD600 results were shown in Fig2C and FigS5B. Comparing these data, the growth rate was different in each medium and data reproducibility was also quite different. Especially in YG medium the data reproducibility was the worst. As Fig. S5A showed, Δags1Δgtb3 mutant strain formed more visible pellets in YG medium than that in RPMI and AMM. Authors should compare such data and draw a conclusion for the best growth condition (medium selection and time for growth) of the proposed strategy. Otherwise, such variations will greatly limit the use of this strategy.

We have revised the text to describe the best conditions for the proposed strategy in lines 354—376.

4, The strategy that described in this manuscript based on a Δags1Δgtb3 mutant strain. As the authors and many other publications noted, such mutant strain has very different cell wall architecture, which means it responds differently as a wildtype strain. In addition, mutant A. fumigatus strains that lack alpha-glucan and GAG are both less virulent. Therefore, it would be hard to tell if the outcomes from this strategy could also be useful for the clinical important strains, which should not be alpha-glucan and GAG defective strains.

We have explained the outcomes of this strategy as follows.

Line 390—399: Fungi, especially filamentous fungi, are phenotypically heterogeneous in their growth. When filamentous fungi form pellets in liquid culture, oxygen reaches only 200 μm from the pellet surface. Therefore, cellular conditions seem to differ considerably between the surface and the interior of the pellet. The hyphae of Δ*ags1*Δ*gtb3* are easily dispersed in liquid culture and thus seem to have relatively

constant cellular metabolism, although metabolic differences between apical and subapical cells of hyphae are hardly avoidable. Dispersed hyphae of Δ*ags1*Δ*gtb3* in culture could be useful to analyze cellular responses such as autophagy, metabolic changes in the presence of antifungal agents, and responses to alteration of growth conditions.

At present, we are investigating culture conditions that prevent pellet formation in the parental strain, which would be useful for testing clinical isolates. The perspectives are described in the manuscript as follows.

Line 407—410: Establishing culture conditions that prevent pellet formation of a strain with an intact cell wall structure could expand the application of growth monitoring by optical density. Understanding the biochemical and physicochemical properties of α-1,3-glucan and GAG will contribute to finding suitable culture conditions.

5 Other strategies have also be used for testing drug sensitivity of filamentous fungi, such as testing colony growth on drug containing solid medium, or testing the pellets diameter in a shaken liquid culture. To validate the application of the proposed strategy in this manuscript, authors should compare these different methods and discuss how the proposed method may overwhelm other methods.

We have revised the manuscript to compare our method with conventional methods as follows.

Line 377—389: Monitoring growth by optical density is superior to that by conventional methods for several reasons: 1) growth monitoring is quantitative and continuous. During drug testing based on CLSI M38-A2, growth has to be observed visually. The mutant with dispersed hyphae would allow establishment of automated drug screening for *Aspergillus*. 2) Fungicidal and fungistatic drugs could be selected

using our strategy. We propose to screen anti-*Aspergillus* drugs from a drug library, although drugs that do not inhibit germination but disorder hyphal extension, such as echinocandin, might be hard to select using our method. Recently, Beattie and Krysan reported that the release of intracellular adenylate kinase from hyphal cells is a sensitive readout to detect cell lysis and is useful for screening antifungal reagents against *A. fumigatus*. In combination with the adenylate kinase method, our strategy may allow selection of anti-*Aspergillus* drugs with various spectra by monitoring optical density of fungal culture.

Minor point:

1 The title of the manuscript was not straightforward to represent the work in this study.

We have changed the title as follows:

Quantitative monitoring of mycelial growth of *Aspergillus fumigatus* in liquid culture by optical density

2 line 208, gene names are not italic

Plain text is correct in this case because the whole heading is in italics.

3 there is a typo in line 322, "he" should be "the"

Corrected.

4 Did author test the GAG content in gtb3 single deletion strain? It would be very helpful to show that gtb3 directly regulated GAG formation in A. fumigatus.

We have added the data on GalN content of Δ*gtb3* to Fig. 1C.

5 In their previous work, authors have generated other alpha-glucan and GAG defective A nidulans and A oryzae strains. Authors may consider to test their strategy with more strains to validate this idea.

We have monitored the growth of the *Aspergillus oryzae* mutant lacking both alpha-1,3-glucan and GAG, and added Figure S7. As expected, our strategy was applicable to this *A. oryzae* mutant. We have revised the text (lines 202–205 in the Materials and Methods, lines 292–297 in the Results, and Table 1). Unfortunately, the *Aspergillus nidulans* mutant is not available to me at my current institute.

Reviewer #2

Incorporate all the comments indicated in the manuscript.

Line 92: The abbreviation should be indicated in bracket as it is for the 1st time.

We have added the names of YG and RPMI media in full on lines 92–94.

Line 100: Why 37^{0}?

To simply quantify growth, we cultured the fungi at 37°C. To evaluate drug sensitivity, we cultured them at 35°C according to the protocols of CLSI M38-A2.

Line 233: Why here? This should be in the discussion part unless otherwise the Results and Discussion parts merged together.

We have moved the sentence to the Discussion section (lines 340–343).

Line 256—259: More evidences from previous researchers that support the current finding should be required in the discussion part.

We have explained the supposed mechanism of the increase in AI-Glc and AI-GlcN in Δ*ags1*Δ*gtb3* and cited appropriate references (lines 347–353).

Line 314: The discussion part is very short and not well expressed. In paragraph 1, 2 and 4 you only present the finding and not well interpreted and do not compared with previous findings. Generally the discussion part require major revision

We have substantially revised the Discussion.

October 4, 2021

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Japan

Re: Spectrum00063-21R1 (Quantitative monitoring of mycelial growth of *Aspergillus fumigatus* in liquid culture by optical density)

Dear Dr. Yoshitsugu Miyazaki:

Cell wall rearrangements were shown to lead to differences in antifungal susceptibility. Both reviewers suggested to perform additional tests using common cell wall stress agents and conditions (T, pH, etc.) or different medium. Furthermore, the a-1,3 beta glucan labeling with GFP should be added to the manuscript, as suggested by reviewer 1. Visualization of the labeling under different conditions (different medium or cell wall stress) would further strengthen the manuscript findings. Please include the requested modifications to the revised version.

Thank you for submitting your manuscript to Microbiology Spectrum. When submitting the revised version of your paper, please provide (1) point-by-point responses to the issues raised by the reviewers as file type "Response to Reviewers," not in your cover letter, and (2) a PDF file that indicates the changes from the original submission (by highlighting or underlining the changes) as file type "Marked Up Manuscript - For Review Only". Please use this link to submit your revised manuscript - we strongly recommend that you submit your paper within the next 60 days or reach out to me. Detailed information on submitting your revised paper are below.

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Sincerely,

Slavena Vylkova

Editor, Microbiology Spectrum

Journals Department
American Society for Microbiology
1752 N St., NW
Washington, DC 20036
E-mail: spectrum@asmusa.org

Reviewer comments:

Reviewer #1 (Comments for the Author):

The authors have addressed most of my concerns during the revision. Especially the elaborated Discussion pointed out the potential advantages and drawbacks of the proposed strategy. I have only one concern left regarding the α-1,3-glucan compensation in YG medium. It is unclear why the result of α-1,3-glucan labeling with GFP was not shown in the manuscript. Author should add such data in the revised manuscript.
Moreover, if re-formation of α-1,3-glucan was the reason for the pellet in YG medium. This result suggested that other factors, especially the medium composition, may challenge the proposed strategy. Beauvais has previously generated a triple A. fumigatus deletion stain that had no α-1,3-glucan at all. And there should be no compensation of α-1,3-glucan in this strain. I would suggest the authors to further construct a new mutant stain based on this triple deletion strain. This would further warranty the compatibility of the proposed strategy.

Reviewer #3 (Comments for the Author):

First, I am an additional reviewer after the first review. The manuscript entitled "Quantitative monitoring of the mycelial growth of Aspergillus fumigatus in liquid culture by optical density" written by Miyazawa K et al. describes a method to monitor hyphal growth of pathogenic filamentous fungus, A. fumigatus, showing age1 and gtb3 double mutant using absorbance OD600 as an indicator. This is also the first study to demonstrate that Gtb3 is involved in biosynthesizing glycosaminoglycans (GAGs). The method was used to assess the effects of antifungal drugs used in clinical treatment, and the results were consistent with those based on the method described in CLSI M38-A2. Monitoring growth by absorbance allows for rapid screening of antifungal drugs. Additionally, this study was robustly conducted and had no technical problems. However, I think this study needs to be revised on several points before publication.

1. I feel uncomfortable with the choice of the word "biomass." I think the term is inappropriate to describe the tiny weight of fungus.
2. The cell wall of age1 and bgtb3 double mutant may be thinner than the parental strain, making it easier for antifungal agents to penetrate it. Considering such influence, I think the number of antifungal agents' examples in the experiment is too small. Therefore, please present additional data that have been widely verified, such as the effects of chemical compounds (except the antifungal agents shown here) and the effects of some stress conditions (temperature, osmotic pressure and pH etc).
3. In the discussion section (line 407), you have said that "establishing culture conditions that prevent pellet formation helps screen antifungal drugs using clinical isolate strain," but this has nothing to do with your findings. Rather, if inhibitors of alpha-glucan and GAG biosynthesis are discovered, you could perform the similar experiment with the inhibitor on a clinical strain. Therefore, I think you should discuss this point.

Staff Comments:

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Response to reviewers

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We have added images of mycelial cells labeled with AGBD–GFP. The AfS35 strain was clearly labeled with AGBD–GFP along the outline of the cells. The Δ*ags1*Δ*gtb3* strain cultured in YG medium (pellet formed) was clearly labeled with AGBD–GFP in the septa and along the hyphal outline. These results suggest that hyphal pellet formation in Δ*ags1*Δ*gtb3* cultured in YG medium depended on α-1,3-glucan synthesized by *ags2* and/or *ags3*. We regret not having constructed the mutant with a triple deletion of α-1,3-glucan synthase genes because of the need for several transformations and the tight revision schedule, but we have labeled the *Aspergillus oryzae* Δ*agsA*Δ*agsB*Δ*agsC*Δ*sphZ*Δ*ugeZ* (AGΔ-GAGΔ) strain with AGBD–GFP. Both the YG- and AMM-cultured AGΔ-GAGΔ cells were scarcely labeled. In both media, AGΔ-GAGΔ hyphae were dispersed. These results support the assumption that the induction of α-1,3-glucan synthesis contributes to pellet formation in YG medium. We have revised the text in lines 220–223 in the

Materials and Methods, lines 318−325 in the Results, and lines 386−390 in the Discussion, and added Figure S9.

Reviewer #3 (Comments for the Author):

First, I am an additional reviewer after the first review. The manuscript entitled "Quantitative monitoring of the mycelial growth of Aspergillus fumigatus in liquid culture by optical density" written by Miyazawa K et al. describes a method to monitor hyphal growth of pathogenic filamentous fungus, A. fumigatus, showing age1 and gtb3 double mutant using absorbance OD600 as an indicator. This is also the first study to demonstrate that Gtb3 is involved in biosynthesizing glycosaminoglycans (GAGs). The method was used to assess the effects of antifungal drugs used in clinical treatment, and the results were consistent with those based on the method described in CLSI M38-A2. Monitoring growth by absorbance allows for rapid screening of antifungal drugs. Additionally, this study was robustly conducted and had no technical problems. However, I think this study needs to be revised on several points before publication.

1. I feel uncomfortable with the choice of the word "biomass." I think the term is inappropriate to describe the tiny weight of fungus.

In response to your suggestion, we have replaced “biomass” with “mycelial weight” (lines 30, 80, 304, 305, 308, and 381).

2. The cell wall of age1 and gtb3 double mutant may be thinner than the parental strain, making it easier for antifungal agents to penetrate it. Considering such influence, I think the number of antifungal agents' examples in the experiment is too small. Therefore, please present additional data that have been widely verified, such as the effects of chemical compounds (except the antifungal agents shown here) and the effects of some stress conditions (temperature, osmotic pressure and pH etc).

We have evaluated the growth of the *A. fumigatus* AfS35, Δ*ags1*, Δ*gtb3*, and

Δ*ags1*Δ*gtb3* strains under stress. The growth was similar among the four strains under several temperature, osmotic stress, and pH conditions. Congo red and calcofluor white are effective inhibitors of the growth of α-1,3-glucan- and/or GAG-deficient strains, which are consistent with our previous reports in *A. nidulans* and *A. oryzae* (Yoshimi et al., PloS One, 2013, doi:10.1371/journal.pone.0054893; Yoshimi et al., J. Appl. Glycobiol., 2017, doi:10.5458/jag.jag.JAG-2017_004; Miyazawa et al., Front. Microbiol., 2019, doi:10.3389/fmicb.2019.02090). We have added these results as Figure S5 and revised the text (lines 97−104 in the Materials and Methods and lines 256−267 in the Results).

3. In the discussion section (line 407), you have said that "establishing culture conditions that prevent pellet formation helps screen antifungal drugs using clinical isolate strain," but this has nothing to do with your findings. Rather, if inhibitors of alpha-glucan and GAG biosynthesis are discovered, you could perform the similar experiment with the inhibitor on a clinical strain. Therefore, I think you should discuss this point.

We have revised the text (lines 430−433 in the Discussion).

November 17, 2021

Dr. Yoshitsugu Miyazaki
National Institute of Infectious Diseases
Department of Chemotherapy and Mycoses
Tokyo 162-8640
Japan

Re: Spectrum00063-21R2 (Quantitative monitoring of mycelial growth of *Aspergillus fumigatus* in liquid culture by optical density)

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Thank you for submitting your paper to Spectrum.

Sincerely,

Slavena Vylkova
Editor, Microbiology Spectrum

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Supplemental Material: Accept