

Biosynthesis of β -(1 $\rightarrow$ 5)-Galactofuranosyl Chains of Fungal-Type and O-Mannose-Type

Galactomannans within the Invasive Pathogen *Aspergillus fumigatus*

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23

24 ABSTRACT

25 The pathogenic fungus *Aspergillus fumigatus* contains galactomannans localized
26 on the surface layer of its cell walls, which are involved in various biological processes.
27 Galactomannans comprise α -(1→2)-/ α -(1→6)-mannan and
28 β -(1→5)-/ β -(1→6)-galactofuranosyl chains. We previously revealed that GfsA is a
29 β -galactofuranoside β -(1→5)-galactofuranosyltransferase involved in the biosynthesis of
30 β -(1→5)-galactofuranosyl chains. Here, we clarified the entire biosynthesis of
31 β -(1→5)-galactofuranosyl chains in *A. fumigatus*. Two paralogs exist within *A. fumigatus*:
32 GfsB and GfsC. We show that GfsB and GfsC, in addition to GfsA, are β -galactofuranoside
33 β -(1→5)-galactofuranosyltransferases by biochemical and genetic analyses. GfsA, GfsB,
34 and GfsC can synthesize β -(1→5)-galactofuranosyl oligomers up to lengths of 7, 3, and 5
35 galactofuranoses within an established *in vitro* highly efficient assay of
36 galactofuranosyltransferase activity. Structural analyses of galactomannans extracted from
37 the strains $\Delta gfsB$, $\Delta gfsC$, $\Delta gfsAC$, and $\Delta gfsABC$ revealed that GfsA and GfsC synthesized all
38 β -(1→5)-galactofuranosyl residues of fungal-type and O-mannose-type galactomannans,

and GfsB exhibited limited function in *A. fumigatus*. The loss of β -(1 $\rightarrow$ 5)-galactofuranosyl residues decreased the hyphal growth rate and conidia formation ability as well as increased the abnormal hyphal branching structure and cell surface hydrophobicity, but this loss is dispensable for sensitivity to antifungal agents and virulence toward immune-compromised mice.

45 **IMPORTANCE**

46 β -(1→5)-galactofuranosyl residues are widely distributed in the subphylum
 47 Pezizomycotina of the phylum Ascomycota. Pezizomycotina includes many plant and animal
 48 pathogens. Although the structure of β -(1→5)-galactofuranosyl residues of galactomannans
 49 in filamentous fungi was discovered long ago, it remains unclear which enzyme is
 50 responsible for biosynthesis of this glycan. Fungal cell wall formation processes are
 51 complicated, and information concerning glycosyltransferases is essential for their
 52 understanding. In this study, we show that GfsA and GfsC are responsible for the
 53 biosynthesis of all β -(1→5)-galactofuranosyl residues of fungal-type and O-mannose-type
 54 galactomannans. The data presented here indicates that β -(1→5)-galactofuranosyl residues
 55 are involved in cell growth, conidiation, polarity, and cell surface hydrophobicity. Our new
 56 understanding of β -(1→5)-galactofuranosyl residue biosynthesis provides important novel
 57 insights into the formation of the complex cell wall structure and the virulence of the
 58 subphylum Pezizomycotina.

59

60 INTRODUCTION

61 The cell wall of the pathogenic fungus *Aspergillus fumigatus* comprises several
62 kinds of polysaccharides including chitin, β -(1→3)-glucan, β -(1→3)-/ β -(1→4)-glucan,
63 α -(1→3)-glucan, galactosaminogalactan, and galactomannans (GMs) (1–3). These
64 polysaccharides are complexly intertwined to form the three-dimensional structure of cell
65 walls (1, 2). GMs are polysaccharides comprising D-mannose (Man) and D-galactofuranose
66 (Gal_f), localized on the surface layer of cell walls (2), and distinguished into fungal-type
67 galactomannan (FTGM) and O-mannose-type galactomannan (OMGM) (4). FTGM includes
68 core-mannan, a structure wherein α -(1→2)-mannotetraose is linked with α -(1→6)-linkage
69 from 9 to 10, and β -(1→5)-/ β -(1→6)-galactofuran side chains (5, 6). FTGM binds to a
70 glycosylphosphatidylinositol anchor for transportation from the Golgi apparatus to the cell
71 surface (7), and the transported FTGM is incorporated into the β -(1→3)-glucan–chitin core
72 of the cell wall by *DFG* family proteins (8). OMGM structure comprises
73 β -(1→5)-/ β -(1→6)-galactofuranosyl chains bonded to an O-Man-type glycan with a
74 structure wherein Man is bonded to serine/threonine of a protein as a basic skeleton (6, 9).

Information on GM biosynthesis has been more thoroughly investigated recently (3, 10, 11). CmsA/Ktr4 has been reported to be an α -(1→2)-mannosyltransferase involved in the biosynthesis of the α -(1→2)-mannan backbone of FTGM (12, 13). In the gene-disrupted strain of *cmsA/ktr4* and/or its homolog *cmsB/ktr7*, pronounced hyphal elongation suppression and conidia formation failure were observed (12, 13). Moreover, the $\Delta cmsA/ktr4$ mutant was significantly less virulent than the parental strain (13). These data indicate that FTGM is crucial for normal cell growth and virulence (12, 13). GfsA is firstly identified as a galactofuranosyltransferase involved in the biosynthesis of OMGM galactofuranosyl residues (14). GfsA is a β -galactofuranoside β -(1→5)-galactofuranosyltransferase also involved in the biosynthesis of FTGM galactofuran side chains (4). However, in the $\Delta gfsA$ strain of *A. fumigatus*, the β -(1→5)-galactofuranosyl residue was not completely lost (4). The biosynthesis of the remaining β -(1→5)-galactofuranosyl residues remain unclear. Therefore, we focused on clarifying which residual β -(1→5)-galactofuranosyl residues are biosynthesized. There are two paralogs GfsB and GfsC in *A. fumigatus*. We evaluated whether GfsB and GfsC are responsible for biosynthesis of the remaining

β -(1→5)-galactofuranosyl residues. We obtained recombinant proteins of GfsA, GfsB, and GfsC to elucidate galactofuranoside chain biosynthesis activity *in vitro* using an established highly efficient assay of galactofuranosyltransferase activity. Furthermore, to investigate the function of *gfs* family proteins *in vivo*, we analyzed the structure of GM extracted from single, double, and triple gene-disruptants of *gfsA*, *gfsB*, and *gfsC*. In this study, we aimed to clarify the biosynthesis and function of β -(1→5)-galactofuranosyl residues in *A. fumigatus*.

RESULTS

Features of GfsB and GfsC in *A. fumigatus*. The $\Delta gfsA$ disruptant exhibited reduction of the content of β -(1→5)-galactofuranosyl residues within FTGM and OMGM (4); however, these residues remained within the galactomannan fractions. To determine the enzyme synthesizing the remaining β -(1→5)-galactofuranosyl residues, we focused on *gfsA* paralogs, termed *gfsB* (Afu4g13710 in *A. fumigatus*; Af293/AFUB_070620 in *A. fumigatus* A1163) and *gfsC* (Afu4g10170/AFUB_067290). Comparison of cDNA and genome sequences revealed that no introns were present in *gfsB* and *gfsC*, similarly to *gfsA* (4, 14). Analysis of

secondary structures using TMHMM revealed that GfsB and GfsC have putative transmembrane domains (GfsB: amino acids 13–35, GfsC: amino acids 23–42) at their *N*-termini, suggesting that both GfsB and GfsC are type II membrane proteins, indicating GfsB and GfsC localization at the Golgi apparatus like GfsA (3, 14). GfsB and GfsC have a conserved metal-binding DXD motif (GfsB: amino acids 237–239, GfsC: amino acids 240–242).

Enzymatic function of GfsA, GfsB, and GfsC. We previously constructed an *Escherichia coli* strain expressing a recombinant GfsA protein. GfsC was successfully expressed as a soluble protein using a cold-shock expression vector and GfsB was obtained as a soluble fused NusA protein using an *E. coli* expression system. Recombinant 6× His-tagged GfsA, GfsB, and GfsC proteins were purified by Ni⁺ affinity chromatography and analyzed using SDS-PAGE (Fig. S1). The NusA tag of GfsB was cleaved with a HRV 3C protease and removed by Ni-agarose. GfsA, GfsB, and GfsC were visualized as bands close to their predicted respective molecular weights of 57.9, 50.3, and 52.0 kDa. For the galactofuranosyltransferase assay it is essential to use UDP-Gal_f as a sugar donor; however, it

120 is difficult to obtain as it is not commercially available. Thus, we biochemically synthesized
 121 UDP-Gal_f using Glf, a UDP-galctopyranose (Gal_p) mutase derived from *E. coli*, followed by
 122 HPLC purification (15, 16). This purified UDP-Gal_f was used for the assay within our
 123 previous study (4, 14). Because enzymatic equilibrium of the reversible enzyme Glf is
 124 inclined to be >93% of the mixture (16), generating much UDP-Gal_f was difficult. To solve
 125 this problem, we attempted to improve the galactofuranosyltransferase assay (Fig. 1A).
 126 When a small amount of UDP-Gal_f generated is consumed by galactofuranosyltransferase,
 127 Glf regenerates UDP-Gal_f to maintain equilibrium (Fig. 1A). Glf oxidizes FADH₂ to FAD
 128 when converting UDP-Gal_p to UDP-Gal_f. Therefore, reducing FAD to FADH₂ is essential for
 129 a continuous reaction (Fig. 1A). Sodium dithionite (SD) was used for re-reduction of FADH₂
 130 from FAD. Dithionite ion plays a role as a driving horse for proceeding to
 131 galactofuranosylation by FADH₂ reduction from FAD within the continuous reaction, which
 132 continues until UDP-Gal_p is almost lost. Based on this principle, we developed a highly
 133 efficient assay for galactofuranosyltransferase activity using Glf and Gfs proteins (Fig. 1A).
 134 Chemically synthesized 4-methylumbelliferyl-β-D-galactofuranoside (4MU-β-D-Gal_f) or

135 *p*-nitrophenyl- β -D-galactofuranoside (pNP- β -D-Gal_f) was used as acceptor substrate (17, 18).
 136 When commercially available pNP- β -D-Gal_f was used as an acceptor substrate instead of
 137 4MU- β -D-Gal_f, pNP- β -D-Gal_f was not detected by UV300 absorbance, suggesting
 138 pNP- β -D-Gal_f variance within the structure by SD (19) (Fig. S2 (d) and (e)). Although
 139 NADH/NADPH instead of SD could be used as a reducing reagent against FAD, SD
 140 promoted galactofuranosyltransferase reaction more effectively than NADH/NADPH (Fig.
 141 S2 (c) and (f)).

142 GfsA-lacking fraction showed no new peak generation, but fractions with GfsA
 143 showed six new peaks at 18.0, 20.4, 23.4, 26.5, 30.2, and 34.3 min (defined as AG2–AG7,
 144 respectively; Fig. 1B, *upper panels*). GfsB-lacking fraction showed no new peak generation,
 145 but fractions with GfsB had two new peaks at 18.0 and 20.4 min (BG2 and BG3,
 146 respectively; Fig. 1B, *middle panels*). GfsC-lacking fraction showed no new peak generation,
 147 but fractions with GfsC had four new peaks at 18.0, 20.4, 23.4, and 26.5 min (CG2–CG5,
 148 respectively; Fig. 1B, *bottom panels*). Table 1 shows the mass-to-charge ratios (*m/z*) of
 149 enzymatic products of GfsA, GfsB, and GfsC as identified by LC/MS. The differences of

each peak were calculated as 162.1, indicating that a hexose molecule is continuously attached and each peak was identical to the theoretical molecular mass of the molecule sequentially added to 4MU- β -D-Gal_f by Gal_f.

To further determine chemical structure, we collected >1 mg of AG3, BG2, and CG3 with HPLC and analyzed the sample using ¹H-NMR (Fig. 2) with 4MU- β -D-Gal_f as a control. Chemical shift values for H-1 position of the Gal_f residue in t-Gal_f- β -(1→5)-Gal_f- β -(1→5)-Gal_f-D- β -4MU structures are 5.22 (signal A), 5.20 (signal B), and 5.79 (signal C) ppm from the non-reducing end, according to previous reports (4, 6). Signals for AG3, BG2, and CG3 were in agreement with the reported chemical shift values (Fig. 2). To obtain further evidence for glycosidic linkage, we collected 500 μ g of AG3, AG4, BG2, CG3, and CG4 using HPLC and analyzed the sample using methylation analysis for each compound. This sample was methylated then hydrolyzed, and subsequently analyzed by GC-MS (Fig. 3). The retention times for t-Gal_f→, 5-Gal_f1→ and 6-Gal_f1→ were 16.36, 18.40, and 19.56 min, respectively, under these analysis conditions (6, 20). AG3, AG4, BG2, CG3, and CG4 displayed a peak at 16.36 min (Fig. 3), indicating presence of terminal Gal_f

165 residues. In addition, AG3, AG4, BG2, CG3, and CG4 had a peak at 18.40 but not 19.56 min
166 (Fig. 3), indicating that the all added Gal_f residue was attached to the C-5 position of the first
167 Gal_f residue. These results indicate that GfsB and GfsC are also
168 β-(1→5)-galactofuranosyltransferases and that GfsA, GfsB, and GfsC could not transfer a
169 Gal_f residue to the C-6 position in contrast to GlfT2, the bacterial
170 β-(1→5)-/β-(1→6)-galactofuranosyltransferase (21, 22).

171

172 **Role of GfsB and GfsC in GM biosynthesis.** To clarify the function of the *gfs* family *in vivo*,
173 we constructed $\Delta gfsB$, $\Delta gfsC$, $\Delta gfsC::C$, $\Delta gfsAC$, and $\Delta gfsABC$ strains (Fig. S3, S4 and S5).
174 To identify the effect of gene disruption on the structure of GMs, those extracted from the
175 mycelia of *A. fumigatus* strains were purified by cetyl trimethyl ammonium bromide
176 precipitation with boric acid buffer. The GMs designated as FTGM+OMGM contain both
177 FTGM and OMGM (4); these were analyzed by ¹³C-NMR spectroscopy (Fig. 4). Signals at
178 107.87 ppm and 108.70 ppm of the ¹³C-NMR spectra represent the C-1 positions of the
179 underlined Gal_f residue within the structure of Gal_f-β-(1→5)-Gal_f-(1→(β-(1→5)-Gal_f)) and

180 β -Gal_f-β-(1→6)-Gal_f-(1→ (β-1→6)-Gal_f), respectively, as according to previous reports (6,
181 23). The signal intensity of β-(1→5)-Gal_f was higher than that of β-(1→6)-Gal_f in the
182 ¹³C-NMR chart of A1151-FTGM+OMGM. Within Δ*gfsB*-FTGM+OMGM there was little
183 difference from A1151-FTGM+OMGM (Fig. 4). In contrast, the intensity of signal of
184 β-(1→5)-Gal_f was inversed in the ¹³C-NMR chart of Δ*gfsC*-FTGM+OMGM, indicating that
185 the amount of β-(1→5)-Gal_f was decreased in the FTGM+OMGM fraction of Δ*gfsC* strains
186 (Fig. 4). The signal intensity of β-(1→5)-Gal_f was recovered in the ¹³C-NMR chart of
187 Δ*gfsC*::*C*-FTGM+OMGM (Fig. 4). Interestingly, signals of β-(1→5)-Gal_f in the ¹³C-NMR
188 chart of Δ*gfsAC*-/Δ*gfsABC*-FTGM+OMGM were not detected, indicating that β-(1→5)-Gal_f
189 disappeared within the FTGM+OMGM fractions of Δ*gfsAC* and Δ*gfsABC* strains. GC-MS
190 analyses of O-methylalditol acetates derived from methylation analyses of FTGM+OMGMs
191 were performed for the A1151, Δ*gfsB*, Δ*gfsC*, Δ*gfsAC*, Δ*gfsABC*, and Δ*gfsC*::*C* strains
192 (Table 2). The ratio of the 5-O-substituted Gal_f residue (5-Gal_f1→) of Δ*gfsC* (2.16% ±
193 0.19%) was lower than that of A1151 (16.31% ± 0.84%); however, the ratio of 5-Gal_f1→ of
194 Δ*gfsB* (15.37% ± 0.71%) was comparable with that of A1151 (Table 2). Interestingly, signals

for the 5-Gal β 1 $\rightarrow$ of $\Delta gfsAC$ or $\Delta gfsABC$ were not detected within these FTGM+OMGM fractions (Table 2). These results clearly indicate that β -(1 $\rightarrow$ 5)-galactofuranosyl residues disappeared within both $\Delta gfsAC$ and $\Delta gfsABC$. Next, the FTGM galactofuran side chain was prepared and separated by gel filtration chromatography to analyze its length (Fig. 5). FTGM+OMGM fractions were treated with 0.15 M of trifluoroacetic acid at 100°C for 15 min. The resultant samples were applied to gel filtration chromatography to separate the obtained galactofuran side chain (Fig. 5). Consequently, sugar chains consisting of up to 6 monosaccharides were detected in the fractions of A1151, $\Delta gfsA$ and $\Delta gfsC$ strains (Fig. 5). Conversely, only monosaccharide was detected in the $\Delta gfsAC$ strain fraction, indicating that elongation by β -(1 $\rightarrow$ 5)-galactofuranosyl residues had not occurred (Fig. 5). These observations clearly indicate that all β -(1 $\rightarrow$ 5)-galactofuranosyl residues of FTGM and OMGM in *A. fumigatus* are biosynthesized by GfsA and GfsC.

Phenotypic analyses of disruptant *gfs* family genes. The colony phenotypes of disruptant

strains were observed following 3 days of growth at 37°C/50°C on minimal medium (Fig. 6).

The colony growth rates of the disruptant strains are shown in Table 3. The colony growth

rate of the $\Delta gfsA$ strain decreased to 85.2% as compared with that of the A1151 strain at 37°C

(Table 3). In contrast, colony growth rate percentages of $\Delta gfsB$ and $\Delta gfsC$ strains were

comparable with that of the A1151 strain at 37°C (Table 3). Growth rate percentages of

$\Delta gfsAC$ and $\Delta gfsABC$ strains were reduced to 68.4% and 67.8% at 37°C, and to 86.4% and

84.0% at 50°C, respectively (Table 3). When quantifying the number of formed conidia at

37°C, the percentage of the $\Delta gfsA$ strain decreased to 50.9% compared with that of the

A1151 strain. The conidiation efficiencies of $\Delta gfsAC$ and $\Delta gfsABC$ were reduced to

approximately 32.1% and 25.4% of that of the A1151 strain (Table 4). In contrast, the

conidiation efficiency of the $\Delta gfsB$ and $\Delta gfsC$ strains did not obviously decrease (Table 4).

These results are consistent with the facts that GfsA and GfsC have redundant enzymatic

functions and GfsB can only synthesize short β -(1→5)-galactofuranosyl oligomers. These

results indicate that the β -(1→5)-galactofuranosyl residues plays an important role in conidia

formation and hyphal growth.

224 It was reported that hyphae branching was increasing within the $\Delta glfA$ strain (24).
 225 Therefore, we observed hyphae in $\Delta gfsAC$ and $\Delta gfsABC$ strains to determine whether
 226 abnormal hyphae branching was also formed in these strains (Fig. 7A). We did observe
 227 abnormal hyphae branching at a high frequency, indicating that deficient of
 228 β -(1 $\rightarrow$ 5)-galactofuranosyl residues causes an increase of abnormal hyphae branching.
 229 Reportedly, lack of the Gal_L-containing sugar chains from the cell causes increased cell
 230 surface hydrophobicity (24). To confirm increasing cell surface hydrophobicity in $\Delta gfsAC$
 231 and $\Delta gfsABC$ strains, whether the amount of adherence of latex beads to the mycelium
 232 increased as observed within the $\Delta glfA$ strain was determined; this adherence was clearly
 233 increased (Fig. 7B), indicating that β -(1 $\rightarrow$ 5)-galactofuranosyl residues are involved in cell
 234 surface hydrophobicity in *A. fumigatus*.

235

236 **Sensitivity to antifungal agents and virulence of β -(1 $\rightarrow$ 5)-galactofuranosyl**
 237 **residue-deficient strains.** Next, we tested the sensitivity of the A1151, $\Delta gfsC$, $\Delta gfsAC$, and
 238 $\Delta gfsABC$ strains to the widely used clinical antifungal agents micafungin (MCFG),

caspofungin (CPFG), amphotericin B (AMPH-B), flucytosine (5-FC), fluconazole (FLCZ), itraconazole (ITCZ), voriconazole (VRCZ), and miconazole (MCZ) (Table 5). Sensitivities of the mutants to antifungal agents were almost identical to those of A1151. The $\Delta gfsABC$ strain exhibited only slightly greater sensitivity to AMPH-B and MCZ as compared with the A1151 strain (Table 5). We also examined the role of β -(1 $\rightarrow$ 5)-galactofuranosyl residues in pathogenesis using a murine infection model (Fig. 8). First, the virulence of A1151, $\Delta gfsC$ and $\Delta gfsC::C$ strains were tested within immune-compromised mice. Survival rates did not differ between A1151, $\Delta gfsC$ and $\Delta gfsC::C$ infections (Fig. 8A). Virulence of A1151, $\Delta gfsC$, $\Delta gfsAC$, and $\Delta gfsABC$ strains were also tested (Fig. 8B). In the aspergillosis model, virulence of the $\Delta gfsAC$ and $\Delta gfsABC$ strains were comparable with that of the A1151 strain (Fig. 8B), indicating that a lack of β -(1 $\rightarrow$ 5)-galactofuranosyl residues did not influence survival rates of immunosuppressed mice.

DISCUSSION

253 We previously characterized that GfsA is the β -galactofuranoside
254 β -(1 $\rightarrow$ 5)-galactofutanosyltransferase (4). However, the β -(1 $\rightarrow$ 5)-galactofuranosyl oligomer
255 synthesized with GfsA could only be confirmed to generate up to 3 sugars in the previous
256 reaction system due to a lack of commercially available UDP-Gal_f (4). In this study, we
257 showed that GfsA could synthesize β -(1 $\rightarrow$ 5)-galactofuranosyl oligomers up to lengths of 7
258 monosaccharides (Fig. 1, *upper panels*). In addition, we showed that GfsB and GfsC also
259 could transfer β -Gal_f to the 5 position of the hydroxy group of the terminal
260 β -galactofuranosyl residue up to 3 and 5 monosaccharides lengths, respectively (Fig. 1,
261 *middle and bottom panels*). AG4, BG2 and CG3 accumulated within the assay of GfsA, GfsB
262 and GfsC, respectively (Fig. 1). This indicates that GfsA is a more suitable enzyme for
263 synthesizing longer β -(1 $\rightarrow$ 5)-galactofuranosyl oligomers compared with GfsB and GfsC.
264 This result is consistent with the fact that *gfsA* disruption had greatest impact in the
265 disruptants of *gfs* family genes (Fig. 6). We subsequently proposed structures of the
266 fungal-type- and O-mannose-type galactomannans in the $\Delta gfsAC$ strain (Fig. 9).

267 One problematic issue for assaying galactofuranosyltransferases is that the sugar
268 donor UDP-Gal_f is not commercially available. Errey et al. described relatively easily
269 synthesizing UDP-Gal_f using flexible enzymatic and chemo-enzymatic approaches (25).
270 However, obtaining and retaining the chemically unstable UDP-Gal_f remains complicated
271 (26). We thus attempted to establish a galactofuranosyltransferase assay using a continuous
272 reaction of sugar-nucleotide conversion and sugar transfer with UDP-galactopyranose
273 mutase and galactofuranosyltransferase. Rose et al. previously performed a method to detect
274 galactofuranosyltransferase activity via continuous reaction using NADH for the reduction
275 of FAD (27). In our hands galactofuranosylation proceeded even when NADH/NADPH was
276 used instead of SD, but was more efficient with SD versus NADH/NADPH (Fig. S2). This
277 established method could measure galactofuranosyltransferase activity without UDP-Gal_f. In
278 addition, since a sufficient amount of purified product can be separated and purified, this is
279 advantageous for structural analysis of the enzymatic product (Figs. 2 and 3). This method
280 will likely be useful for functional analysis of other galactofuranosyltransferases.

The growth phenotypes of the $\Delta gfsA$ strain are less severe than that of the $\Delta gfsAC/\Delta gfsABC$ strains (Fig. 6, Tables 3 and 4). Deletion of *gfsB* and *gfsC* did not result in any growth defect of *A. fumigatus* (Fig. 6, Tables 3 and 4). Very recently, similar results have been observed within disrupted strains of *gfs* family genes in *A. niger* (28), suggesting that existence of a common functional relationships of the *gfs* family proteins for *A. niger* and *A. fumigatus*. We clarified that the phenotypic abnormalities occurring in the $\Delta gfsAC$ strain were due to defects in β -(1 $\rightarrow$ 5)-galactofuranosyl residues via analysis of the sugar chain structure of the $\Delta gfsAC$ strain.

Several galactofuranosyl sugar chain-absent mutants have been reported in *A. fumigatus*; whole galactofurnosyl sugar chains are absent within $\Delta glfA$ and $\Delta glfB$ strains (24, 29, 30). These absent Gal_f residues caused decreased growth rates, abnormal hyphae branching, thinner cell walls, increased susceptibility to several antifungal agents and increased adhesive phenotype as compared with the parental strain (24, 29, 30). The phenotypes of the $\Delta gfsAC/\Delta gfsABC$ strains were similar in some aspects to the $\Delta glfA$ strain, but not identical. The latter showed stronger inhibition of hyphal growth and conidia

formation compared with $\Delta gfsAC/\Delta gfsABC$ (Fig. 6). This is because galactofuranosyl residues are β -(1 $\rightarrow$ 5)-linked, β -(1 $\rightarrow$ 2)-linked, β -(1 $\rightarrow$ 3)-linked, and β -(1 $\rightarrow$ 6)-linked sugars (5, 6, 10). These Gal_f residues, except β -(1 $\rightarrow$ 5)-galactofuranosyl, are found in glycosyl phosphoinositolceramides (GIPC), FTGM, OMGM, and other sugar chains (5, 6, 10, 31–33) and might be involved in biological events. Only the β -(1 $\rightarrow$ 5)-galactofuranosyl residues disappear in the $\Delta gfsAC/\Delta gfsABC$ strains, thus it seems reasonable that they exhibit less influence than the $\Delta glfA$ strain, wherein all Gal_f-containing sugar chain is lost. However, abnormal branching of the hyphae and cell surface hydrophobicity were not significantly different between the $\Delta gfsAC$, $\Delta gfsABC$, and $\Delta glfA$ strains (Fig. 7), indicating that GM β -(1 $\rightarrow$ 5)-galactofuranosyl residues' functions are heavily involved in normal polarity of the hyphae and cell surface hydrophobicity.

The presence of the β -(1 $\rightarrow$ 6)-galactofuranosyl moiety has been reported in the galactofuran side chain of FTGM and OMGM in *A. fumigatus*. We, therefore, predicted that if the β -(1 $\rightarrow$ 5)-galactofuranosyl residues disappeared so would the β -(1 $\rightarrow$ 6)-galactofuranosyl residues. However, upon disappearance of the

311 β -(1→5)-galactofuranosyl residues the β -(1→6)-galactofuranosyl residues remained
 312 detectable within the ^{13}C -NMR of GMs from the $\Delta gfsAC$ strain (Fig. 4). This strongly
 313 suggests the existence of a β -(1→6)-galactofuranosyl oligomer and/or polymer other than the
 314 β -(1→6)-galactofuranosyl moiety of the FTGM galactofuran side chain.
 315 β -(1→6)-Galactofuranosyl polymer was found in *Fusarium* sp., but not in *A. fumigatus* (34–
 316 36).

317 In the mouse infection model of invasive aspergillosis, the lack of GM
 318 β -(1→5)-galactofuranosyl residues exhibited no significant differences in virulence for the
 319 A1151, $\Delta gfsAC$ and $\Delta gfsABC$ strains. These findings were consistent with Lamarre's
 320 findings that disruption of *glfA* has no effect on virulence (24). In contrast, Schmalhorst et al.
 321 reported that disruption of *glfA* resulted in attenuated virulence in mouse model of invasive
 322 aspergillosis (29). Recently, Koch et al. showed that the survival rate of $\Delta glfA$ strain
 323 (Schmalhorst's strain) decreased slightly more gradually compared with the wild strains
 324 using zebrafish embryo model (37). They explained that the attenuated pathogenicity of the
 325 $\Delta glfA$ strain might be caused by decreased germination rate or hyphal growth rate (37). These

differences in virulence might be due to varying genetic backgrounds of the strains used or differing protocols of pathogenicity tests, necessitating further detailed analysis to understand the involvement of β -(1 $\rightarrow$ 5)-galactofuranosyl sugar chains in pathogenicity.

This study broadens our understanding of the biosynthesis of β -(1 $\rightarrow$ 5)-galactofuranosyl residues in *A. fumigatus* and their important role in cell wall formation. However, β -(1 $\rightarrow$ 6)-galactofuranosyltransferases that transfer β -galactofuranose to galactofuranosyl residues have not been identified in *A. fumigatus*. Additionally, β -(1 $\rightarrow$ 2)-, β -(1 $\rightarrow$ 3)-/ β -(1 $\rightarrow$ 6)-galactofuranosyltransferases transferring β -galactofuranose to mannosyl residues remain unknown. Our findings regarding the biosynthesis of β -(1 $\rightarrow$ 5)-galactofuranosyl residue provide important novel insights into the formation of the complex cell wall structure and the virulence of the subphylum Pezizomycotina. Future studies will be needed to identify other galactofuranosyltransferases and clarify the individual functions of each Gal₇-containing oligosaccharide.

MATERIALS AND METHODS

Microorganisms and growth conditions. *A. fumigatus* strains used in this study are listed in Table S1. *A. fumigatus* A1160 and A1151 were obtained from the Fungal Genetics Stock Center (FGSC) (38). Strains were grown on minimal medium (MM). Standard transformation procedures for *Aspergillus* strains were used. Plasmids were amplified in *E. coli* DH5 α . *E. coli* strain Rosetta-gami B (DE3) purchased from Merck Millipore (Merck Millipore, Germany) was used for protein expression. Colony growth rates were measured as described previously (14).

Construction of GfsB and GfsC expression vector. All PCR reactions were performed using Prime STAR GXL DNA Polymerase (Takara Bio, Otsu, Japan). The pCold™ II (Takara Bio) and pET50b-Amp plasmids for protein expression in *E. coli* were used. The pET50b-Amp is a plasmid constructed by replacing the kanamycin resistance gene of pET50b with an ampicillin resistance gene, and was constructed as follows: DNA region of pET50b except for the kanamycin resistance gene was amplified by PCR using pET50b plasmid as a template for the primer pairs, pET50b-Amp-F and pET50b-Amp-R. The

356 ampicillin resistance gene was amplified by PCR using pET15b plasmid as a template for the
357 primer pairs Amp-gene-F and Amp-gene-R. The obtained DNA fragments were ligated
358 using an In-fusion HD cloning Kit (Takara Bio) to yield pET50b-Amp. Plasmids useful for
359 expression of *gfsB* and *gfsC* were constructed as follows: total RNA was extracted from *A.*
360 *fumigatus* A1160 strain mycelia grown in MM for 18 h using TRIzol Reagent (Thermo
361 Fisher Scientific, MA, USA) according to the manufacturer's instructions. Single-stranded
362 DNA was synthesized by M-MLV Reverse Transcriptase (NIPPON GENE, Tokyo, Japan)
363 using oligo-dT-18 primers. *gfsB* and *gfsC* were amplified using PCR with single-stranded
364 DNA as a template for primer pairs pET50b-AfGfsB-F and pET50b-AfGfsB-R for *gfsB*,
365 pCold2-AfGfsC-F and pCold2-AfGfsC-R for *gfsC*, respectively. The amplified fragments
366 were inserted into the *Nde* I site of pCold™ II to yield pCold2-AfGfsC, and the *Sma* I site of
367 pET50b-Amp to yield pET50b-Amp-AfGfsB using the In-fusion HD cloning Kit. The
368 constructed plasmids were transformed into Rosetta-gami B (DE3) cells.
369

Protein purification, quantification, and electrophoresis. GfsA protein was expressed in Rosetta-gami B (DE3) cells harboring the plasmids pET15b-AfGfsA (4). Protein expression and purification for GfsA and GfsB were performed as described previously (4). Rosetta-gami B (DE3) cells harboring the plasmids pCold2-AfGfsC were used for protein expression of GfsC, which was performed according to the manufacturer's protocol for the pCold DNA cold-shock expression system. The NusA tag of GfsB was cleaved with a HRV 3C protease at 4°C (Takara Bio) and removed by Ni-agarose. Protein concentrations were determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific), and purified proteins were analyzed by SDS-PAGE to assess purity and molecular weight. Glf protein was obtained with the ASKA clone as previously described (4, 39). Purified Glf was visualized as a band close to the predicted molecular weights of 45.0 kDa (Fig. S1).

Synthesis of *p*-nitrophenyl β -D-galactofuranoside (pNP-Gal) and 4-methylumbelliferyl β -D-galactofuranoside (4MU-Gal). Para-nitrophenyl β -D-galactofuranoside (pNP-Gal) was chemically synthesized as described previously (18, 40) or purchased (Toronto Research

Chemicals, Toronto, Canada). 4-methylumbelliferyl β -D-galactofuranoside (4MU-Gal_f) was chemically synthesized as follows (41, 42): 4-methylumbelliferon (50.0 mmol) and BF₃•Et₂O (50.0 mmol) was added to a solution of per-benzoylated galactofuranose (10.0 mmol) with 4A molecular sieves in CH₃CN (50 mL) at 0°C (41). The reaction mixture was stirred at 0°C for 1 h followed by 23°C for 24 h. Next, the mixture was filtered through a Celite pad and the residue was diluted with EtOAc, washed with sat. aq. NaHCO₃ solution and brine, dried over MgSO₄, and concentrated *in vacuo* to dryness, producing a mixture of 4-methylumbelliferyl 2,3,4,6-tetra-*O*-benzoyl- β -D-galactofuranoside. A 28% aq. NH₃ solution was added to the aforementioned mixture in CH₃OH at 0°C, the resulting solution was stirred at this temperature for 1 h and then at 23°C for 24 h. The reaction solution was concentrated. The target material was purified by silica-gel column chromatography (CHCl₃:CH₃OH, 4/1) to give 4-methylumbelliferyl β -D-galactofuranoside (4MU-Gal_f) as a yellow solid (1.80 mmol).

Continuous enzymatic reaction assay. Standard assays were performed with 1.5 mM

400 4MU- β -D-Gal_f acceptor substrate, 40 mM UDP-galactopyranose, purified Glf protein
 401 (UDP-galactopyranose mutase from *Escherichia coli*: 15.8 μ g), 40 mM sodium dithionite
 402 (SD), and purified GfsA (4.5 μ g), GfsB (4.5 μ g), or GfsC (4.5 μ g) proteins in a total reaction
 403 volume of 20 μ L. The mixtures were incubated at 30°C for 16 h and the reaction was stopped
 404 by heat (99°C) for 5 min. The supernatants were analyzed by HPLC with an amino column
 405 Shodex Asahipak NH2P-50 4E (250 x 4.6 mm, Showa Denko, Tokyo, Japan) as previously
 406 described (4). 4-Methylumbelliferyl and *p*-nitrophenyl derivatives were detected by 300 nm
 407 of absorbance. The mass spectra of the enzymatic products of GfsA, GfsB, and GfsC were
 408 determined using the Exactive Plus Orbitrap Mass Spectrometer (Thermo Fisher Scientific).

409

410 **Construction of Δ *gfsB* and Δ *gfsC* gene disruption strains.** *A. fumigatus* A1151/A1160
 411 was used as parental strain (Table S1); *gfsB* were disrupted in the A1151 strain by *ptrA*
 412 insertion; *gfsC* was also disrupted in the A1160 strain by *AnpyrG* insertion. DNA fragments
 413 for gene disruption were constructed using a “double-joint” PCR method as described
 414 previously (43). The 5'- and 3'-flanking regions (approximately 1.0–1.1 kb each) of each

gene were PCR amplified from genomic DNA with the following primer pairs (Table S2):

AFUB_070620-1/AFUB_070620-2 and AFUB_070620-3/AFUB_070620-4 for *gfsB*

disruption; AFUB_067290-1/AFUB_067290-2 and AFUB_067290-3/AFUB_067290-4 for

gfsC. *ptrA* and *AnpyrG* used as selective markers were amplified using plasmids pPTR-I

(Takara Bio) and pSH1 (14) as template, respectively, and the primer pairs ptrA-5/ptrA-6 or

pyrG-5/pyrG-6. The three amplified fragments were purified and mixed and a second PCR

was performed without specific primers to assemble each fragment, as the overhanging

chimeric extensions act as primers. A third PCR was performed with the nested primer pairs

AFUB_070620-7/AFUB_070620-8 for *gfsB* or AFUB_067290-7/AFUB_067290-8 for *gfsC*

and the products of the second PCR as a template to generate the final deletion construct. The

amplified final deletion constructs were purified using the Fast Gene Gel/PCR Extraction Kit

(NIPPON GENE) and used directly for transformation. Transformants were grown on MM

plates containing 0.6 M KCl as an osmotic stabilizer under appropriate selection conditions

and single colonies were isolated twice before further analysis. Disruption of target genes

was confirmed by PCR with these primer pairs: AFUB_070620-1/ptrA-R and

ptrA-F/AFUB_070620-4 for *gfsB*, AFUB_067290-1/ptrA-R and ptrA-F/AFUB_067290-4 or
AFUB_067290-1/pyrG-R and pyrG-F/AFUB_067290-4 for *gfsC* (Fig. S3).

Construction of the complementary strain of $\Delta gfsC$ using *gfsC*. *A. fumigatus* $\Delta gfsC$ strain
was used as the parental strain (Table S1). The relevant region of *gfsC* was PCR amplified
from genomic DNA using the primer pair AfgfsC-complement-1/AfgfsC-complement-2
(Table S2). The relevant region of the *AnpyrG* was PCR amplified from pSH1 using the
primer pair AfgfsC-complement-3/AfgfsC-complement-4 (Table S2). *ptrA* used as selective
markers were amplified using the pPTR-I plasmid as a template and the primer pair
ptrA-5/ptrA-6. The three amplified fragments were purified and mixed, and a second PCR
was performed. A third PCR was performed using the nested primer pair
AfgfsA-complement-7/AfgfsA-complement-8 and the products of the second PCR as a
template to generate the final DNA construct. Correct replacement of the DNA fragments for
gene complementation was confirmed by PCR using the primer pairs
AfgfsA-complement-1/ptrA-R and ptrA-F/AfgfsA-complement-4 (Fig. S4).

445

446 **Construction of double and triple gene disruption strains.** *A. fumigatus* $\Delta gfsA$ was used
447 as a parental strain (Table S1) to construct double and triple gene disruption strains. Genes
448 were disrupted in *A. fumigatus* by *ptrA/hph* insertion; *gfsC* was disrupted in strain $\Delta gfsA$ by
449 *ptrA* insertion to construct strain $\Delta gfsAC$. Next, *gfsB* was disrupted in strain $\Delta gfsAC$ by *hph*
450 insertion to construct strain $\Delta gfsABC$. Primer pairs
451 AFUB_067290-1/AFUB_067290-2(*gfsC::ptrA*),
452 AFUB_067290-3(*gfsC::ptrA*)/AFUB_067290-4, *ptrA*-5/*ptrA*-6 and
453 AFUB_067290-7/AFUB_067290-8 were used to construct a deletion cassette for $\Delta gfsC$.
454 Primer pairs AFUB_070620-1/AFUB_070620-2(*gfsB::hph*),
455 AFUB_070620-3(*gfsB::hph*)/AFUB_070620-4, *hph*-5/*hph*-6 and
456 AFUB_070620-7/AFUB_070620-8 were used to construct a deletion cassette for $\Delta gfsB$. *hph*
457 was amplified by PCR using pAN7-1 as template (44) and the primers *hph*-5 and *hph*-6
458 (Table S2). Target gene disruption was confirmed using PCR with primer pairs
459 AFUB_096220-1/*pyrG*-R and *pyrG*-F/AFUB_096220-4 for *gfsA*, AFUB_070620-1/*hph*-R

and hph-F/AFUB_070620-4 for *gfsB*, and AFUB_067290-1/ptrA-R and ptrA-F/AFUB_067290-4 for *gfsC* (Fig. S5).

Methylation analysis and Nuclear magnetic resonance (NMR) spectroscopy. GMs were prepared using a previously described method (4, 14). Glycosidic linkage was analyzed using a previously described method (4, 6). NMR experiments were performed as previously described (4, 6, 14). Proton and carbon chemical shifts were referenced relative to internal acetone at δ 2.225 and 31.07 ppm, respectively.

Analysis of conidiation efficiency and surface adhesion. Conidiation efficiency was analyzed as described previously (14). Hyphal surface adhesion assay was performed as described previously with slight modifications (24, 45). The 0.5- μ m diameter polystyrene beads (Sigma) were diluted 1:100 in sterile phosphate buffered saline (PBS). Mycelia were grown for 18 h at 37°C with shaking at 127 rpm in MM liquid medium, harvested into PBS-containing polystyrene beads for 1 h, and then washed five times with PBS. Mycelia

images were collected using a microscope equipped with a digital camera.

Drug susceptibility testing and mouse model of pulmonary aspergillosis.

Drug susceptibility was tested in triplicates as described previously (12, 46, 47). The mouse model of pulmonary aspergillosis was generated as per a previously described method with slight modifications (48). In each experiment, A1151, $\Delta gfsC$, and $\Delta gfsC::C$ or A1151, $\Delta gfsC$, $\Delta gfsAC$, and $\Delta gfsABC$ strains were used to infect immunosuppressed mice (10 or 11 mice per group). Outbreed male ICR mice were housed in sterile cages (5 or 6 per cage) with sterile bedding and provided with sterile feed and drinking water containing 300 μ g/ml tetracycline hydrochloride to prevent bacterial infection. Mice were immunosuppressed with cyclophosphamide (200 mg per kg of body weight), which was intraperitoneally administered on days -4, -2, 2, and 5, or -4, -2, and 3 (day 0: infection). Cortisone acetate (200 mg per kg of body weight) was injected on day -1 for immunosuppression. Mice were infected by intratracheal instillation of 3×10^5 conidia in 30 μ l of PBS. Mice were weighed and visually inspected every 24 h from the day of infection. On recording 30% body weight

reduction, the mouse was regarded as dead and euthanized. Prism statistical analysis package was used for statistical analysis and survival curve drawing (GraphPad Software Inc., CA, USA).

Ethics statement. The institutional animal care and use committee of Chiba University approved the animal experiments (Permit Number: DOU28-376 and DOU29-215). All efforts were made to minimize suffering in strict accordance with the principles outlined by the Guideline for Proper Conduct of Animal Experiments.

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697 **FIGURE LEGENDS**

698 **Figure 1. *In vitro* method for measuring galactofuranosyltransferase activity using a**

699 **continuous reaction.** (A) Schematic diagram of continuous reaction in

700 galactofuranosyltransferase activity assay. Glf is UDP-galctopyranose (Gal_p) mutase derived

701 from *E. coli* to generate UDP-Gal_f from UDP-Gal_p. The enzymatic equilibrium of a

702 reversible Glf enzyme is skewed >93% to UDP-Gal_p. When a small amount of UDP-Gal_f

703 generated is consumed by galactofuranosyltransferase, Glf reverts to UDP-Gal_f to maintain

704 equilibrium. Glf oxidizes FADH₂ to FAD when converting from UDP-Gal_p to UDP-Gal_f.

705 Therefore, reducing FAD to FADH₂ is essential for a continuous reaction. The dithionite ion

706 plays a role as a driving horse for proceeding to galactofuranosylation by reduction of

707 FADH₂ from FAD within the continuous reaction. (B) Chromatograms of *in vitro* assay of

708 galactofuranosyltransferase activity of GfsA, GfsB, and GfsC. Enzyme activities were

709 assayed as described in the Materials and Methods. 4.5 µg of purified GfsA, GfsB, or GfsC

710 were used as enzyme. Chromatograms indicate typical results of the assay with/without

711 GfsA, GfsB, or GfsC (*right and left panels, respectively*). The assays lacking GfsA, GfsB, or

712 GfsC showed no novel peak generation (*left panels*), but in contrast, fractions containing
 713 GfsA, GfsB, or GfsC did have new products (defined as AG2-AG7 for GfsA; BG2 and BG3
 714 for GfsB; CG2-CG5 for GfsC; *right panels*). Retention times were 18.0 min for AG2, BG2,
 715 and CG2, 20.4 min for AG3, BG3, and CG3, 23.4 min for AG4 and CG4, 26.5 min for AG5
 716 and CG5, 30.2 for AG6, and 34.3 min for AG7. Gal_f, galactofuranose; Gal_p, galactopyranose;
 717 Glf, UDP-galactopyranose mutase from *E. coli*; UDP, uridine diphosphate; FAD, flavin
 718 adenine dinucleotide; 4MU, 4-methylumbelliferyl.

719 **Figure 2. ¹H-NMR analyses of enzymatic products of GfsA, GfsB, and GfsC using**
 720 **4MU-β-D-Gal_f as an acceptor substrate.** ¹H-NMR charts for 4MU-β-D-Gal_f, AG3, BG2,
 721 and CG3. The 5.8 ppm signal was detected in the ¹H-NMR chart for 4MU-β-D-Gal_f. The
 722 chemical shift values of BG2 of the H-1 position of the underlined Gal_f residue in the
 723 Gal_f-β-(1→5)-Gal_f-β-4MU and Gal_f-β-(1→5)-Gal_f-β-4MU structures are 5.22 and 5.79 ppm,
 724 respectively, according to previous reports (4, 6, 17, 18). The chemical shift values of AG3
 725 and CG3 of the H-1 position of the underlined Gal_f residue in the
 726 Gal_f-β-(1→5)-Gal_f-β-(1→5)-Gal_f-β-4MU, Gal_f-β-(1→5)-Gal_f-β-(1→5)-Gal_f-β-4MU and

Gal_f-β-(1→5)-Gal_f-β-(1→5)-Gal_f-β-4MU structures are 5.22, 5.20 and 5.79 ppm, respectively, according to previous reports (4, 6, 17, 18).

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Figure 3. Methylation analyses of enzymatic products of GfsA, GfsB, and GfsC using 4MU-β-D-Gal_f as an acceptor substrate. 500-μg samples of AG3, AG4, BG2, CG3, and CG4 were analyzed. The retention times under these analysis conditions for t-Gal_f→, 5-Gal_f1→ and 6-Gal_f1→ were 16.36, 18.40, and 19.56 min, respectively (4, 6, 20). The asterisk indicates an artificial peak that appears frequently.

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Figure 4. ¹³C-NMR analyses of purified FTGM+OMGM fractions from A1151, ΔgfsB, ΔgfsC, ΔgfsAB, ΔgfsABC, and ΔgfsC::C strains. The signals at 107.87 ppm and 108.70 ppm represent a C-1 chemical shift of the underlined Gal_f residue in the Gal_f-β-(1→5)-Gal_f-β-(1→5)-Gal_f (β-(1→5)-Gal_f) and Gal_f-β-(1→5)-Gal_f-β-(1→6)-Gal_f (β-(1→6)-Gal_f) structures, respectively (6, 20). The carbon chemical shifts were referenced relative to internal acetone at 31.07 ppm, respectively. OMGM, O-mannose-type

galactomannan; FTGM, fungal-type galactomannan; FTGM+OMGM, total GM (FTGM + OMGM).

Figure 5. Analysis of galactofuran side chain length of fungal-type galactomannan.

Galactofuran side chain was prepared and separated by gel filtration chromatography. FTGM+OMGM fractions were treated with 0.15 M of trifluoroacetic acid at 100°C for 15 min. The resultant samples were applied to gel filtration chromatography using a Bio-Gel P-2 (2 × 90 cm) column and dH₂O as eluent. The partial acid hydrolysis product of dextran was used as a molecular weight marker. The eluted sugar was detected using the phenol-sulfuric acid method. G1, Glucose; G2, Maltose; G3, Maltotriose; G4, Maltotetraose; G5, Maltopentaose; G6, Maltohexaose; G7, Maltoheptaose.

Figure 6. Colony phenotype comparison of A1151, $\Delta gfsA$, $\Delta gfsB$, $\Delta gfsC$, $\Delta gfsAB$,

$\Delta gfsABC$, $gfsA::A$ and $\Delta gfsC::C$ strains. Strain colony images are shown for A1151,

$\Delta gfsA$, $\Delta gfsB$, $\Delta gfsC$, $\Delta gfsAB$, $\Delta gfsABC$, $gfsA::A$, and $\Delta gfsC::C$. Conidia were incubated on

minimal medium at 37°C (*left*) or 50°C (*right*) for 3 days.

Figure 7. Morphology of the A1151, $\Delta glfA$, $\Delta gfsAC$, and $\Delta gfsABC$ strains. (A) Hyphae morphology of the A1151, $\Delta glfA$, $\Delta gfsAC$, and $\Delta gfsABC$ strains. (B) Hyphae hydrophobicity of A1151, $\Delta glfA$, $\Delta gfsAC$, and $\Delta gfsABC$ strains. Hydrophobicity is indicated by adherence of latex beads to the hyphae.

Figure 8. β -(1 $\rightarrow$ 5)-galactofuranosyl residues are dispensable for virulence in a mouse model of invasive pulmonary aspergillosis. (A) Infection with A1151, $\Delta gfsC$ and $\Delta gfsC::C$ strains. Outbred ICR mice (male; 5 weeks of age; n=11) were immune-compromised via intraperitoneal injection of cyclophosphamide (200 mg/kg) at days -4, -2, 2, and 5. Cortisone acetate was also administered subcutaneously at a concentration of 200 mg per kg of body weight on day -1. Mice were infected intratracheally with 3.0×10^5 conidia in a volume of 30 μ L for each strain (A1151, $\Delta gfsC$ and $\Delta gfsC::C$ strains) on day 0. (B) Mouse infection with A1151, $\Delta gfsC$, $\Delta gfsAC$, and $\Delta gfsABC$ strains. Outbred ICR mice (male; 5

weeks of age; n=10–11) were immune-compromised via intraperitoneal injection of cyclophosphamide (200 mg/kg mouse) at days –4, –2, 2, and 3. Cortisone acetate was also administrated subcutaneously at a concentration of 200 mg per kg of body weight on day –1. Mice were infected intratracheally with 3.0×10^5 conidia in a volume of 30 μ L for each strain (A1151, $\Delta gfsC$, $\Delta gfsAC$, and $\Delta gfsABC$) on day 0.

Figure 9. Proposed GM structures in $\Delta gfsAC$ strain. (A) Typical structure of FTGM, and (B) OMGM in *A. fumigatus*. (C) Proposed structure of FTGM in the $\Delta gfsAC$ strain. (D) Proposed structure of OMGM in the $\Delta gfsAC$ strain.

Figure S1. SDS-PAGE analysis of purified recombinant GfsA, GfsB, GfsC, and Glf

proteins. Purified recombinant GfsA (5.0 µg), GfsB (3.0 µg), GfsC (5.0 µg), and Glf (5.0 µg)

were separated by 5%–20% SDS-PAGE and stained with Coomassie brilliant blue, revealing

bands of approximately 57.9 kDa (GfsA), 50.3 kDa (GfsB), 52.0 kDa (GfsC), and 45.0 kDa

(Glf).

Figure S2. Effects of reducing agent and acceptor substrate on

galactofuranosyltransferase assay. Chemically synthesized

4-methylumbelliferyl-β-D-galactofuranoside (4MU-β-D-Gal_f) or

p-nitrophenyl-β-D-galactofuranoside (pNP-β-D-Gal_f) were used acceptor substrate. The

chromatogram of standard galactofuranosyltransferase assay containing the purified GfsA

protein (4.5 µg), purified 15.8 µg of Glf protein, 40 mM UDP-Gal_p, 1 mM Mn²⁺, 1.5 mM

4MU-β-D-Gal_f, and 40 mM sodium dithionite (SD) at 30°C for 16 h (a); 40 mM SD was

omitted from the standard assay (b); 40 mM NADH was added to the standard assay instead

of 40 mM SD (c), pNP-β-D-Gal_f was used as an acceptor substrate instead of 4MU-β-D-Gal_f

(d), or pNP- β -D-Gal_f instead of 4MU- β -D-Gal_f and 40 mM SD was omitted from the standard assay (e); and 40 mM NADPH was added to the standard assay instead of 40 mM SD (f).

Figure S3. Construction of the strains $\Delta gfsB$, $\Delta gfsC$ (*ptrA*), and $\Delta gfsC$ (*AnpyrG*).

Chromosomal maps of strains $\Delta gfsB$ (a) and $\Delta gfsC$ (b), and primers used for confirmation. The positions of the primers are indicated by arrows. Electrophoretic analyses of products amplified by PCR using the primer pairs AFUB_070620-1/*ptrA*-R (5'-region) and *ptrA*-F/AFUB_070620-4 (3'-region) for $\Delta gfsB$ (c), and AFUB_067290-1/*pyrG*-R (5'-region) and *pyrG*-F/AFUB_067290-4 (3'-region) for $\Delta gfsC$ (d). M: DNA size markers; Gene Ladder Wide 2 (Nippon Gene, Tokyo, Japan).

Figure S4. Construction of the $\Delta gfsC$ complementary strains $\Delta gfsC::C$. (a) Schematic

representation of $\Delta gfsC$ complementation with *gfsC*. *gfsC* (P), *gfsC* promoter; *gfsC* (T), *gfsC* terminator; *gfsC*, open reading frame of *gfsC*. The positions of the primers are indicated by

arrows. (b) Confirmation of correct recombination of *gfsC* using PCR analysis. Electrophoretic analysis of products amplified by PCR are shown. M, DNA size markers; Gene Ladder Wide 2; Lane 1, DNA fragment (2.6 kbp) amplified using PCR and the primers A_{gfsC}-complement-7 and ptrA-R; lane 2, DNA fragment (1.1 kb) amplified using PCR and the primers ptrA-F and A_{gfsC}-complement-8.

Figure S5. Construction of the strains $\Delta gfsAC$ and $\Delta gfsABC$. (a) Chromosomal maps of strains $\Delta gfsAC$ (a) and $\Delta gfsABC$ (b), and primers used for confirmation. The positions of the primers are indicated by arrows. Electrophoretic analyses of products amplified by PCR using the primer pairs AFUB_096220-1/pyrG-R (5' region of *gfsA*), pyrG-F/AFUB_096220-4 (3' region of *gfsA*), AFUB_067290-1/ptrA-R (5' region of *gfsC*), and ptrA-F/AFUB_067290-4 (3' region of *gfsC*), for $\Delta gfsAC$ (c), AFUB_096220-1/pyrG-R (5' region of *gfsA*, pyrG-F/AFUB_096220-4 (3' region of *gfsA*), AFUB_070620-1/hygB-R (5' region of *gfsB*), hygB-F/AFUB_070620-4 (3' region of *gfsB*), AFUB_067290-1/ptrA-R

828 (5' region of *gfsC*), and ptrA-F/AFUB_067290-4 (3' region of *gfsC*), for $\Delta gfsABC$ (d). M:

829 DNA size markers; Gene Ladder Wide 2.

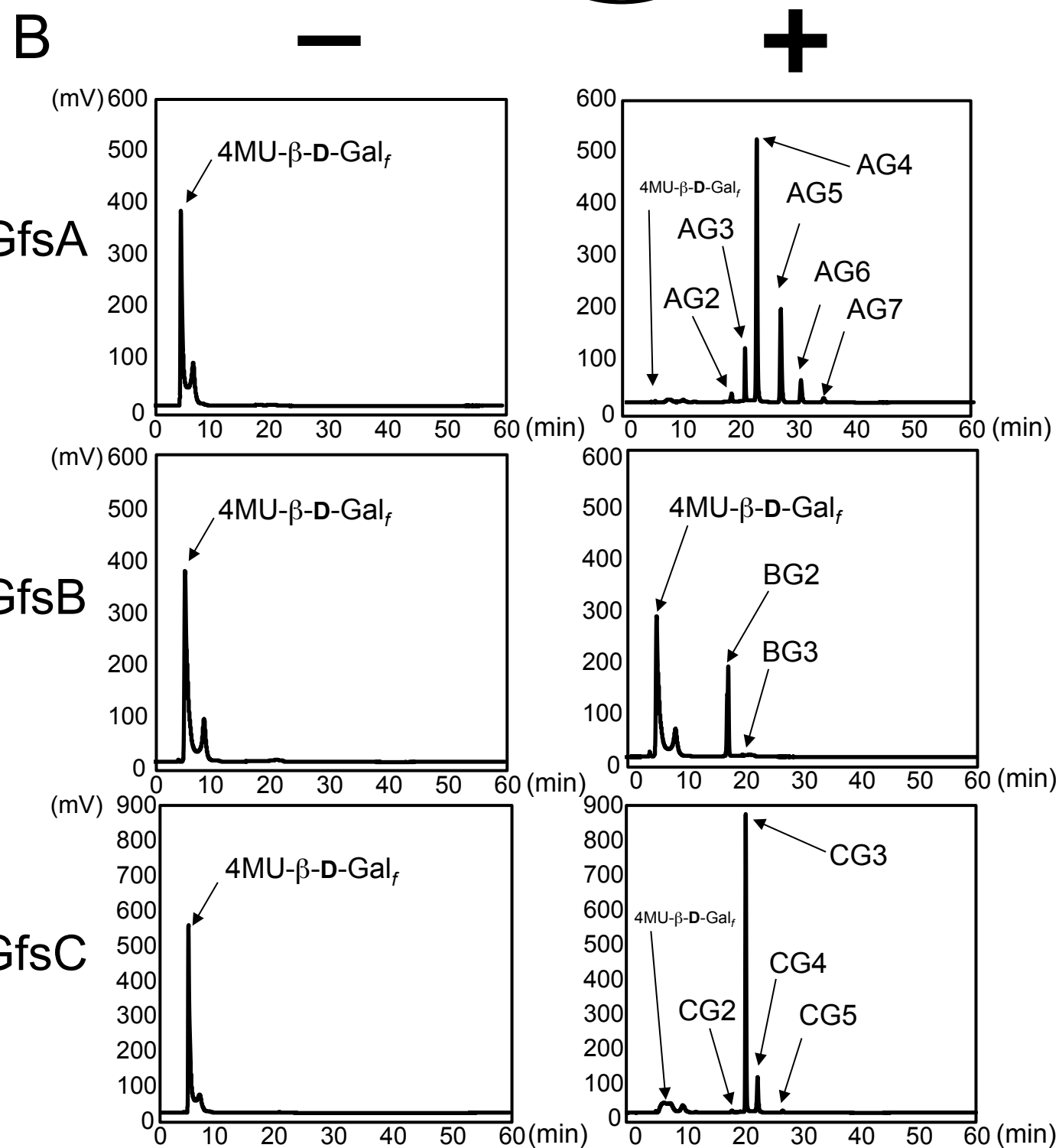
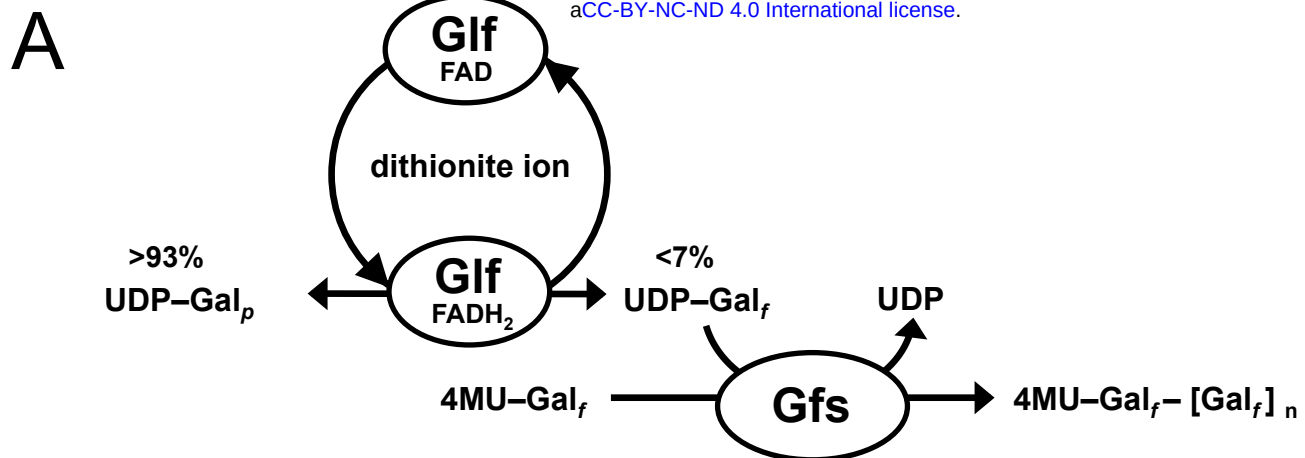
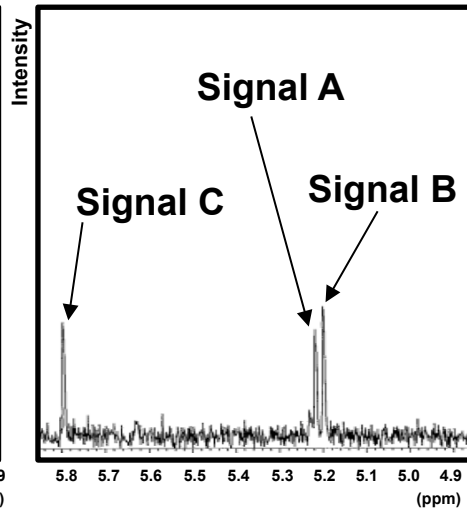
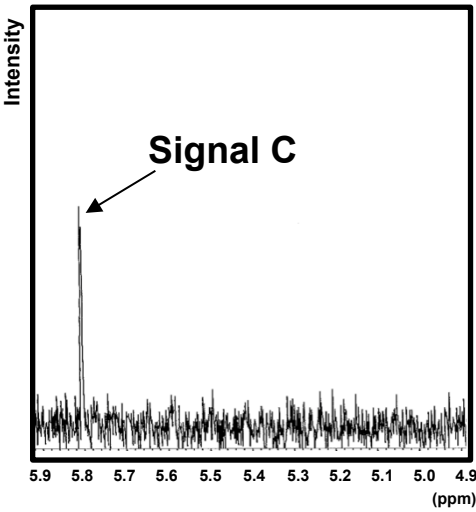


Fig. 1

GfsA

4MU-Gal_f

AG3

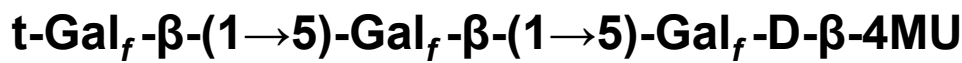
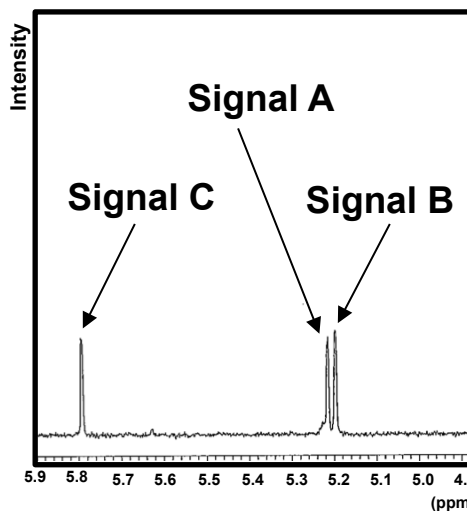
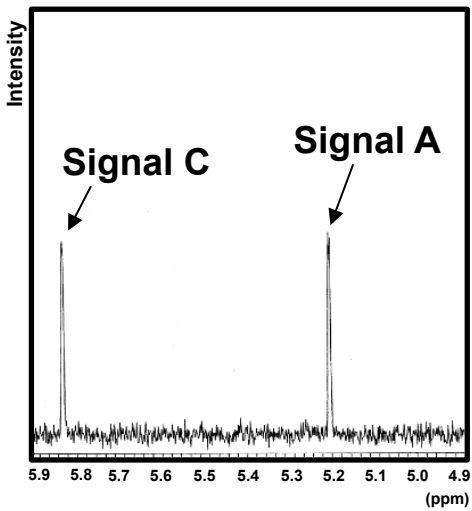


GfsB

GfsC

BG2

CG3



Signal A

Signal B

Signal C

Fig. 2

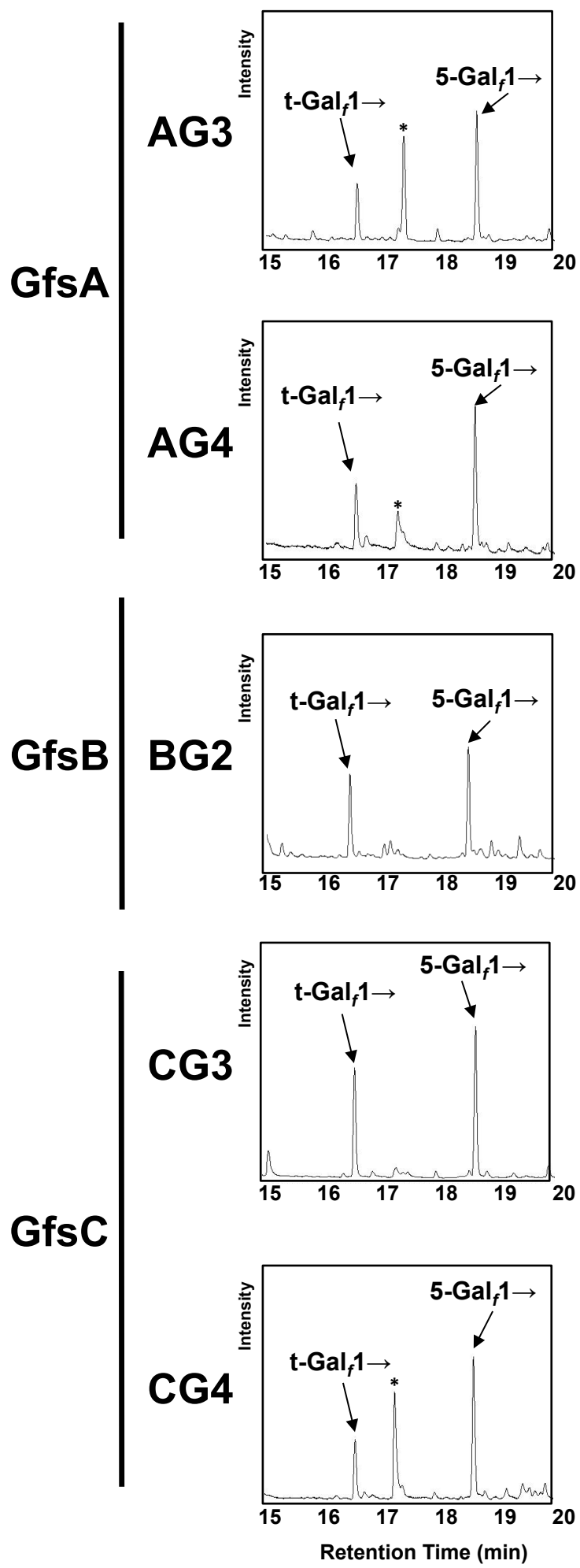


Fig. 3

^{13}C -NMR

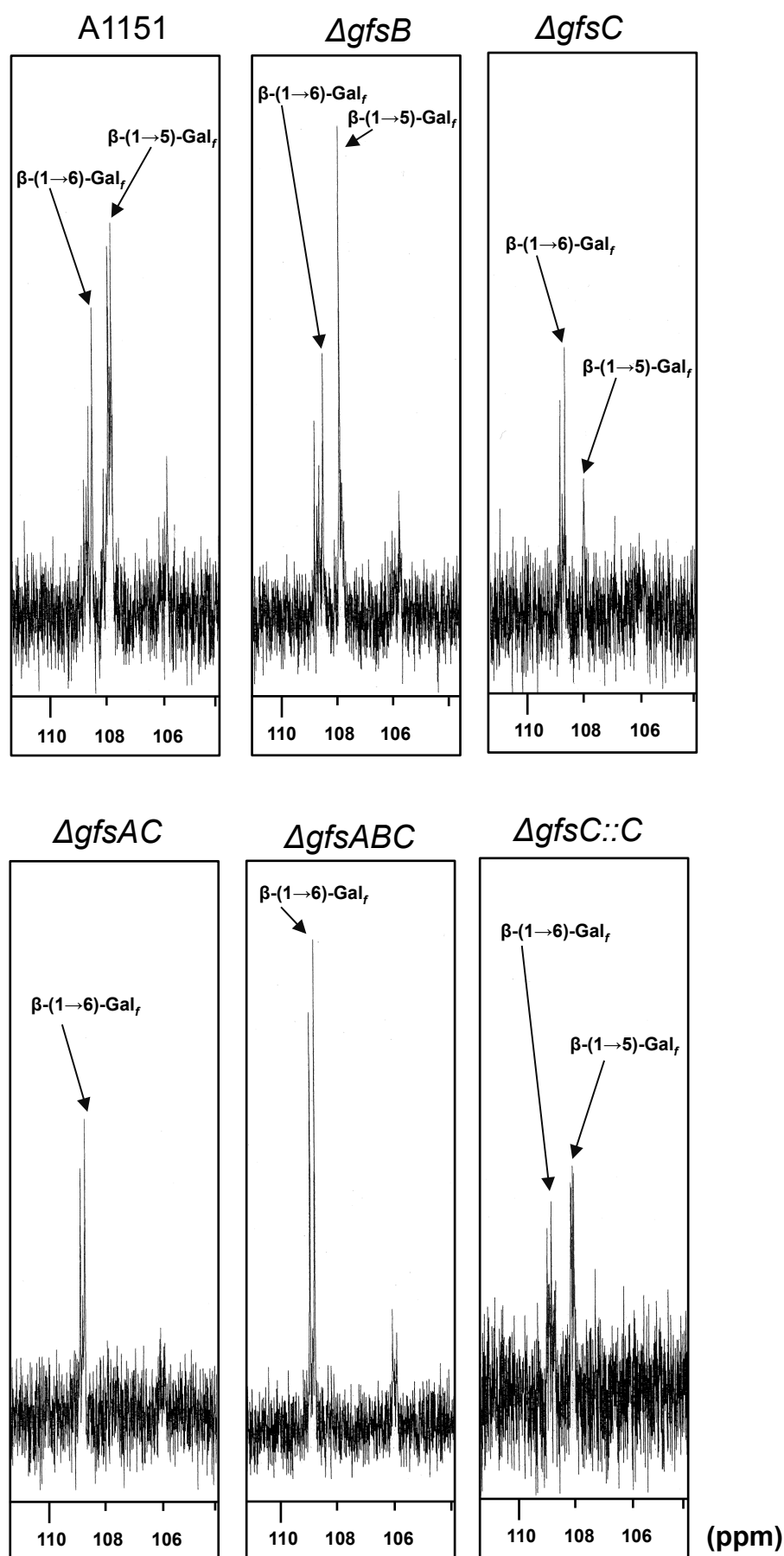
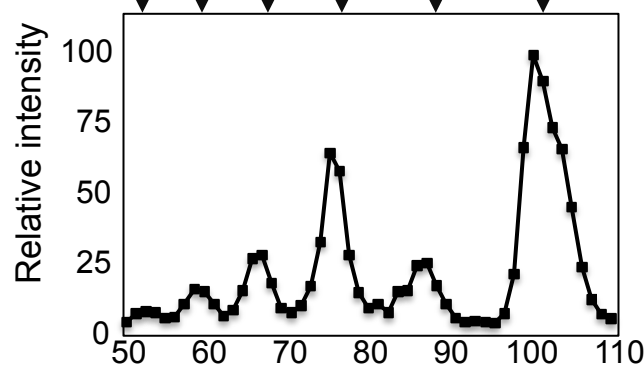


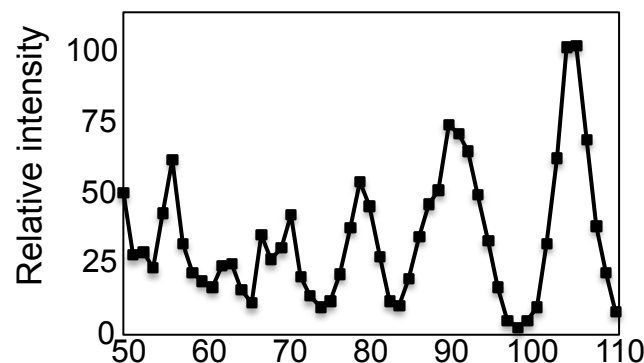
Fig. 4

G6 G5 G4 G3 G2 G1

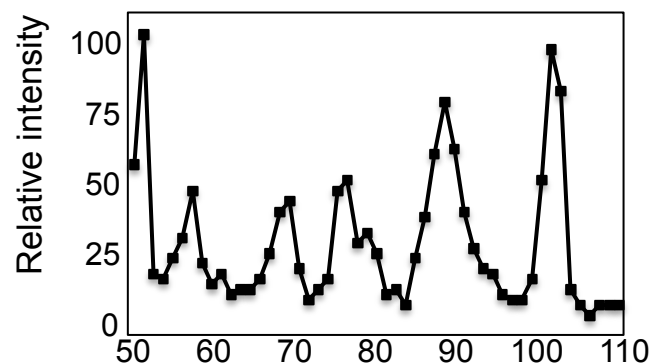
Dextran



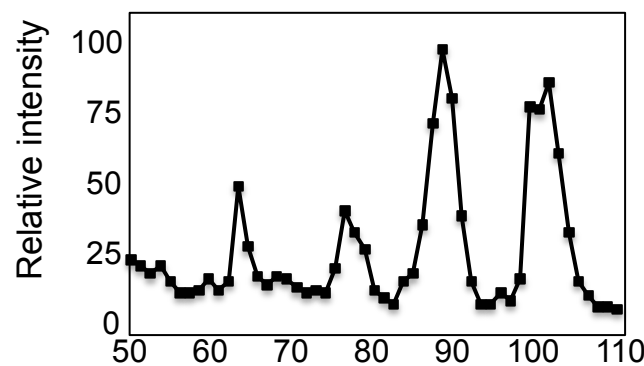
A1151



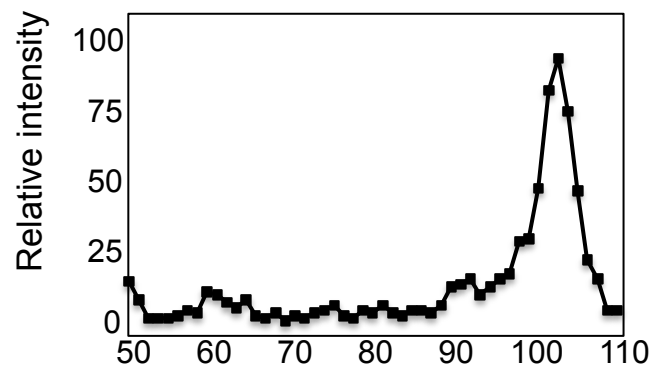
$\Delta gfsA$



$\Delta gfsC$



$\Delta gfsAC$



(Fraction No.)

Fig. 5

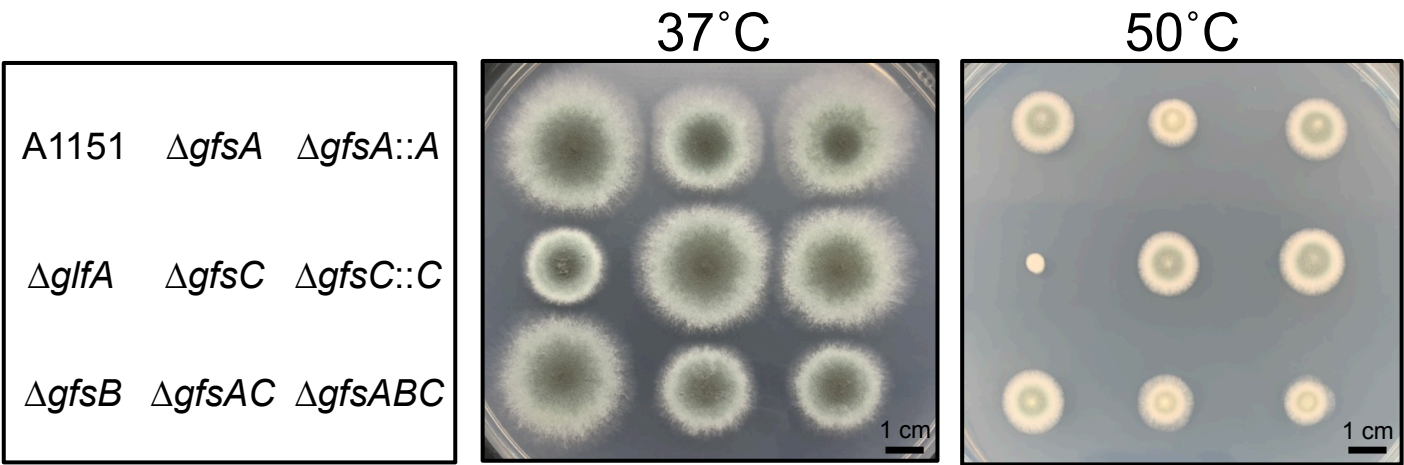


Fig. 6

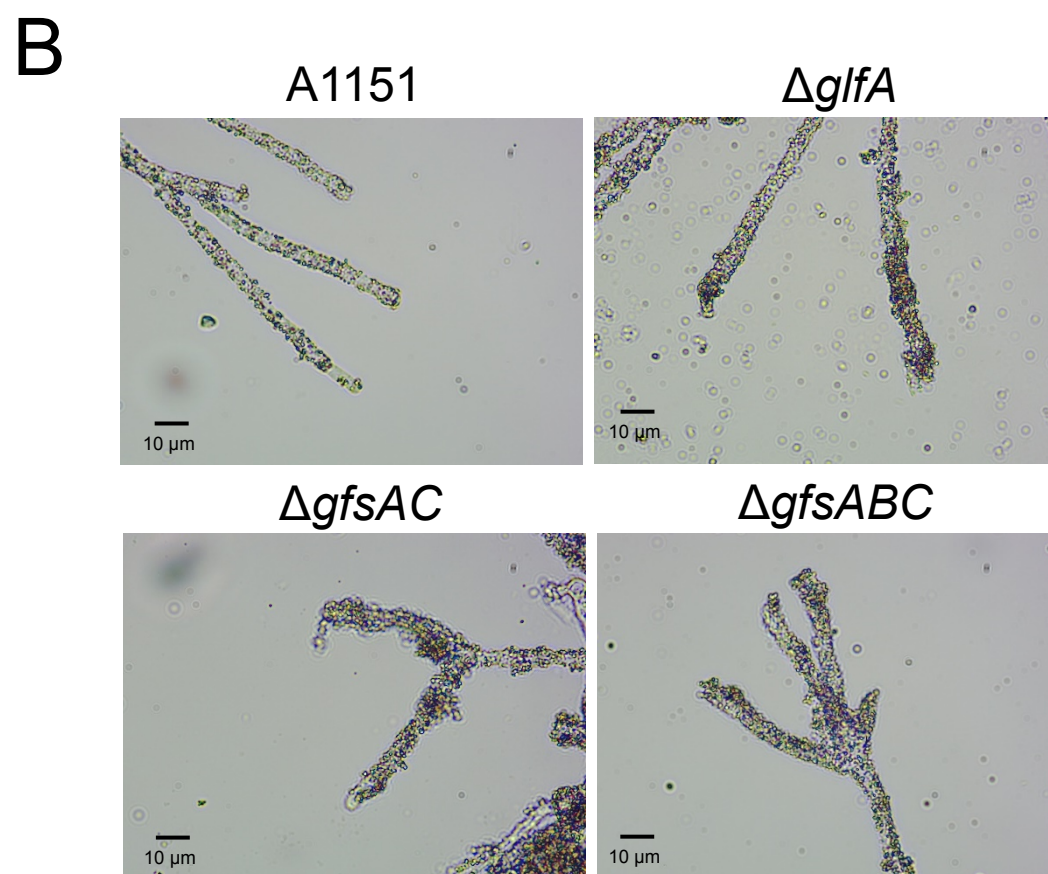
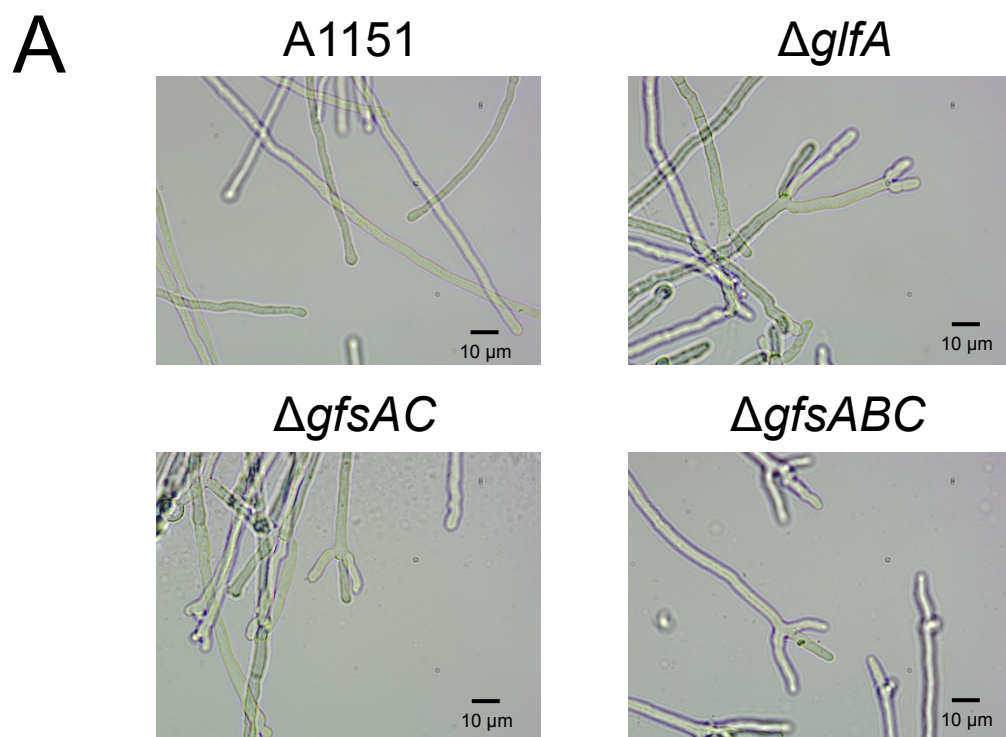
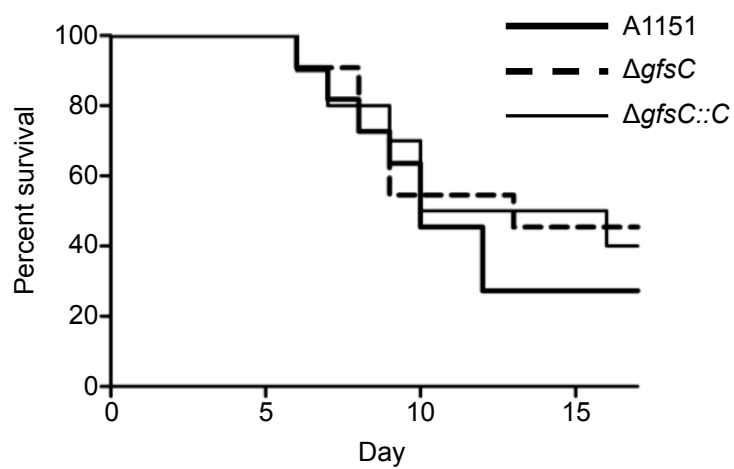


Fig. 7

A



B

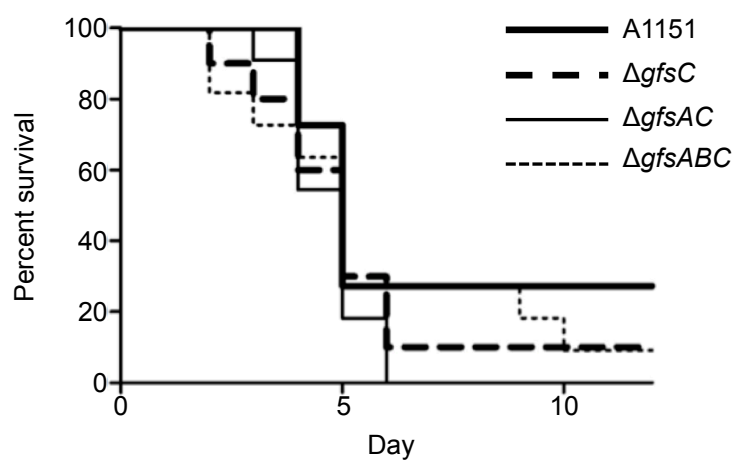


Fig. 8

Fungal-type galactomannan (FTGM)

O-mannose-type galactomannan (OMGM)

A1151

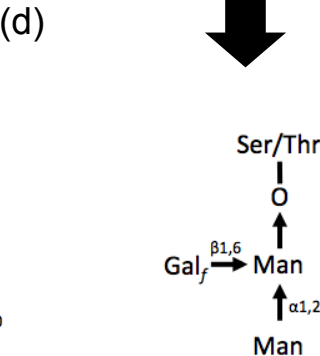
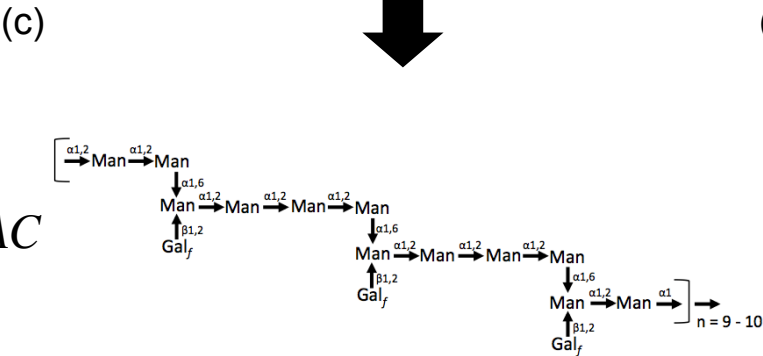
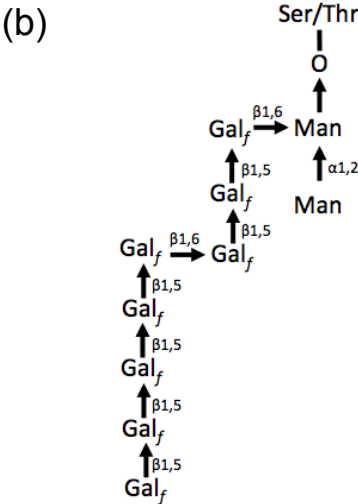
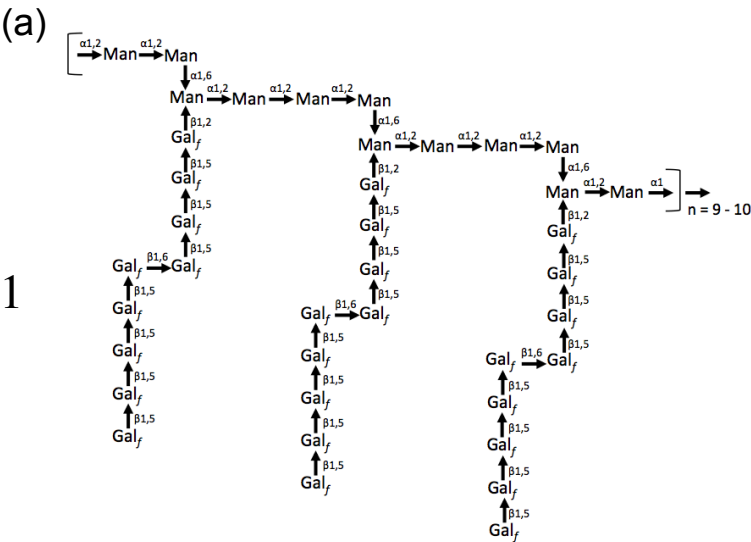


Fig. 9

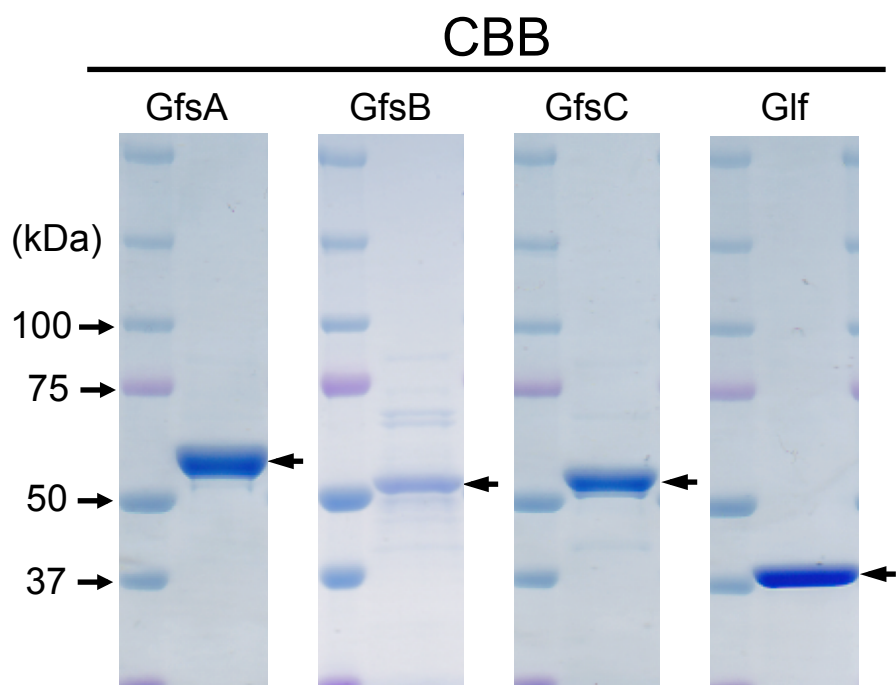


Fig. S1

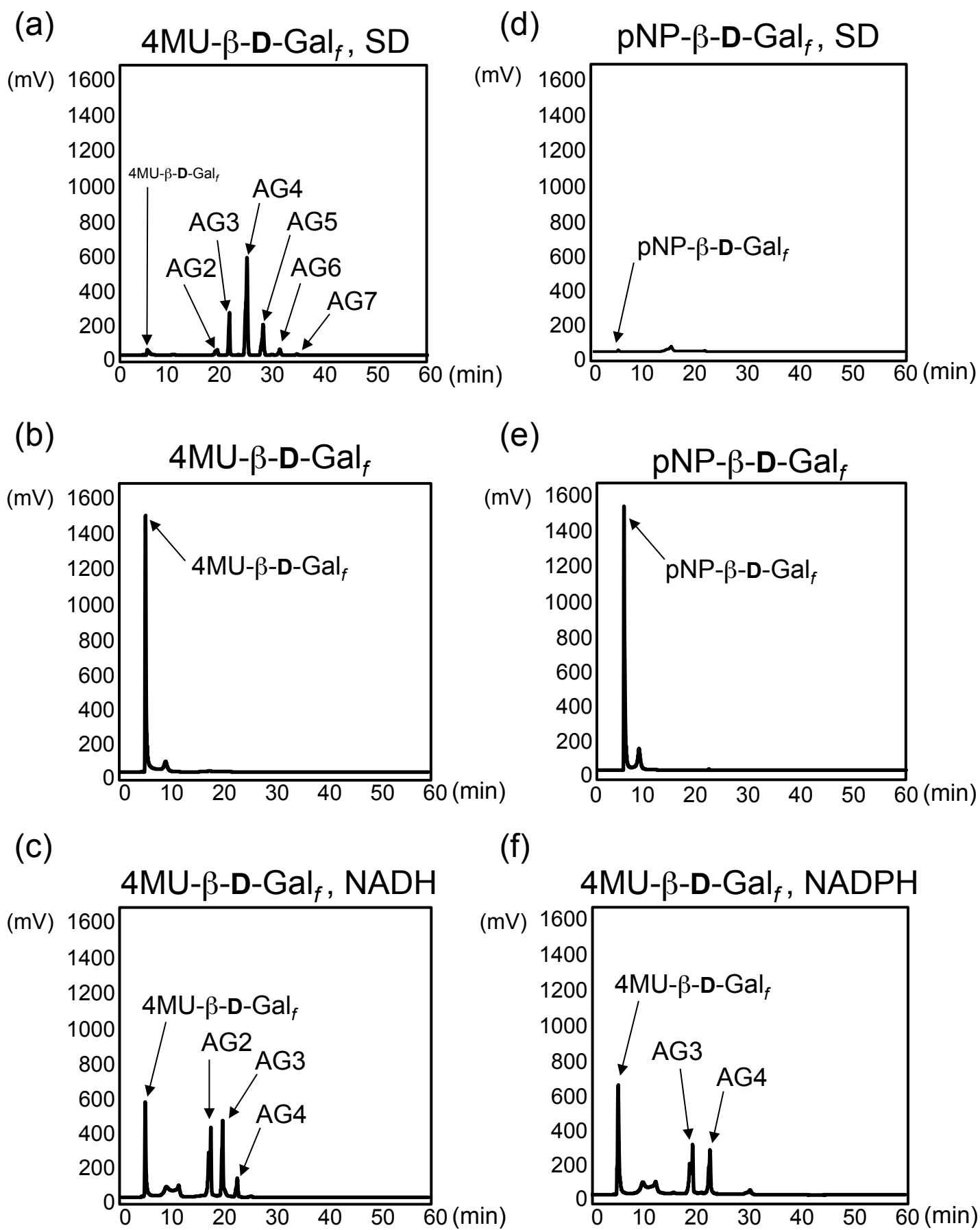
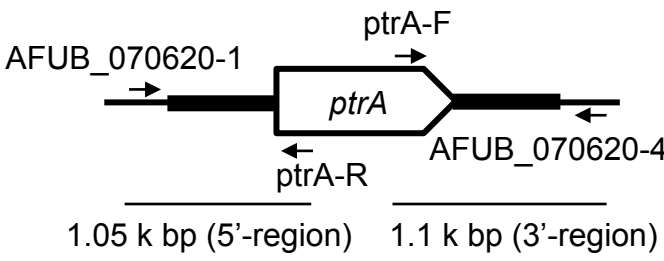
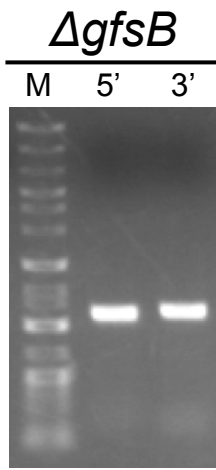


Fig. S2

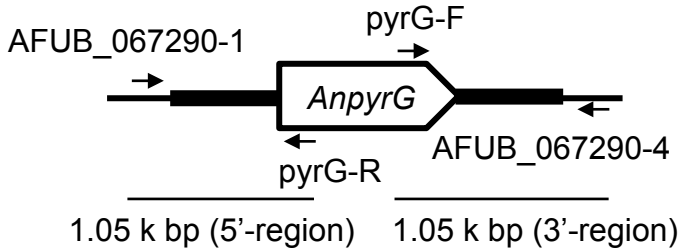
(a) *ΔgfsB*



(c)



(b) *ΔgfsC*



(d)

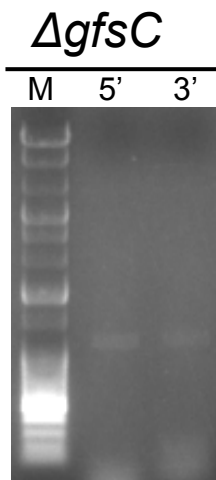


Fig. S3

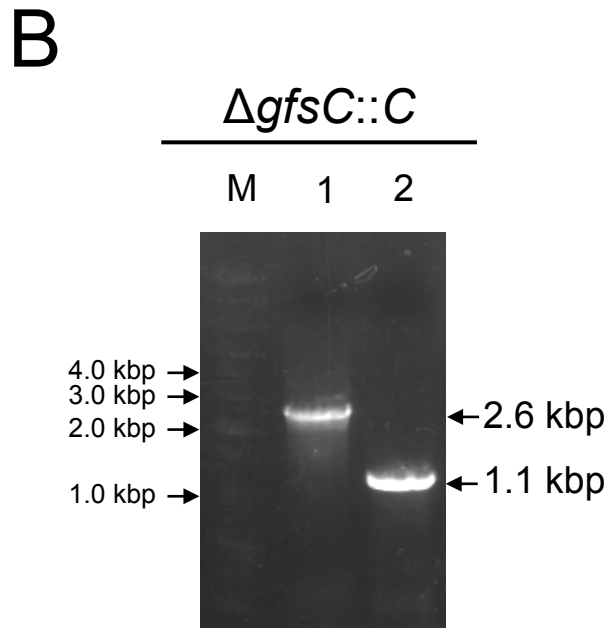
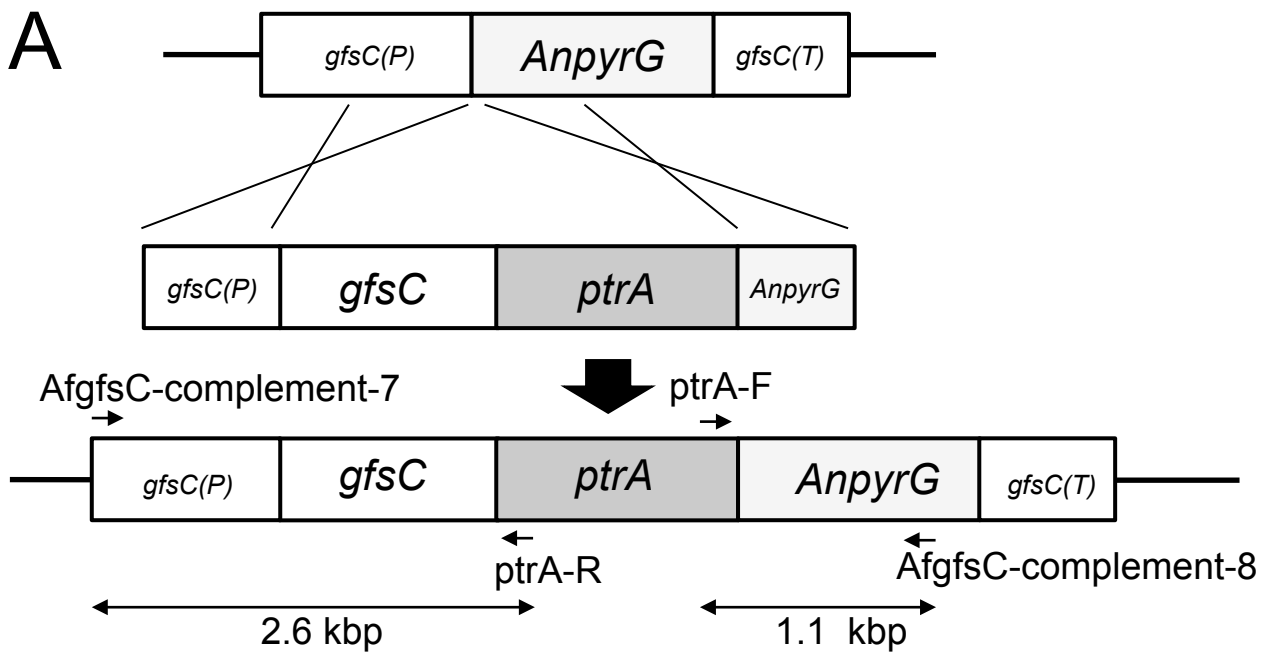
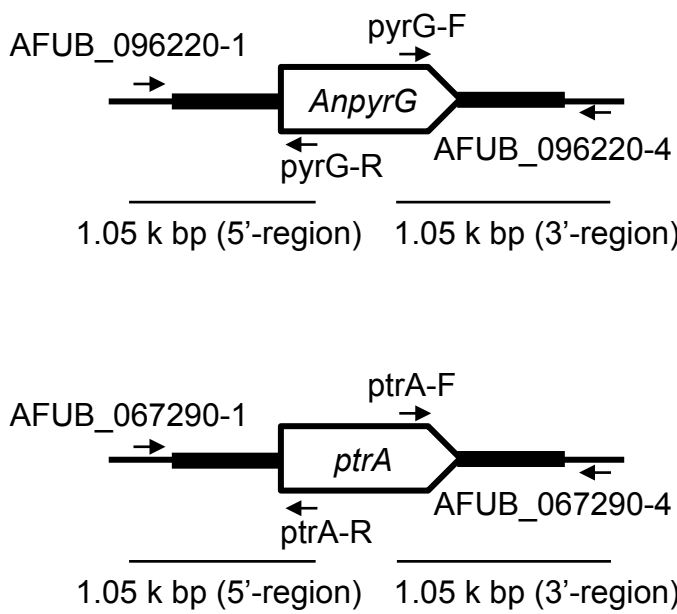
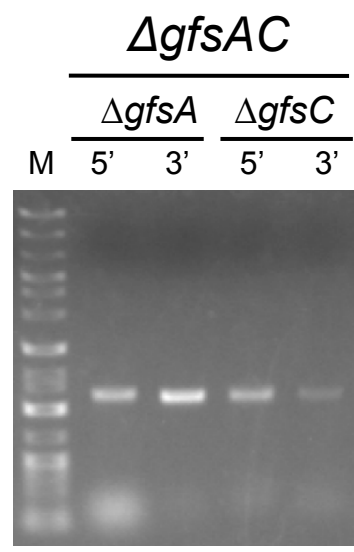


Fig. S4

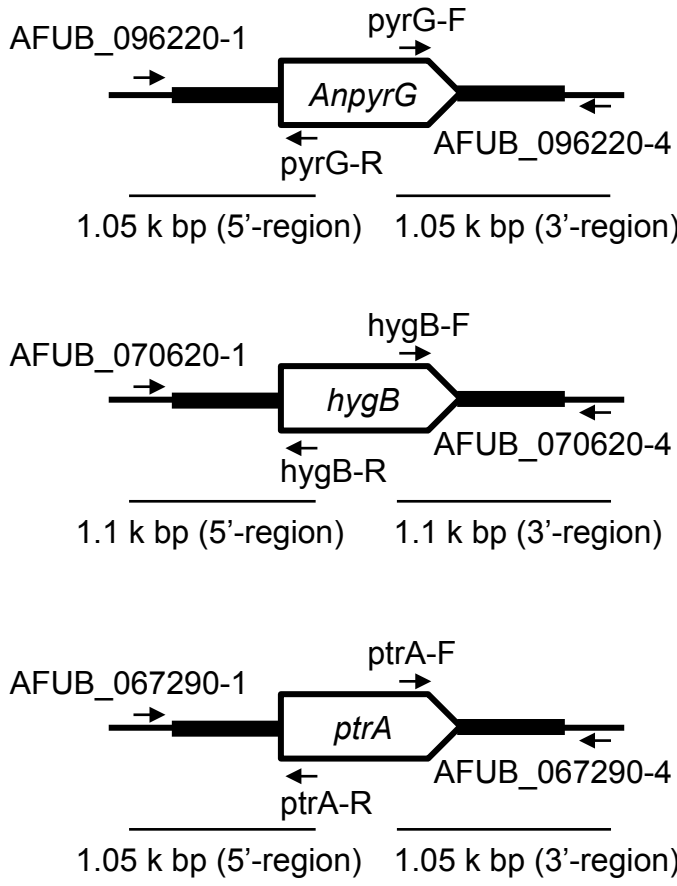
(a) *ΔgfsAC*



(c)



(b) *ΔgfsABC*



(d)

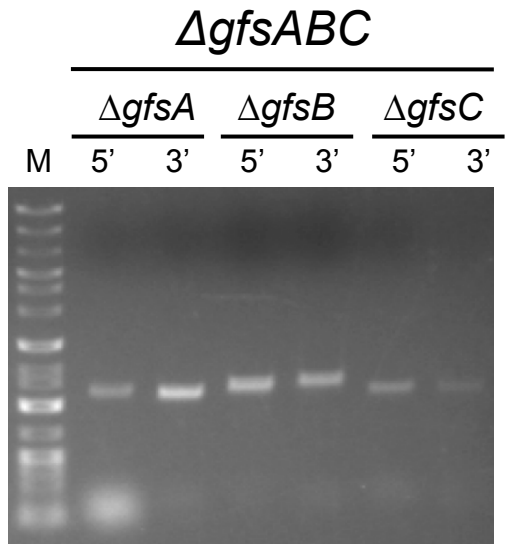


Fig. S5

Table 1. List of mass-to-charge ratios (m/z) of enzymatic products of GfsA, GfsB, and GfsC identified by LC/MS.

	Product name	Molecular mass (calculated)	Mass spectrum [M – H] ⁺ (m/z)	Suggested structure
	4MU-β-D-Gal	338.31	339.11	
GfsA	AG2	500.45	501.16	Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	AG3	662.59	663.21	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	AG4	824.73	825.27	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	AG5	986.87	987.32	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	AG6	1149.01	1149.37	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	AG7	1311.15	1311.42	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
GfsB	BG2	500.45	501.16	Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	BG3	662.59	663.22	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
GfsC	CG2	500.45	501.16	Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	CG3	662.59	663.21	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	CG4	824.73	825.27	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU
	CG5	986.87	987.32	Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -β-(1→5)-Gal ₇ -D-β-4MU

Products of GfsA were AG2, AG3, AG4, AG5, AG6, and AG7; those of GfsB were BG2 and BG3; and for GfsC were CG2, CG3, CG4, and CG5. Mass-to-charge ratios (m/z) of the products were determined by LC/MS with positive ion mode electrospray ionization (ESI).

Table 2. GC-MS analysis of O-methylalditol acetates derived from methylation analyses of galactomannans.

<i>O</i> -methylalditol acetate	Sugar linkage	A1151	$\Delta gfsB$	$\Delta gfsC$	$\Delta gfsAC$	$\Delta gfsABC$	$\Delta gfsC::C$
2,3,4,6-Me4-Man	tMan _p 1→	17.01 ± 1.05	21.19 ± 3.48	22.89 ± 2.96	26.50 ± 1.00	23.13 ± 5.45	16.32 ± 1.83
3,4,6-Me3-Man	2-Man _p 1→	29.80 ± 2.28	27.89 ± 2.34	26.69 ± 1.25	25.23 ± 5.11	25.04 ± 2.39	21.35 ± 3.15
2,3,4-Me3-Man	6-Man _p 1→	12.95 ± 1.66	12.76 ± 1.54	16.42 ± 1.09	16.80 ± 1.87	16.39 ± 1.24	8.50 ± 0.74
3,4-Me2-Man	2,6-Man _p 1→	8.36 ± 0.86	7.14 ± 1.33	8.25 ± 1.28	7.62 ± 0.92	8.99 ± 1.26	4.06 ± 0.40
2,3,5,6-Me4-Gal	tGal _p 1→	13.76 ± 3.33	14.29 ± 2.04	19.97 ± 1.27	20.94 ± 2.35	22.77 ± 1.09	26.86 ± 1.51
2,3,6-Me3-Gal	5-Gal _p 1→	16.31 ± 0.84	15.37 ± 0.71	2.16 ± 0.19	N. D.	N. D.	20.18 ± 2.12
2,3,5-Me3-Gal	6-Gal _p 1→	1.81 ± 0.70	1.34 ± 0.24	3.62 ± 0.44	2.91 ± 0.68	3.68 ± 0.51	2.73 ± 0.87

Table 3 Colony growth rate of the WT, $\Delta gfsA$, $\Delta gfsB$, $\Delta gfsC$, $\Delta gfsAC$, $\Delta gfsABC$, $\Delta gfsA::A$, and $\Delta gfsC::C$ strains.

	A1151	$\Delta gfsA$	$\Delta gfsB$	$\Delta gfsC$	$\Delta gfsAC$	$\Delta gfsABC$	$\Delta gfsA::A$	$\Delta gfsC::C$
37°C	0.75 ± 0.06 (100%)	0.63 ± 0.04 (85.2%)	0.73 ± 0.10 (98.6%)	0.76 ± 0.08 (102.0%)	0.51 ± 0.03 (68.4%)	0.50 ± 0.05 (67.8%)	0.78 ± 0.04 (104.1%)	0.76 ± 0.06 (101.9%)
50°C	0.30 ± 0.04 (100%)	0.24 ± 0.03 (81.2%)	0.30 ± 0.03 (100.5%)	0.27 ± 0.11 (90.9%)	0.26 ± 0.03 (86.4%)	0.25 ± 0.03 (84.0%)	0.31 ± 0.03 (103.2%)	0.37 ± 0.03 (124.3%)

Table 4 Number of formed conidia of the WT, $\Delta gfsA$, $\Delta gfsB$, $\Delta gfsC$, $\Delta gfsAC$, $\Delta gfsABC$, $\Delta gfsA::A$, and $\Delta gfsC::C$ strains.

Strain name	Number of formed conidia (conidia/mm ²)	Percentage of formed conidia to WT strain
A1151	$3.1 \times 10^5 \pm 9.6 \times 10^4$	100%
$\Delta AfgfsA$	$1.6 \times 10^5 \pm 9.1 \times 10^3$	50.9%
$\Delta AfgfsB$	$3.0 \times 10^5 \pm 5.2 \times 10^4$	95.9%
$\Delta AfgfsC$	$2.8 \times 10^5 \pm 3.0 \times 10^4$	90.9%
$\Delta AfgfsAC$	$1.0 \times 10^5 \pm 5.7 \times 10^3$	32.1%
$\Delta AfgfsABC$	$7.9 \times 10^4 \pm 3.5 \times 10^3$	25.4%
$\Delta AfgfsA::A$	$2.6 \times 10^5 \pm 3.5 \times 10^4$	82.6%
$\Delta AfgfsC::C$	$3.8 \times 10^5 \pm 3.4 \times 10^4$	120.3%

Table 5 Sensitivity of the WT, $\Delta cmsA$, $\Delta cmsB$, and $\Delta cmsAB$ strains to antifungal agents ($\mu\text{g/mL}$).

	MCFG	CPFG	AMPH-B	5-FC	FLCZ	ITCZ	VRCZ	MCZ
A1151	0.015	0.25	1	>64	>64	0.5	0.5	2
$\Delta AfgfsC$	0.015	0.25	1	>64	>64	0.5	0.5	1-2
$\Delta AfgfsAC$	0.015	0.25	1	>64	>64	0.25-0.5	0.5-1	1-2
$\Delta AfgfsABC$	0.015	0.25	0.5	>64	>64	0.5	0.5	1

micafungin (MCFG), caspofungin (CPFG), amphotericin B (AMPH-B), flucytosine (5-FC), fluconazole (FLCZ), itraconazole (ITCZ), voriconazole (VRCZ), and miconazole (MCZ).

Table S1 *Aspergillus* strains used in this study

Strains	Genotype	Source of reference
<i>A. fumigatus</i> A1151	<i>KU80::pyrG</i>	da Silva Ferreira, 2006; FGSC
<i>A. fumigatus</i> A1160	<i>KU80::pyrG, pyrG⁻</i>	da Silva Ferreira, 2006; FGSC
<i>A. fumigatus</i> Δ <i>glfA</i>	<i>KU80::AfpyrG, glfA::ptrA</i>	Komachi, 2013 (ref)
<i>A. fumigatus</i> Δ <i>gfsA</i>	<i>KU80::AfpyrG, pyrG⁻, AfgfsA::AnpyrG</i>	Komachi, 2013 (ref)
<i>A. fumigatus</i> Δ <i>gfsB</i>	<i>KU80::AfpyrG, AfgfsB::ptrA</i>	This study
<i>A. fumigatus</i> Δ <i>gfsC</i>	<i>KU80::AfpyrG, pyrG⁻, AfgfsC::AnpyrG</i>	This study
<i>A. fumigatus</i> Δ <i>gfsAC</i>	<i>KU80::AfpyrG, pyrG⁻, AfgfsA::AnpyrG, AfgfsC::ptrA</i>	This study
<i>A. fumigatus</i> Δ <i>gfsABC</i>	<i>KU80::AfpyrG, pyrG⁻, AfgfsA::AnpyrG, AfgfsB::hph, AfgfsC::ptrA</i>	This study
<i>A. fumigatus</i> Δ <i>gfsA::A</i>	<i>KU80::pyrG, AfgfsA::AnpyrG, ΔAfgfsA::AfgfsA-<i>ptrA</i></i>	Katafuchi, 2017 (ref)
<i>A. fumigatus</i> Δ <i>gfsC::C</i>	<i>KU80::AfpyrG, pyrG⁻, AfgfsC::AnpyrG, ΔAfgfsC::AfgfsC-<i>ptrA</i></i>	This study

Table S2 Oligonucleotides used in this study

Oligonucleotide primers	Sequence
AFUB_070620-1	5'- CCGTCGTCATTACAGAGC -3'
AFUB_070620-2	5'- TCGTTACCAATGGGATCCCGTTAGATGTCGCCTGCTTGCAG -3'
AFUB_070620-3	5'- ATGCAAGAGCGGCTCATCGCGCCTTCTGTCCGAGTTCTTT -3'
AFUB_070620-2(<i>gfsB::hph</i>)	5'- GGAAGATCCAGGCACCGGGTTAGATGTCGCCTGCTTGCAG -3'
AFUB_070620-3(<i>gfsB::hph</i>)	5'- AAAACGCGTTTCGGGTTTACCCGCCCTTCTGTCCGAGTTCTTT -3'
AFUB_070620-4	5'- TAGCCGGGGTGGAAATTCG -3'
AFUB_070620-7	5'- AGCTTTGAGCGTTTCTTGGG -3'
AFUB_070620-8	5'- GCTTCAGTGCCAACGAGAGTG -3'
AFUB_067290-1	5'- TTCGTAGTCGTTGGAACCTCTC -3'
AFUB_067290-2	5'- GGCGTGATAGCGTTGAAGGGGTATGTGTTCCGCTTCCCTCG -3'
AFUB_067290-3	5'- AGGTTCTTTTGTGGCTGGGACCCACACTGAGATGATTTGTGG -3'
AFUB_067290-2(<i>gfsC::ptrA</i>)	5'- TCGTTACCAATGGGATCCCGTATGTGTTCCGCTTCCCTCG -3'
AFUB_067290-3(<i>gfsC::ptrA</i>)	5'- ATGCAAGAGCGGCTCATCGCCCCACACTGAGATGATTTGTGG -3'
AFUB_067290-4	5'- TGCCGCTTCTGTTCCTGTG -3'
AFUB_067290-7	5'- TTGTCTTTGCCACTGTCGTTTC -3'
AFUB_067290-8	5'- GCATATCCCATCCCCATGAC -3'
AFUB_096220-1	5'- TACGCCGCTTGCTACTTGG -3'
AFUB_096220-4	5'- GCCAAATTCAATAGTGCACGC -3'
AfGfsC-complement-1	5'- GGAACCTCTCCAATTGTCTAATCG -3'
AfGfsC-complement-2	5'- TCGTTACCAATGGGATCCCGTACGCCATCTTCCAGATCAATTC -3'
AfGfsC-complement-3	5'- ATGCAAGAGCGGCTCATCGCCCTTCAACGCTATCACGCC -3'
AfGfsC-complement-4	5'- AGACATGATGGCGGTTCTCC -3'
AfGfsC-complement-7	5'- TTGGCACTGTCGTTTCTCC -3'
AfGfsC-complement-8	5'- AAGCTGGAAGTGGGATGGCT -3'
pyrG-5	5'- CCCTTCAACGCTATCACGCC -3'
pyrG-6	5'- TCCAGCCACAAAGGAACCT -3'
ptrA-5	5'- GGGATCCCATTTGGTAACGA -3'
ptrA-6	5'- CGATGAGCCGCTCTTGCAT -3'
hph-5	5'- CCGGTGCCTGGATCTTCCT -3'
hph-6	5'- GGTAACCCGAAACGCGTTTAT -3'
pyrG-F	5'- GATCTACCCCTTGGAAACGA -3'
pyrG-R	5'- GACCATCGTGGGCAATTGGT -3'
ptrA-F	5'- CATATGTAATGGCTGTGTCCCG -3'
ptrA-R	5'- TTTAGCTTTGACCGGTGAGC -3'
hph-F	5'- CCGCGGGATCCACTTAACG -3'
hph-R	5'- GTCTCTCCGCATGCCAGAAA -3'
pET50b-AfGfsB-F	5'- ATCATCATCATCATAGCTCCAGACCTGCTAGTCCCTC -3'
pET50b-AfGfsB-F	5'- GTACCGAGCTCCATATCACCCAGATGTAGGTTTCCAG -3'
pCold2-AfGfsC-F	5'- ATCATCATCATCATAGCCGCGCGGTATATGC -3'
pCold2-AfGfsC-R	5'- GTACCGAGCTCCATACTACGCCATCTTCCAGATC -3'
pET50b-Amp-F	5'- GAATTAATTCATGAGCGGATA -3'
pET50b-Amp-R	5'- AACACCCCTTGTTACTGT -3'
Amp-gene-F	5'- AATACAAGGGGTGTTATGAGTATTCAACATTTCCGT -3'
Amp-gene-R	5'- CTCATGAATTAATCTTACCAATGCTTAATCAGTG -3'