Velvet activated McrA plays a key role in cellular and metabolic development in *Aspergillus nidulans*

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McrA is a key transcription factor that functions as a global repressor of fungal secondary metabolism in *Aspergillus* species. Here, we report that *mcrA* is one of the VosA-VelB target genes and McrA governs the cellular and metabolic development in *Aspergillus nidulans*. The deletion of *mcrA* resulted in a reduced number of conidia and decreased mRNA levels of *brlA*, the key asexual developmental activator. In addition, the absence of *mcrA* led to a loss of long-term viability of asexual spores (conidia), which is likely associated with the lack of conidial trehalose and increased β-(1,3)-glucan levels in conidia. In supporting its repressive role, the *mcrA* deletion mutant conidia contain more amounts of sterigmatocystin and an unknown metabolite than the wild type conidia. While overexpression of *mcrA* caused the fluffy-autolytic phenotype coupled with accelerated cell death, deletion of *mcrA* did not fully suppress the developmental defects caused by the lack of the regulator of G-protein signaling protein FibA. On the contrary to the cellular development, sterigmatocystin production was restored in the Δ*fibA* Δ*mcrA* double mutant, and overexpression of *mcrA* completely blocked the production of sterigmatocystin. Overall, McrA plays a multiple role in governing growth, development, spore viability, and secondary metabolism in *A. nidulans*.

Most filamentous fungi produce a high number of asexual spores to survive and propagate in the environment1. Fungal spores are rapidly distributed in the air affecting humankind in a variety of ways. Spores can act as the major infectious agent in both animals and humans, and the inhaled fungal spores can cause invasive infectious diseases in immunocompromised individuals2. Fungal spores are also easily widespread in the air and germinate in the crops causing economic losses3. In addition, spore formation in some filamentous fungi is closely linked to the biosynthesis of secondary metabolites, such as mycotoxins4,5.

The genus *Aspergillus* is one of the most important fungal genera as some species can cause diseases in humans (*A. fumigatus*), produce the most potent carcinogen in nature aflatoxins (*A. flavus*), and are used for the food and pharmaceutical industries (*A. oryzae* and *A. niger*)3,6. *Aspergillus* species use asexual sporulation (called conidiation) as a main reproductive mode7. *Aspergillus* species produce a specialized asexual reproductive structure called conidiophore, which bears numerous chains of asexual spores called conidia8,9. A conidiophore is composed of varying cell types and the process of its production is precisely regulated by multiple positive and negative regulators10. Some of these developmental regulators are thought to be conserved in *Aspergillus* species, and they have been extensively studied in the model fungus *A. nidulans*11,12.

The asexual life cycle of *A. nidulans* can be divided in two stages; vegetative growth and conidiation8. During vegetative growth, fungal spores germinate, leading to the formation of the undifferentiated hyphae. In the early growth phase, the FadA- and GanB-mediated heterotrimeric G protein signaling pathways activate spore germination and vegetative growth, but repress conidiation and sterigmatocystin (ST) production1. During the

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early vegetative growth phase, key negative regulators such as NsdD, VosA, and SfgA cooperatively repress expression of brlA, the essential activator for the initiation of conidiation, until the acquisition of the developmental competence33–36. Under the appropriate conditions, it is speculated that these negative regulators are displaced from the brlA promoter, and upstream positive regulators induce brlA mRNA expression, thereby the fungus begins conidiation and forms conidiophores37.

BrA is a C2H2 transcription factor (TF) that activates abaA, which in turn activates watA17–21. These genes consist of the conserved central cascade of conidiation in Aspergillus species and they control expression of thousands of asexual developmental genes32,22–25. The process of the spore formation and maturation is governed by WatA and the VosA-VelB complex15,24–28. These regulators cooperatively control expression of spore-specific genes for conidia formation and integrity, and confer feed-back negative regulation of brlA24,25,28,29.

VosA and VelB are the violet regulators governing multiple processes including conidiation, spore viability, secondary metabolism, and conidial trehalose biogenesis in A. nidulans. Genome-wide expression and protein-DNA analyses demonstrated that VosA and VelB directly bind to the promoter regions of many genes such as fksA, tpsA, and brlA, and control their expression29,30. Further analyses have led us to define additional VosA-VelB target genes such as vadA (VosA/VelB-Activated Developmental gene)31, mtfA (MultiCluster Regulator A; AN8694)32. Our previous study presented that the expression of mcrA in conidia requires both VosA and VelB30. The mcrA gene encodes a putative TF with a Zn(II)Cys6 domain, which acts as a multicluster negative regulator of fungal secondary metabolism33. Overexpression of mcrA caused repression of secondary metabolite production, whereas deletion of mcrA induced the production of novel secondary metabolites in A. nidulans34. Independent to this study, we have been characterizing the mcrA gene as a direct target of the VosA-VelB heterodimer in conidia and present new findings in the present report. Briefly, the deletion of mcrA resulted in the decreased production of conidia, a rapid loss of conidial viability, and a reduced amount of conidial trehalose, but increased the levels of the conidial β-(1,3)-glucan and secondary metabolites such as ST. Conversely, overexpression of mcrA led to a near complete shut-down of secondary metabolites and the fluffy-autolytic phenotypes. Further genetic studies led us to conclude that McrA represses secondary metabolism downstream of FadA-mediated signaling pathway. We present a genetic model depicting the roles of McrA coordinating cellular and metabolic development in A. nidulans.

Results

Expression and the role of McrA in asexual development of A. nidulans. Previous study described that mRNA levels of mcrA in conidia were drastically low by the lack of VelB or VosA, and a promoter region of mcrA contains a putative VosA response element (VRE), suggesting that McrA is a potential target of the VosA-VelB complex in A. nidulans35. The mcrA ORF composed of 1,453 bp with four exons predicted to encode a 399 aa-length protein that contains a GAL4-like Zn(II)Cys6 domain at the C-terminus. To begin to investigate its function, the levels of mcrA mRNA in the life cycle were investigated. As shown in Figs. 1A and S1A, mcrA mRNA was detectable throughout the life cycle and was high at 48 h after asexual developmental induction.

To investigate the roles of mcrA, we generated the mcrA deletion (ΔmcrA) mutant and the ΔmcrA complemented (C’mcrA) strains, which reintroduced a wild-type copy of the mcrA gene back into the other locus. Wild type (WT), ΔmcrA, and complemented strains were point-inoculated onto a minimal medium (MM) with 1% glucose (MMG) agar and their phenotype was checked. In comparison to WT and complemented strains, the ΔmcrA mutant strain produces abnormal conidiophores and brown colony (Fig. 1B). The deletion of mcrA resulted in a reduced number of conidia and reduced levels of brlA compared to WT and complemented strains (Figs. 1C, D and S1B). These results suggest that McrA plays a key role in asexual development in A. nidulans.

McrA is required for conidial integrity. As the expression of mcrA was activated by VosA-VelB, which controls the conidial viability and integrity, we hypothesized that McrA may play a role in spore survival. To test this hypothesis, WT, ΔmcrA, and C’ strain conidia were collected from 2, 5, 8, 10 and 20 days grown colonies and checked for the viability. As shown in Fig. 2A, the ΔmcrA mutant conidia rapidly lost viability starting from day 5. As trehalose is a key component conferring the long-term spore viability, conidial trehalose amount was tested in the ΔmcrA mutant conidia. The amount of trehalose in ΔmcrA conidia was about twofold less than that of WT or C’ strains (Fig. 2B). Since the absence of vosA increased the levels of β-(1,3)-glucan in conidia, we investigated the β-(1,3)-glucan levels in the ΔmcrA mutant conidia, and found that β-(1,3)-glucan levels in the ΔmcrA conidia were about twofold higher than those of WT and C’ conidia (Fig. 2C). This was corroborated by the finding that ΔmcrA mutant conidia exhibited elevated mRNA levels of fksA, a gene encoding a β-1,3-glucan synthase (Fig. 2D). These results indicate that VosA-VelB-activated McrA is necessary for the governing the integrity of conidia.

The absence of mcra leads to elevated secondary metabolism. Previously, Oakley and colleagues have shown that the McrA is a negative regulator of the production of secondary metabolites in A. nidulans36. We confirmed that the absence of mcra alters the patterns of secondary metabolites and increases ST production in stationary cultures (Fig. 3A, B). We then tested the secondary metabolite patterns in conidia using the high-performance liquid chromatography (HPLC) and found that the ΔmcrA conidia showed about fourfold enhanced production of ST and an unknown metabolite compared to those of WT and complemented strains (Fig. 3C–E). We then tested mRNA levels of aflR encoding an essential Zn(II)Cys6 TF for the activation of the ST gene cluster and found that ΔmcrA conidia exhibited higher levels of aflR mRNA than those of WT and C’ conidia (Fig. 3F). Overall, these results imply that McrA is a key negative regulator of secondary metabolite production in both hyphae and conidia.
Pleiotropic effects caused by overexpression of McrA. To further test the potential role of mcrA in conidiation and secondary metabolism, mcrA overexpression (OE) strains by expressing an ectopic copy of mcrA under the alcA promoter (alcA(p)::mcrA) were generated and checked for their phenotypes. First, we checked ST accumulation under non-inducing (MMG) and inducing (MM with 100 mM threonine, MMT) conditions and found that there were no differences between WT and OE mcrA for ST production in MMG. However, when cultured in MMT, OE mcrA caused the total blockage of ST and other metabolites' production (Fig. 4A). The previous study described that OE mcrA leads to a reduction of fungal growth that might be related to a reduction of secondary metabolite production34. We then cultured WT and OE mcrA strains onto solid MMG and solid MMT and found that OE mcrA led to the fluffy-autolytic phenotype with about tenfold reduction in conidia formation, whereas growth and development of WT and OE mcrA strains were similar in MMG (Fig. 4B,C). We then examined whether OE mcrA-caused fluffy-autolytic phenotypes were coupled with accelerated cell death using the alamarBlue reduction assay and found that, OE mcrA led to dramatically reduced cell viability starting at day 3 compared to WT (Fig. 4D). Taken together, these results imply that McrA plays
an important role not only in the production of ST and other secondary metabolites, but also in fungal growth, conidiation, autolysis, and cell death.

Genetic epistasis between McrA and FlbA. Previous studies found that the FadA Gα-dependent signaling pathway activates vegetative growth while inhibiting development and ST biosynthesis, and that this signaling is attenuated by the regulator of G-protein signaling (RGS) protein FlbA. Loss of flbA function leads to the fluffy-autolytic phenotype coupled with the lack of ST production, accelerated cell death and autolysis. As FlbA functions quite upstream of vegetative signaling, we envisioned that McrA might act downstream of FlbA-controlled pathway and exert the fluffy-autolytic phenotype and blockage of ST production. To test this genetic epistasis, we generated the ΔflbA ΔmcrA double mutant and compared its phenotypes with those of WT and individual single mutant. As shown in Fig. 5B, the ΔflbA ΔmcrA double mutant exhibited a decrease in colony diameter which is similar to the ΔflbA mutant and exhibited partial autolysis in the edge of the colony. In addition, asexual development was not restored in the ΔflbA ΔmcrA double mutant compared to the ΔflbA or ΔmcrA single mutant (Fig. 5C). Likewise, while delayed 1 day compared to the ΔflbA mutant, the ΔflbA ΔmcrA double mutant could not regain cell viability (Fig. 5D). These results imply that mcrA is not essential but adequate to cause the fluffy-autolytic phenotype and accelerated cell death, and that McrA is likely independent to the FlbA-attenuated signaling pathway repressing ST production.
Discussion
Conidial formation and maturation are regulated by three key transcription factors WetA, VosA and VelB. These are highly conserved regulators that control the expression of spore-specific genes in Aspergillus species. Genome-wide expression and protein-DNA analyses identified certain target genes for these TFs. Follow-up studies identified the functions of some target genes and these results provide some clues to elaborate on how these TFs can control spore formation and maturation. For example, the VosA-VelB complex controls the expression of fksA, mtfA, sclB, and vadA, thereby fine-tuning β-glucan synthesis, secondary metabolism, oxidative response, and conidial pigmentation. This study further expands the VosA/VelB-mediated regulatory networks involving McrA in A. nidulans. In conidia, deletion of mcrA leads to decreased conidial viability and trehalose amount but increased β-glucan and ST levels. The ΔmcrA mutant conidia exhibited an intermediate phenotype compared to the ΔvosA mutant and WT conidia. In addition, previous microarray and
Figure 4. Overexpression of *mcrA* causes pleiotropic effects. (A) Amount of ST production in WT (FGSC4) and OEmcrA (THS41; *alcA*(p)::*mcrA*) strains. The supernatant of each strain following 3 days of stationary culture was extracted using chloroform and subjected to TLC analysis. (B) WT (FGSC4) and OEmcrA (THS41; *alcA*(p)::*mcrA*) strains were point inoculated onto solid MMG (non-inducing) or onto solid MMT (inducing) including 0.5% YE medium. Photographs of the cultures at day 5 are also shown. The bottom panels indicate close-up views of the middle of the plates (bar = 100 μm). (C) Quantitative analysis of conidiospore formation of the strains shown in (B). The numbers of conidia per plate were counted in triplicates (**p < 0.01; ***p < 0.001). (D) Relative AB reduction rates of WT and OEmcrA (THS41) strains grown under submerged culture conditions at 37 °C. The percent of alamarBlue (AB) reduction represents the fungal cell viability. The mean values were represented by a bar graph.
Figure 5. Genetic position of McrA action. (A) WT (FGSC4), OEmcrA (THS41; alcA(p)::mcrA), and ΔflbA (TMK15) strains were point inoculated onto solid MMG (non-inducing) or solid MMT (inducing) including 0.5% YE. Photographs of the cultures at day 2 and 5 are shown. (B) WT, ΔmcrA (TMK19), ΔflbA (TMK15), and ΔmcrA ΔflbA (TMK14) strains were point inoculated onto solid MMG. Photographs of the cultures at day 5 are shown. (C) Quantitative analysis of conidiospore formation of the strains shown in (B). The numbers of conidia per plate were counted in triplicates. (D) Relative AB reduction rates of WT, ΔmcrA (TMK19), ΔflbA (TMK15) and ΔmcrA ΔflbA (TMK14) strains grown under submerged culture conditions at 37 °C. The percent of alamarBlue (AB) reduction represents the fungal cell viability. The mean values were represented by a bar graph, respectively. (E) ST analysis by TLC. WT, ΔmcrA (TMK19), ΔflbA (TMK15), and ΔmcrA ΔflbA (TMK14) strains were stationary cultured in liquid MMG at 37 °C for 3 days, and extracted with chloroform and subjected to TLC. ST standard (15 μg) was loaded as a positive control. The arrow indicates ST.
Chromatin-Immuno-Precipitation (ChIP) followed by microarray (ChIP-chip) analyses proposed that VosA may directly bind to the mcrA promoter and regulate mcrA expression in conidia, that is, McrA is probably a direct target of VosA in conidia.

McrA is a multifunctional regulator controlling certain gene expression involved in biosynthesis of secondary metabolites. We revealed that, in conidia, the loss of mcrA increased the mRNA level of aflR, proposing the role of McrA in repressing conidial secondary metabolites. Furthermore, McrA is needed for proper biogenesis of conidial trehalose and downregulation of fksA in conidia, thereby governing the integrity of spores.

Importantly, we have shown McrA’s new role in vegetative growth, autolysis, and cell death in A. nidulans. Autolysis is a naturally occurring phenomenon that is reported as enzymatic self-degradation of the cells, affected by nutrient limitation, aging, and other factors. Previous studies have noted that FlbA, an RGS protein, negatively regulates vegetative growth by turning off the FadA-mediated G protein to cAMP-dependent protein kinase (PKA) signaling cascade. The absence of flbA leads to prolonged the activation of FadA-mediated G protein signaling, resulting in the autolytic phenotypes. Our double deletion analysis has shown that the deletion of mcrA leads to a slight delay in the autolytic phenotypes but could not suppress the elevated cell death caused by ΔflbA. On the contrary, the removal of mcrA restored ST production in the ΔflbA mutant, proposing that McrA represses ST biosynthesis downstream of the FlbA-controlled FadA-PKA signaling route.

Collectively, we suggest a genetic model showing the multiple roles of McrA in A. nidulans (Fig. 6). During vegetative growth, McrA represses secondary metabolites production acting downstream of the FadA (Ga) and SfaD::GpgA (Gjb) mediated signaling pathway, which is attenuated by the RGS protein FlbA. McrA may have a potential to activate hyphae growth and autolysis in parallel to the FadA-mediated signaling pathway. McrA is also required for proper expression of brlA and thereby conidiation. It is not yet known whether McrA directly binds to the brlA region or it affects other upstream regulators. During spore formation, the VosA-VelB complex activates the expression of mcrA in conidia, thereby controlling trehalose biogenesis and spore viability, and inhibiting ST production and β-glucan synthesis. While this study demonstrated the complex role of McrA in fungal biology, the detailed molecular mechanisms of McrA action need to be investigated. In this regard, we are in the process of identifying the direct targets of McrA via ChIP-seq analysis using the McrA-FLAG fusion protein.

**Methods**

**Fungal strains and culture conditions.** A. nidulans strains used in this study are listed in Table 1. Fungal strains were grown on solid or liquid minimal medium (MM) with 1% glucose (MMG) with supplements as described previously. To determine the numbers of conidia in WT (FGSC4) and mutant strains, 10⁵ spores
were spread onto solid MMG and incubated at 37 °C for 2 days. The conidia were then collected from the entire plate and counted with the use of a hemocytometer.

To examine the effects of overexpression of mcrA by expressing an ectopic copy of mcrA under the alcA promoter, the 3/4 mcrA mutant, THS41, was screened by Western blot overexpression (OE mcrA)(p) and then introduced into TNJ36. The final flbA deletion construct was amplified with OHS771;OHS772 or OMK611;OMK612, respectively. To generate the ΔflbA deletion construct, amplified from genomic DNA of A. nidulans wild type FGSC4 using OHS767;OHS769 and OMK607;OMK610 (for mcrA), and OMK609;OMK610;OMK614;OMK610 (for flbA). The A. fumigatus pyrG marker was amplified from A. fumigatus AF293 genomic DNA with the primer pair OMK589;OMK590. The final mcrA or flbA deletion construct was amplified with OHS771;OHS772 or OMK611;OMK612, respectively. To generate the ΔflbA ΔmcrA double mutant, 5′ and 3′ flanking regions of each gene were amplified from genomic DNA of A. nidulans strain TMK14 pyrG89;ΔmcrA(p) and TMK15 pyrG89;ΔflbA::pyroA4(p) and ΔmcrA::*pyroA4(p) (for pyroA) with OHS768;OHS770 (for mcrA), and OMK607;OMK613 and OMK614;OMK610 (for flbA). The A. nidulans pyroA+ marker was amplified from FGSC4 genomic DNA with the primer pair ONK395;ONK396. The flbA deletion cassette was introduced into TMK19. Protoplasts were generated using the Vinoflow FCE lysing enzyme (Novozymes)48. At least three independent deletion mutant strains were isolated and confirmed by PCR analysis.

Generation of A. nidulans strains. Oligonucleotides used in this study are listed in Table 2. The double joint PCR (DJ-PCR) method was used to generate the ΔmcrA and ΔflbA mutants. Both 5′ and 3′ flanking regions of each gene were amplified from genomic DNA of A. nidulans FGSC4 using OHS767;OHS769 and OMK607;OMK613 and OMK614;OMK610 (for flbA). The A. fumigatus pyrG marker was amplified from A. fumigatus AF293 genomic DNA with the primer pair OMK589;OMK590. The final mcrA or flbA deletion construct was amplified with OHS771;OHS772 or OMK611;OMK612, respectively. To generate the ΔflbA ΔmcrA double mutant, 5′ and 3′ flanking regions of each gene were amplified from genomic DNA of A. nidulans strain TMK14 pyrG89;ΔmcrA(p) and TMK15 pyrG89;ΔflbA::pyroA4(p) and ΔmcrA::*pyroA4(p) (for pyroA) with OHS768;OHS770 (for mcrA), and OMK607;OMK613 and OMK614;OMK610 (for flbA). The A. nidulans pyroA+ marker was amplified from FGSC4 genomic DNA with the primer pair ONK395;ONK396. The flbA deletion cassette was introduced into TMK19. Protoplasts were generated using the Vinoflow FCE lysing enzyme (Novozymes)48. At least three independent deletion mutant strains were isolated and confirmed by PCR analysis.

To complement the deletion of mcrA, the mcrA locus such as its 2 kb 5′ UTR and coding region was amplified with the primer pair OMK657;OHS878, digested with EcoRI and NotI, and cloned into pHS13, which contains 3/4 pyroA, a 3xFLAG tag, and the trpC terminator. The resulting plasmid pMK23 was then introduced into the recipient ΔmcrA mutant TMK19, in which a single copy mcrA+ is confirmed to be inserted into the pyroA4 locus, to give rise to TMK20.

To generate the alcA(p)::mcrA fusion construct, the mcrA ORF derived from FGSC4 genomic DNA was amplified using the primer pair OH5875;OH5878. The amplicon was double digested with EcoRI and NotI and cloned into pHS3, which has the alcA promoter and the trpC terminator. The resulting plasmids pHSN74 was then introduced into TNJ36. The mcrA overexpression (OE mcrA) mutant, THS41, was screened by Western blot analysis using monoclonal anti-Flag antibody (M2 clone, Sigma-Aldrich).

Nucleic acid manipulation. Genomic DNA isolation was performed as previously described. Total RNA for Northern blot was isolated from each sample using Trizol reagent (Thermo Fisher Scientific) following the protocol provided by the manufacturer’s instructions. For Northern blot analysis, DNA probes were prepared by PCR amplification of the coding region of individual genes with suitable oligonucleotide pairs using WT genomic DNA as a template. Probes were labeled with α-32P-dCTP (PerkinElmer) using the Random Primer DNA Labeling Kit (Takara Bio) and purified by Illustra MicroSpin G-25 columns (GE Healthcare).

For quantitative real-time PCR, complementary DNA was synthesized using the GoScript Reverse Transcription system (Promega) using the total RNA was isolated from each sample using Trizol reagent. qRT-PCR was performed with each gene-specific primer set and iTaq universal SYBR Green supermix (Bio-Rad) and using a CFX96 Touch Real-Time PCR system (Bio-Rad).

Determination of cell viability. For spore viability, WT and mutant strains were inoculated onto MMG and cultured for 2, 5, 8, 10, and 20 days. Conidia were collected from the cultured plates. After then, about 100 conidia were spread onto MMG plates and the plates were then incubated for 48 h. Survival rates were calculated as the ratio of the number of colony forming unit to the number of spores inoculated.

Table 1. Aspergillus strains used in this study. a All A. nidulans strains carry the veA+ allele. b Fungal Genetic Stock Center. c The 3/4 pyroA marker restores pyroA+ when it is integrated into the pyroA locus.
Fungal cell viability was determined by the percent reduction of alamarBlue (Bio-Rad). The alamarBlue assay reagent was placed into each well of a 24-well plate, which has 1 mL of fresh liquid MMG with 0.5% YE and 0.5 mL of individual cultures with an equal amount of the mycelium, at a final concentration of 10% of the reaction volume. After the plate was incubated at 37 °C for 6 h in the dark, the absorbance of each well was detected by A570 and A600 nm wavelength. The reduction percent of alamarBlue was calculated as described.

The TLC plate was exposed to UV of A320 nm, and ST levels were measured. This experiment was performed in triplicate.

High-performance liquid chromatography (HPLC) analysis. The HPLC analysis was performed as previous described. Asexual spores (2 × 10⁵) fungal strains were extracted by adding chlorofom into the vials. The samples were vigorously mixed using a vortex mixer. The organic phase was then separated by centrifugation and transferred to new vials. Each sample was evaporated and resuspended with 0.5 mL of HPLC grade acetonitrile:methanol (50:50, v/v), where the RF value of ST was 0.65. Aluminum chloride (20%) w/v in 95% ethanol) was sprayed onto the TLC plate and the plate was baked at 70 °C for 5 min to enhance the detection of ST. The TLC plate was exposed to UV of A320 nm, and ST levels were measured. This experiment was performed in triplicate.

**Table 2.** Oligonucleotides used in this study. "Tail sequence is in italic. Restriction enzyme site is in bold.

| Name   | Sequence (5’-3’) | Purpose                  |
|--------|------------------|--------------------------|
| OHS767 | TCGAAGAGTTGTCGCCACAGC | 5’ flanking of mcrA       |
| OHS769 | GGCTTTGCGCTGACATCATGACATTCA CATTGGAAAGTGCGGGAGGACAG | 3’ mcrA with AfupyrG tail |
| OHS770 | TTTTGGTGACGACATTCCTGGGACCACTTCTCATGCCAAATCGTCT    | 5’ mcrA with AfupyrG tail |
| OHS768 | AGCACTGTGGATGACAGCTAAC  | 3’ flanking of mcrA       |
| OHS771 | CGACCCCAACTCACCAGGACTCT | 3’ flanking of mcrA  |
| OHS772 | CAATGCTCCTAATGTCTACTGCGG | 3’ not of mcrA |
| OMK607 | TCACATCTCGATGTTCTGTAATG  | 3’ flanking of flbA    |
| OMK613 | GCCCTGGCGCTGATGACATTCTCA TGCCATTGAAAGTGCGGGAGGAG | 3’ flbA with AfupyrG tail |
| OMK614 | ATCGACCGAACCCTAGGATGGTA ACAGTAATTATCTACAGCCGTAGT | 5’ flbA with AfupyrG tail |
| OMK610 | ACTACTCACTACTACACATTTGACT  | 3’ flanking of flbA  |
| OMK611 | TGGTTGATGTTGATGTCAGC       | 3’ not of flbA          |
| OMK612 | TGTAGCTTTGCTCTAAGGAGATGT  | 3’ not of flbA          |
| OMK608 | ACTCTGACGATAGCTG AAGTTGCTGCGGAG | 3’ flbA with AnipyroA tail |
| OMK609 | TGGTTGAGAACAACATCGCCAACAATGTC AGCATGAAATTATCTACAGCCGTAGT | 5’ flbA with AnipyroA tail |
| OHS875 | ATATGAATCTATGTGGACAAATCCGAGCCCG | 5’ mcrA_EcoRI             |
| OHS878 | ATATGGCGGCGGCTGACCAATCCACCGGCT    | 3’ mcrA_NotI           |
| OHS857 | ATATGAATCTGAGTTGATATTCTTGCCTTGGTCTTGG | 3’ mcrA for C_EcoRI |
| OMK589 | GCTGAAAGTCTTAGTACAGCAGCAGAA | 5’ AfupyrG marker       |
| OMK590 | ATCTGCGGAGGATTTGTCGTCAC | 5’ AfupyrG marker       |
| ONK395 | ACTCTCATGTTGGTCTGCGAAAGG   | 5’ AnipyroA marker      |
| ONK396 | TTGTCATCGCTATCGATGTCGATCGG | 3’ AnipyroA marker      |
| OMK578 | TGCGGAGTGGAAAGATGC  | 5’ brlA probe           |
| OMK579 | AGAGTTAACAACCGTAGA       | 3’ brlA probe           |
| OHS5044 | GTAAGGACTGTGACGCCAAC | 5’ actin RT_probe      |
| OHS5045 | AGATCCACATCTGCTGGAAG  | 3’ actin RT_probe      |
| OHS5078 | TGAGGAATTGACCACCGACA | 5’ fksA RT Probe        |
| OHS5079 | GACCAAGGATGACAACAGG   | 5’ fksA RT Probe        |
| OHS5099 | GCGCGGAAGAAGACCTCACA   | 5’ afIR RT Probe        |
| OHS5060 | TGCAATAACTGCGCGAGCAC | 5’ afIR RT Probe        |
acetonitrile:water (60:40, v/v). The flow rate was 0.8 mL/min and ST was detected at a wavelength of 246 nm.

Retention time for ST was approximately 5.6 min. Samples (10 μL) were auto-injected and run in triplicate.

**β-(1,3)-glucan analysis.** The β-(1,3)-glucan concentration in conidia was determined using the Glucatell assay (Associates of Cape Cod). All samples were tested according to the manufacturer's instructions. Briefly, 2-day old conidia were collected with ddH₂O and resuspended in 25 mL of ddH₂O. Each sample was mixed with 25 μL of Glucatell reagent and incubated at 37 °C for 30 min. To stop the reaction, diazo-reagents were added and optical density was determined at 540 nm. The mean rate of optical density change was determined for each well, and the β-(1,3)-glucan concentration was determined by comparison to a standard curve. This assay was performed in triplicate.

**Conidial trehalose analysis.** The amount of conidial trehalose was measured as previously described. Fungal strains were inoculated onto MMG solid and then cultured for 2 days. After culture, 2 × 10⁸ conidia were collected, resuspended in ddH₂O, and incubated at 95 °C for 20 min. The supernatant was separated by centrifugation, transferred into a new tube, mixed with equal volume of 0.2 M sodium citrate (pH5.5), and incubated with trehalase (3 mU, Sigma-Aldrich), which hydrolyzes trehalose to glucose. The amount of glucose produced from trehalose was assayed with a glucose assay kit (Sigma-Aldrich). Samples untreated with trehalase served as negative controls.

**Microscopy.** The colony photographs were taken by using a Sony digital camera (DSC-F28). Photomicrographs were taken using a Zeiss M2 Bio microscope equipped with AxioCam and AxioVision (Rel. 4.8) digital imaging software.

**Statistical analysis.** Statistical differences between WT (or control) and mutant strains were assessed with the use of Student’s unpaired t-test. Data are reported as mean ± standard deviation (SD). P values <0.05 were considered significant.
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Author contributions
MKL, HSP, and JHY conceived and supervised the study; MKL, AFA, HSP, KHH, and JHY designed experiments; MKL, YES, HSP, and AFA performed experiments; MKL, YES, HSP, and JHY analyzed data; MKL, HSP, KHH, and JHY wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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