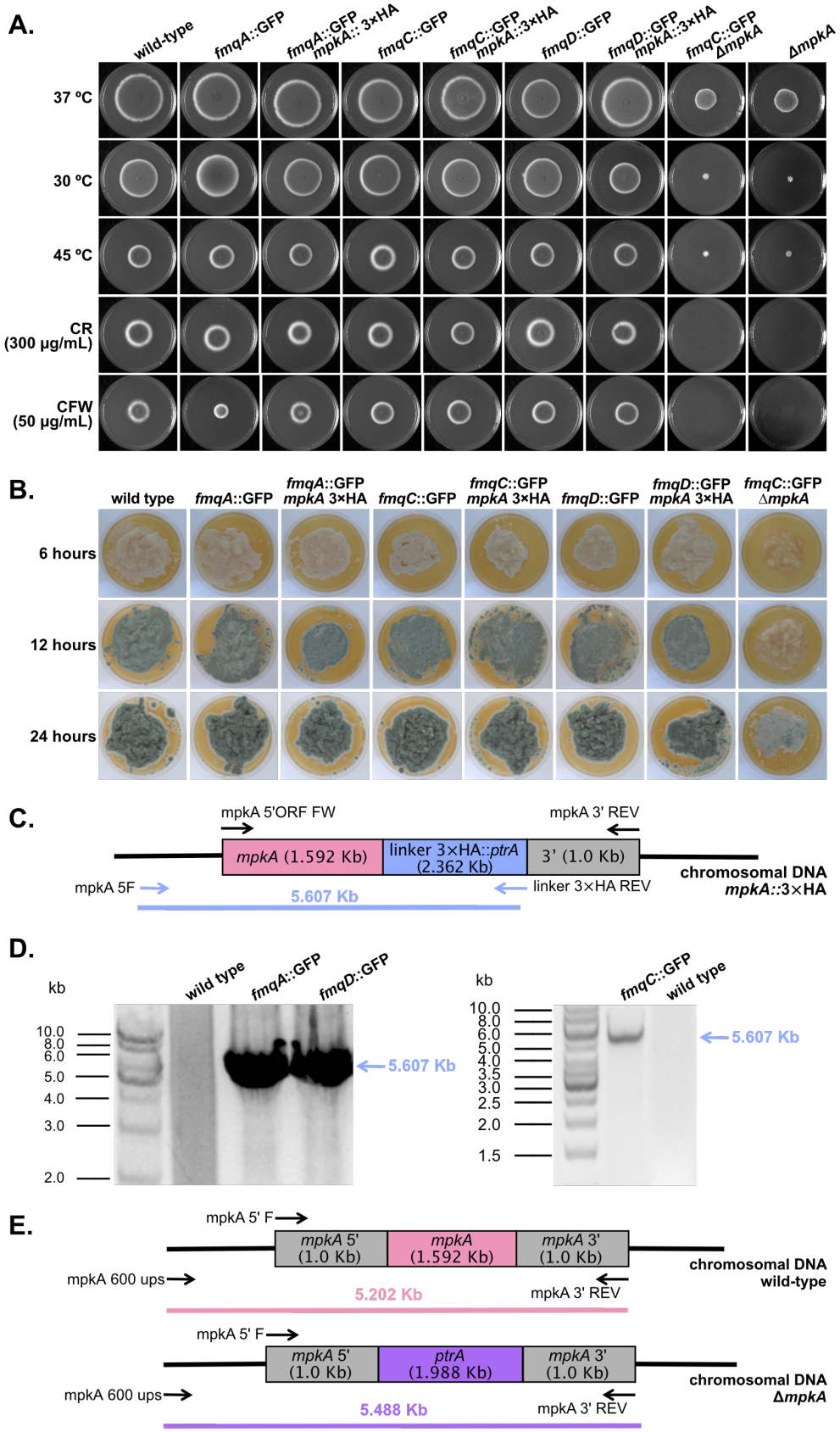
A.
wild-type
fmqA::GFP
fmqA::GFP mpkA:: 3×HA
fmqC::GFP
fmqC::GFP mpkA::3×HA
fmqD::GFP
fmqD::GFP mpkA::3×HA
fmqC::GFP ΔmpkA
ΔmpkA
37 °C
30 °C
45 °C
CR (300 µg/mL)
CFW (50 µg/mL)
B.
wild type
fmqA::GFP
fmqA::GFP mpkA 3×HA
fmqC::GFP
fmqC::GFP mpkA 3×HA
fmqD::GFP
fmqD::GFP mpkA 3×HA
fmqC::GFP ΔmpkA
6 hours
12 hours
24 hours
C.
mpkA 5'ORF FW
mpkA 3' REV
mpkA (1.592 Kb)
linker 3×HA::ptrA (2.362 Kb)
3' (1.0 Kb)
chromosomal DNA mpkA::3×HA
mpkA 5F
5.607 Kb
linker 3×HA REV
D.
kb
wild type
fmqA::GFP
fmqD::GFP
10.0
8.0
6.0
5.0
4.0
3.0
2.0
5.607 Kb
kb
fmqC::GFP
wild type
10.0
8.0
6.0
5.0
4.0
3.5
3.0
2.5
2.0
1.5
5.607 Kb
E.
mpkA 5' F
mpkA 5' (1.0 Kb)
mpkA (1.592 Kb)
mpkA 3' (1.0 Kb)
chromosomal DNA wild-type
mpkA 600 ups
5.202 Kb
mpkA 3' REV
mpkA 5' F
mpkA 5' (1.0 Kb)
ptrA (1.988 Kb)
mpkA 3' (1.0 Kb)
chromosomal DNA ΔmpkA
mpkA 600 ups
5.488 Kb
mpkA 3' REV
F.
mpkA locus
fmqC locus
kb
ΔmpkA fmqC::GFP
wild-type
fmqC::GFP
ΔmpkA fmqC::GFP
wild-type
10.0
8.0
6.0
5.0
4.0
4.0
5.607 Kb
5.488 Kb
5.202 Kb

Figure S1 Graphical representation of the mutated loci for the tagged *A. fumigatus* mutants. (A) Radial growth of the tagged *mpkA::*3×HA *fmqA::*GFP, *fmqC::*GFP or *fmqD::*GFP and Δ*mpkA fmqC::*GFP strains . 1×10^5 conidia of each strain were inoculated on solid YG medium at the indicated temperature and in the presence of cell wall stressors congo red (CR) and calcofluor white (CFW) at the indicated concentrations. Plates were incubated for 72 hours at 37ºC (or the indicated temperature) and photographed. (B) Asexual differentiation of the tagged mutants employed in this study. With the exception of Δ*mpkA* background, for which delayed conidiation is reported previously, no defects in conidiation rate were observed for the other tagged strains. (C) The *mpkA*::3×HA::*ptrA* cassette was amplified and used to transform each *fmqA-C-D::*GFP recipient strain. (C) The pyrithiamine-resistant transformants were selected and validated by PCR with primers mpkA 5F and linker 3×HA REV to confirm the *mpkA* locus replacement by the amplification of the 5.954 kb band in the double-tagged strains. (D) The Δ*mpkA* deletion cassette was amplified from the genomic DNA of the Δ*mpkA* strain using primers mpkA 5F and mpkA 3' REV and transformed into TFYL50.1 strain to generate the Δ*mpkA fmqC::*GFP mutant. (F) The *mpkA* deletion cassette contains the *ptrA* gene as a selectable marker and the gene replacement in this strain mutants was confirmed by PCR using the primers mpkA 600 ups and mpkA 3' REV, which can be used to discriminate the *mpkA* deletion from the wild-type *mpkA* locus by the amplification of the 5.6 kb or 5.2 kb band, respectively.

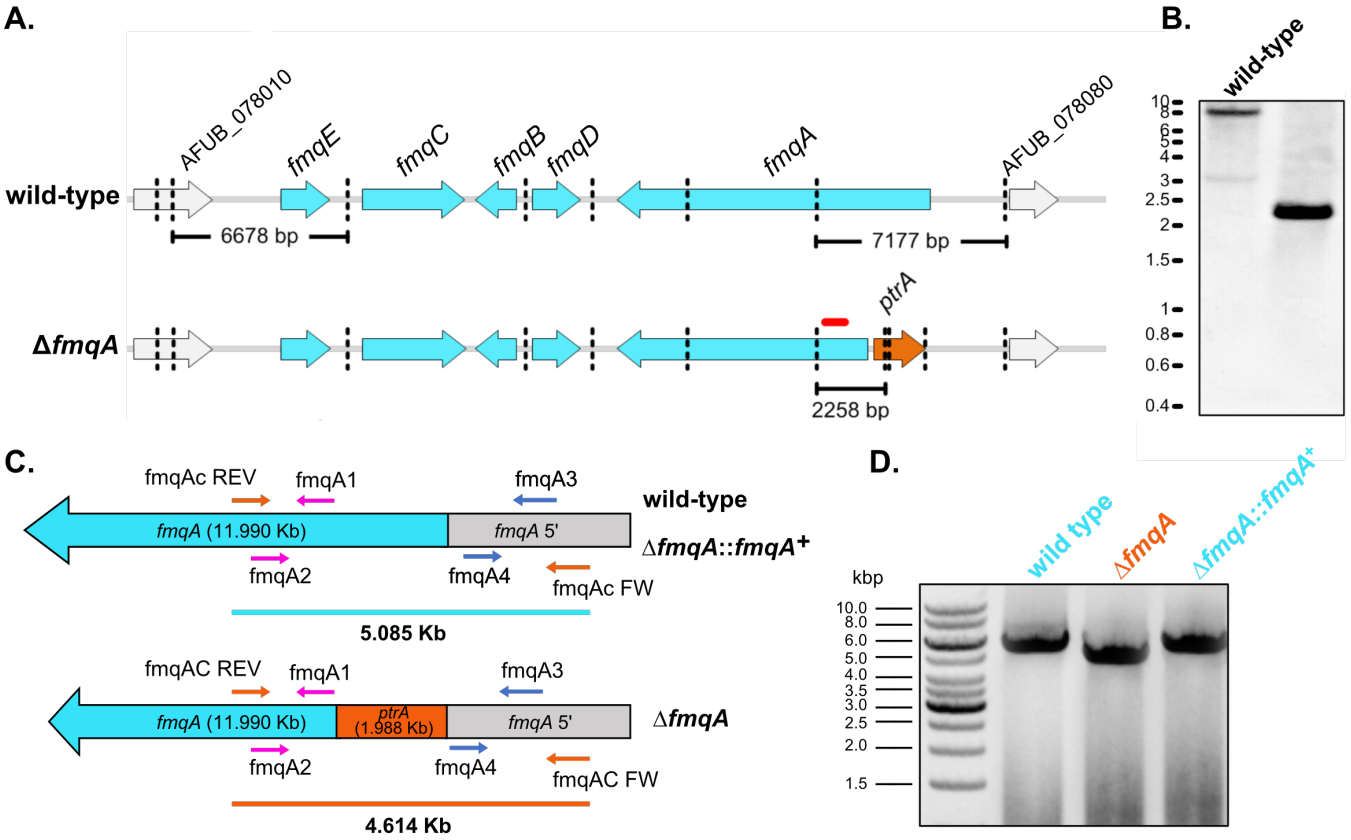
A.
wild-type
AFUB_078010
fmqE
fmqC
fmqB
fmqD
fmqA
AFUB_078080
6678 bp
7177 bp
ΔfmqA
ptrA
2258 bp
B.
wild-type
10
8
6
5
4
3
2.5
2
1.5
1
0.8
0.6
0.4
C.
fmqAc REV
fmqA1
fmqA3
wild-type
fmqA (11.990 Kb)
fmqA 5'
ΔfmqA::fmqA+
fmqA2
fmqA4
fmqAc FW
5.085 Kb
fmqAC REV
fmqA1
fmqA3
fmqA (11.990 Kb)
ptrA (1.988 Kb)
fmqA 5'
ΔfmqA
fmqA2
fmqA4
fmqAC FW
4.614 Kb
D.
wild type
ΔfmqA
ΔfmqA::fmqA+
kbp
10.0
8.0
6.0
5.0
4.0
3.5
3.0
2.5
2.0
1.5
E.
FqC
RT: 0.05 - 14.97
wild-type
ΔfmqA
ΔfmqA:fmqA+
F.
wild-type
ΔfmqA
ΔfmqA::fmqA+
control
30 ºC
48 ºC
CR (75 µg/mL)
CFW (40 µg/mL)
CASP (0.1 µg/mL)

Figure S2 Graphical representation of the mutated *fmqA* loci and FqC analysis. (A) Graphical representation of the mutated *fmqA* locus and respective Southern blot. The genes of the Fq biosynthetic cluster are shown in sky-blue. The cassette used for *fmqA* gene disruption is the orange labeled pyrithiamine (*ptrA*). The probe used for Southern blot analyses is marked as red stripes. (B) The DNA ladder used is indicated (Hyperladder 1Kb, Bioline). The Southern blot was performed by digesting genomic DNA with *Xba*I (dashed bands). (C-D) To complement the Δ*fmqA* strain a co-transformation strategy was employed using the 2495 bp gel-purified hygromycin resistant cassette (*gpdA*(*p)::hph;* not shown) and the *fmqA* coding sequence*,* which was amplified using the fmqAc FW and fmqAc REV oligonucleotides. Complementation was validated by PCR using the primers fmqAc FW and fmqAc REV which yield a band of 5.085 bp after reconstruction of the wild-type *fmqA* locus in the deletion strain. (E) Chromatograms indicating that FqC production is abolished in the Δ*fmqA* strain and fully restored in the complemented Δ*fmqA::fmqA*$^{+}$ strain. FqC was quantified in the mutant strains by HPLC-MS/MS. (F) Δ*fmqA* mutant presents wild-type levels of susceptibility to cell wall-disturbing compounds. The indicated number of conidia was inoculated onto solid YG plates that were supplemented with the following different cell wall perturbing agents: congo red (CR), calcofluor white (CFW) and caspofungin (CASP) and incubated at 37ºC (or the indicated temperature) for 24 hours.

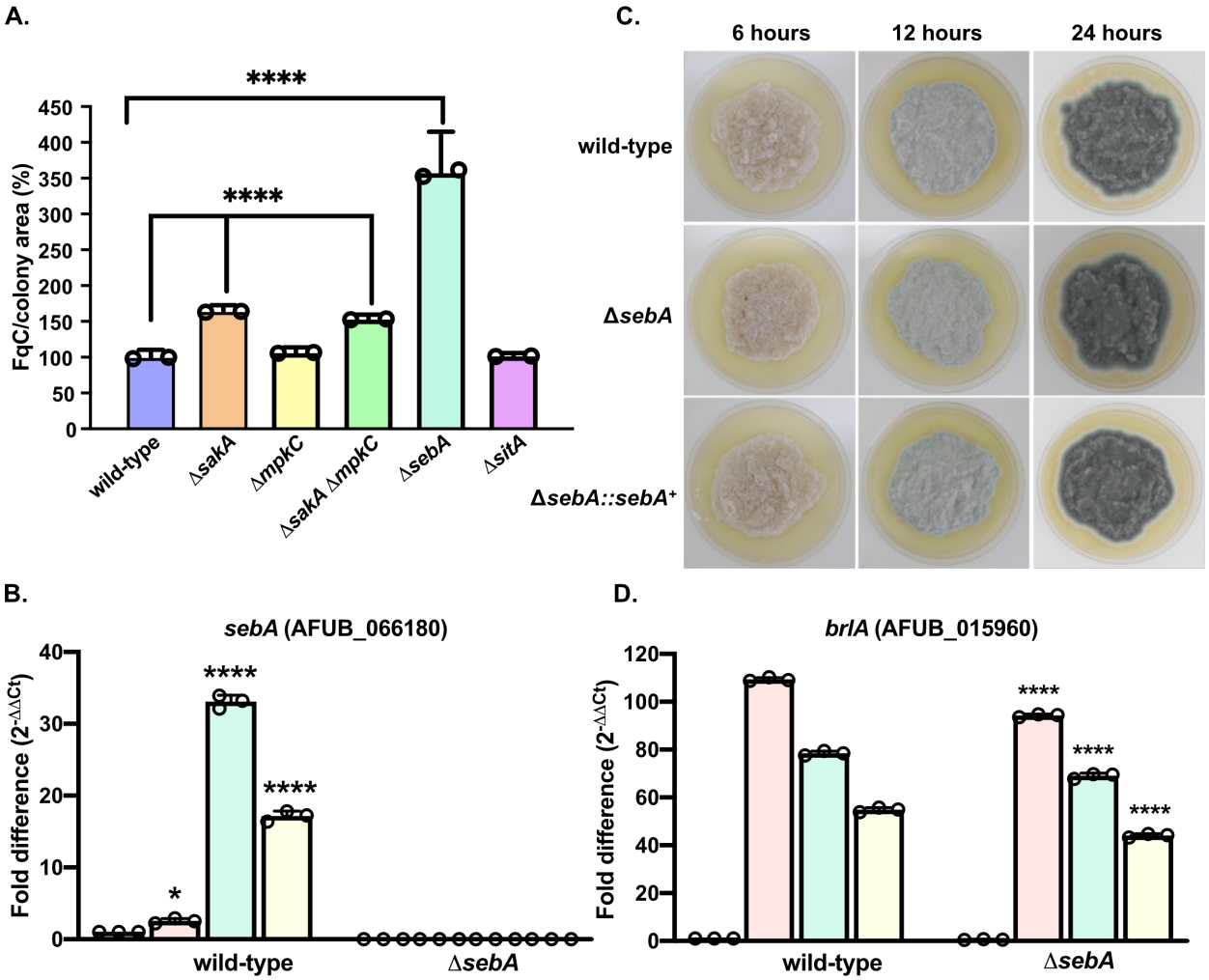

Figure S3 Selected results for the quantification of FqC in a collection of mutant strains and expression levels of *sebA* and *brlA* during the asexual differentiation. (A) Quantification of FqC in mutant strains from the screen of an arbitrary collection of selected *A. fumigatus* mutant strains carrying deletions on kinases, phosphatases and transcription factors involved in different signaling pathways. FqC was quantified by HPLC-MS/MS, n=2 ± SD. One-way ANOVA with Tukey's multiple comparison test relative to wild-type was performed. ****p<0.0001. (B) Induction of asexual differentiation of the wild-type, Δ*sebA* and the complemented strain shows no conidiation defects. The experiment was performed at 37ºC and imagens obtained at the indicated time points. (C-D) Gene expression measured by RT-qPCR of *sebA* and *brlA* in the wild-type and Δ*sebA* strains subjected to synchronized asexual differentiation shown in B. Control

indicates the hyphal state (submerged culture). n=3 ± SD. One-way ANOVA with Sidak's multiple comparison test relative to wild-type at the same time point of differentiation was performed. ****p<0.0001; *p=0.0348.

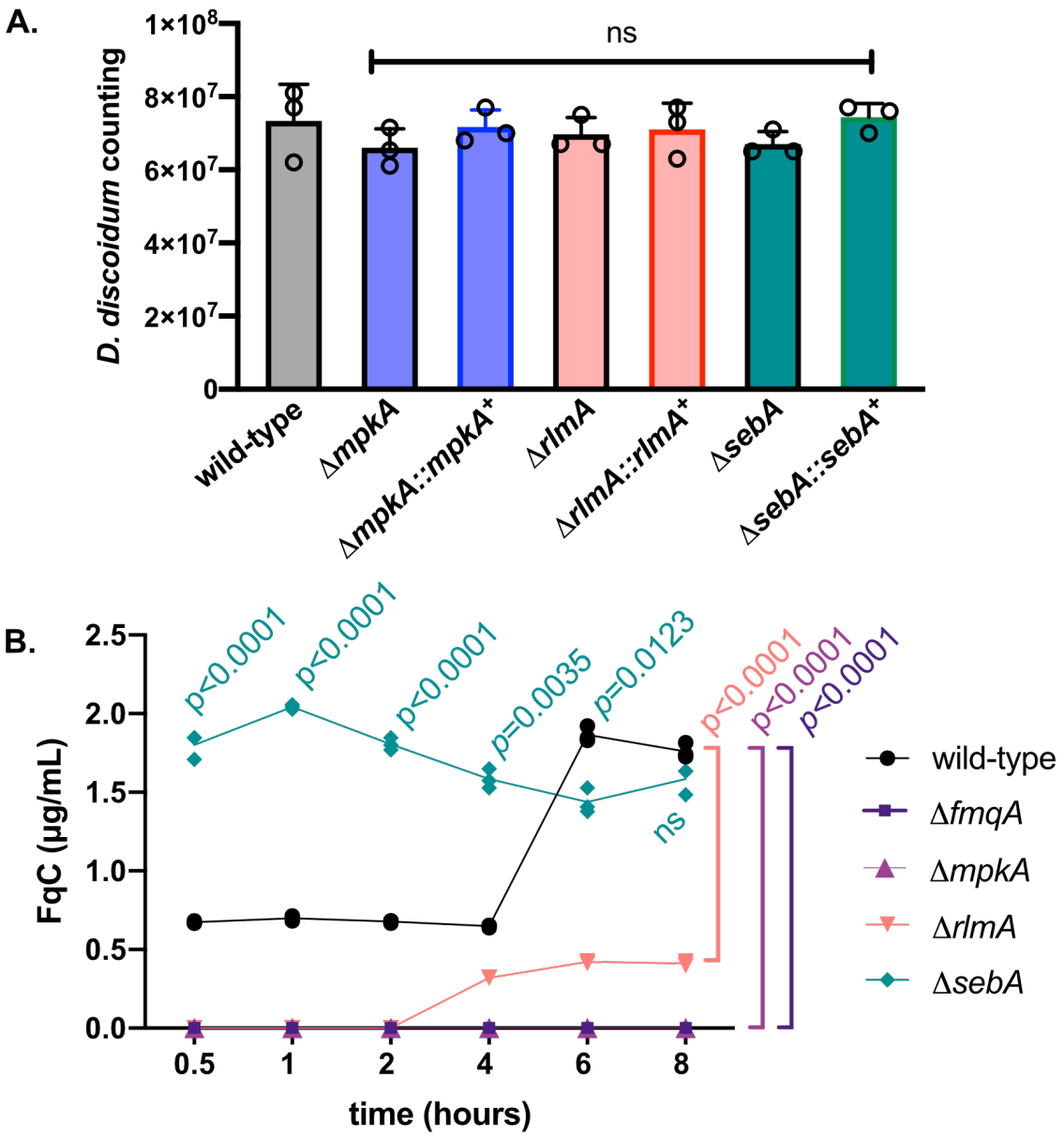

Figure S4 Survival of *D. discoideum* after confrontation with the mutant strains and quantification of FqC release in the culture medium. (A) Viability of *D. discoideum* cells determined 24 h after the confrontation with the wild-type, Δ*mpkA,* Δ*rlmA*, and Δ*sebA* conidia. *A. fumigatus* conidia were coincubated with *D. discoideum* at a MOI of 10:1. After 24 h at 22ºC, viable amoebae cells were counted using a hemocytometer and plotted in the graph. One-way ANOVA with Dunnett's multiple comparison test was performed in comparison to the control condition (n=3 ± SD). (B) FqC accumulated in *A. fumigatus* conidia is released into the culture medium. 2×10^8 conidia of each strain were harvested from plates grown for five days (37ºC) and immediately inoculated into 20 mL of liquid

YG. Conidia suspension was incubated at 37ºC (80 rpm). Samples (1 mL) were collected at the intervals shown in the graphs starting after 30 min of incubation. The sample volume was centrifuged at maximum speed, filtered (0.22 µm) and subjected to HPLC-MS/MS analysis and quantification, as described in the materials and methods section (n=3 ± SD). Two-way ANOVA with Tukey's multiple comparisons test was performed. *p*-values shown at each time point refer to the comparison to the wild-type strain. ns = non-significant.