Table S1. Concentration (µM) of accumulating precursors and pathway end products in engineered strains of *Neosartorya fumigata* (*N.f.*) and *Metarhizium brunneum* (*M.b.*) 10 days post inoculation

Species and pathway end product	Agroclavine	LA[a]	Festuclavine	DHLA[b]	dihydroLAH[c]
N.f. LA	7.76 ± 1.87	0.19 ± 0.08	n.d.[d]	n.d.	n.d.
M.b. LA	0.61 ± 0.06	4.12 ± 0.54	n.d.	n.d.	n.d.
N.f. DHLA	n.d.	n.d.	4.10 ± 0.94	0.11 ± 0.02	n.d.
M.b. DHLA	n.d.	n.d.	0.08 ± 0.01	0.48 ± 0.29	n.d.
M.b. dihydroLAH	n.d.	n.d.	n.d.	0.23 ± 0.09	0.04 ± 0.01

[a]lysergic acid
[b]dihydrolysergic acid
[c]provisional dihydrolysergic acid α-hydroxyethylamide
[d]not detected

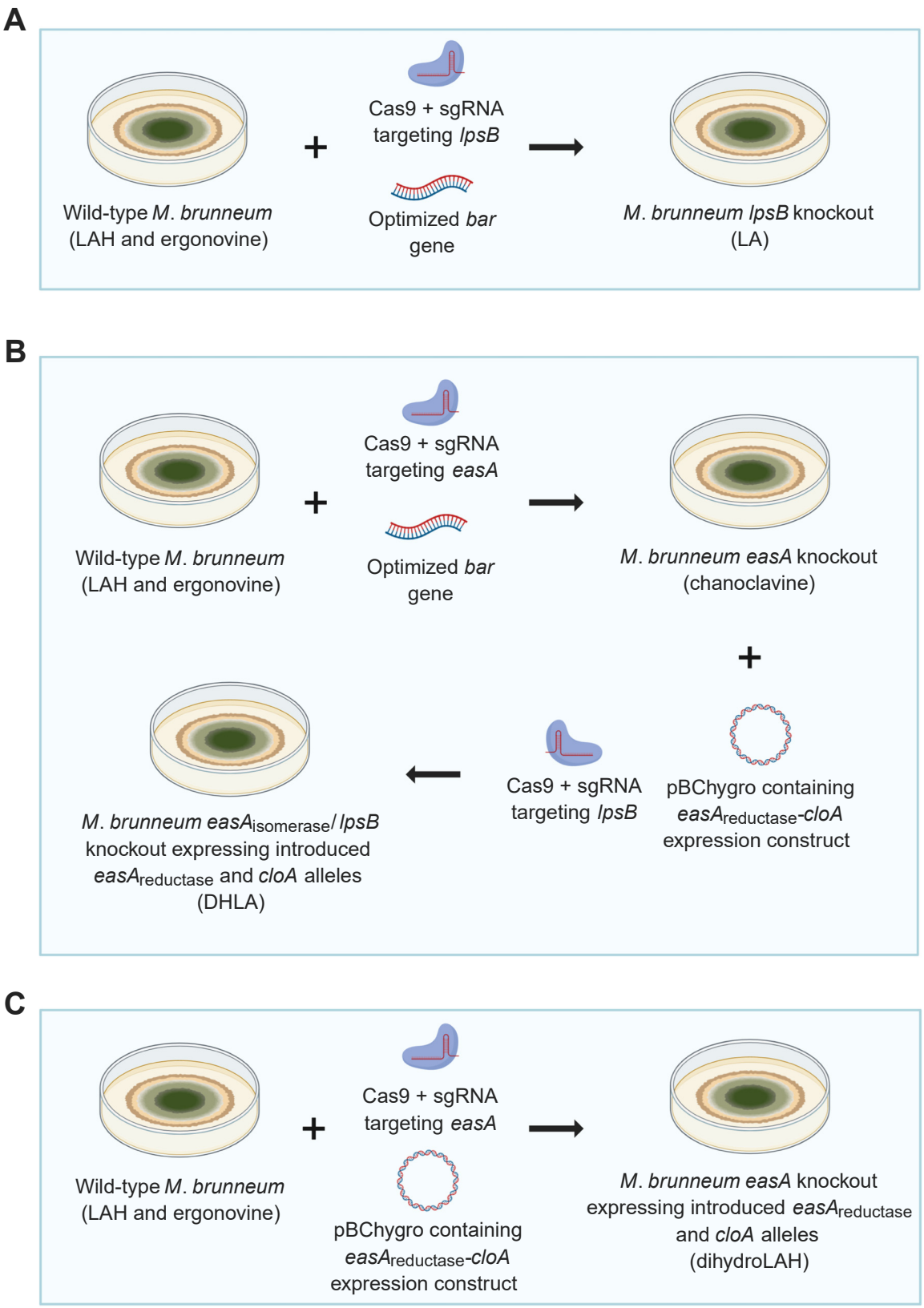

Fig S1. Strategies for engineering mutant strains of *M. brunneum*. Strains are listed with their terminal alkaloid product appearing in parentheses. **(A)** Strategy for engineering the LA-producing strain of *M. brunneum*. **(B)** Strategy for engineering the DHLA-producing strain of *M. brunneum*. **(C)** Strategy for engineering the provisional dihydroLAH-producing strain of *M. brunneum*. LAH, lysergic acid α–hydroxyethylamide; LA, lysergic acid; DHLA, dihydrolysergic acid; dihydroLAH, dihydrolysergic acid α–hydroxyethylamide. Created with BioRender.com.

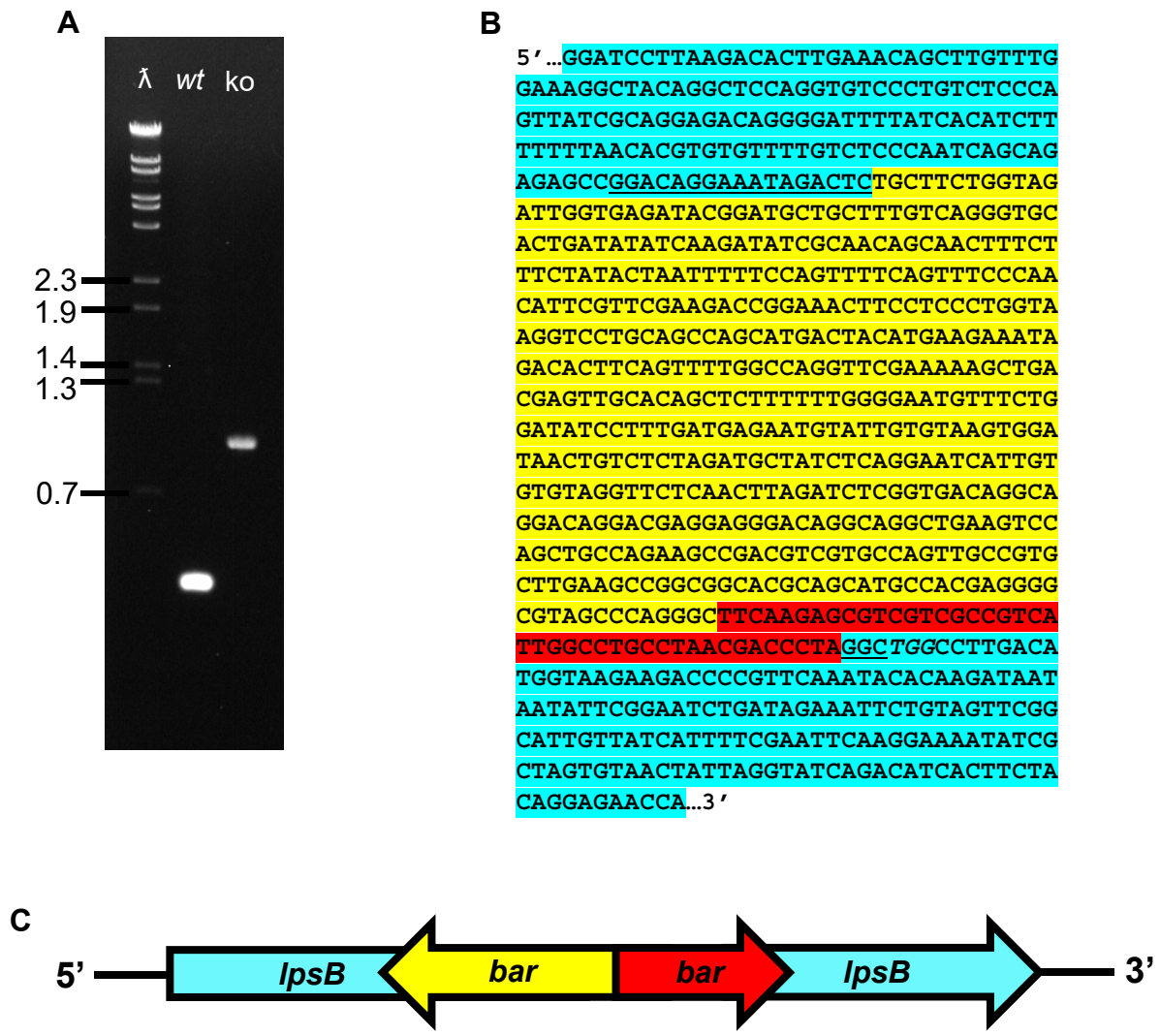

Fig S2. PCR analysis of *M. brunneum lpsB* locus following gene knockout. (A) PCR products from primer combination 7 (listed in Table 1) and genomic DNA isolated from wild type (wt) and *lpsB* knockout (ko) strains of *M. brunneum*. Sizes of relevant fragments from *Bst*EII-digested bacteriophage lambda DNA are indicated to the left. Gel was stained with ethidium bromide. **(B)** DNA sequence of *lpsB* locus after CRISPR mutagenesis. Blue highlight indicates sequences flanking the site of CRIPSR-Cas9 recombination. Yellow highlight represents sequence fragment from optimized *bar* selectable marker. Red highlight represents another truncated fragment of *bar* marker present in the locus. Target sequence is underlined and protospacer-adjacent motif (PAM) is italicized. **(C)** Graphic displaying the orientation and components integrated into the cut sites for this mutant. Two partial fragments of the *bar* marker with different lengths and divergent orientations are shown to have integrated.

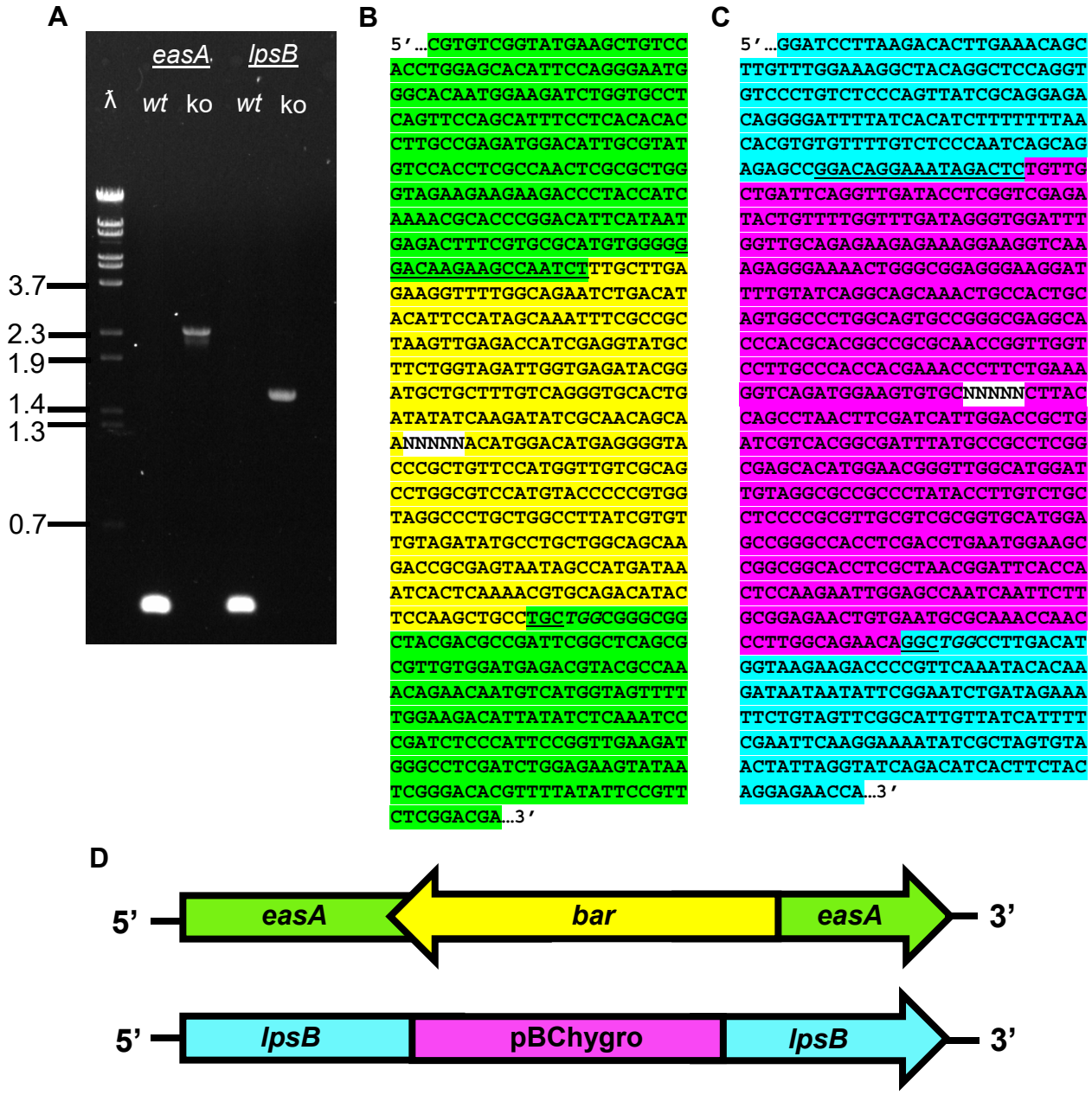

Fig S3. PCR analysis of *M. brunneum easA* and *lpsB* loci following gene knockout and transformation with *bar* marker and dihydro expression construct. (A) PCR products from primer combinations 7 or 8 (Table 1) and genomic DNA isolated from wild type (wt) and DHLA-producing (ko) strains of *M. brunneum*. Sizes of relevant fragments from *Bst*EII-digested bacteriophage lambda DNA are indicated to the left. Gel was stained with ethidium bromide. **(B)** DNA sequence of *easA* locus after CRISPR mutagenesis. Green highlight indicates sequences flanking the site of CRIPSR-Cas9 recombination. Yellow highlight represents bar marker integrated in opposite orientation present in the locus. Target sequence is underlined and protospacer-adjacent motif (PAM) is italicized. **(C)** DNA sequence of *lpsB* locus after CRISPR mutagenesis concomitant with introduction of the dihydro construct. Blue highlight indicates sequences flanking the site of CRIPSR-Cas9 recombination. Pink highlight represents fragment from pBChygro portion of the dihydro expression construct with 612 nt omitted (represented by NNNNN) to simplify presentation. Target sequence is underlined and protospacer-adjacent motif (PAM) is italicized. **(D)** Graphics displaying the orientation and components integrated into cut sites for both mutant loci.

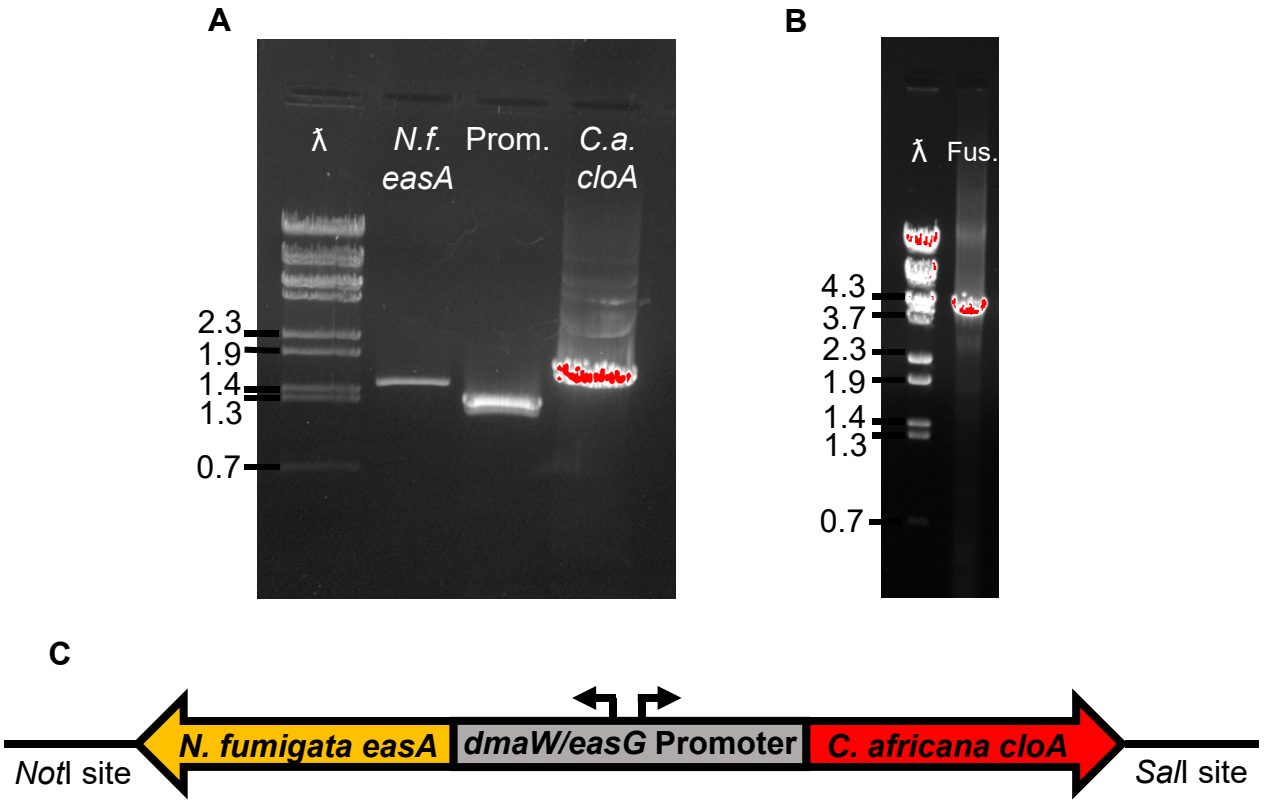

Fig S4. Construction of the dihydro expression construct prior to ligation into pBChygro. (A) Products from primer combinations 2, 3, and 4 (Table 1) prior to fusion PCR. Sizes of relevant fragments from *Bst*EII-digested bacteriophage lambda DNA are indicated to the left of either gel. Gel was stained with ethidium bromide. Red tint indicates saturated pixels detected by imaging software. **(B)** Fusion product from PCR primer combination 5, prior to ligation into pBChygro. Fus., $easA_{reductase}$-promoter-*cloA* fusion product. **(C)** Graphic displaying the orientation and components of the dihydro expression construct.

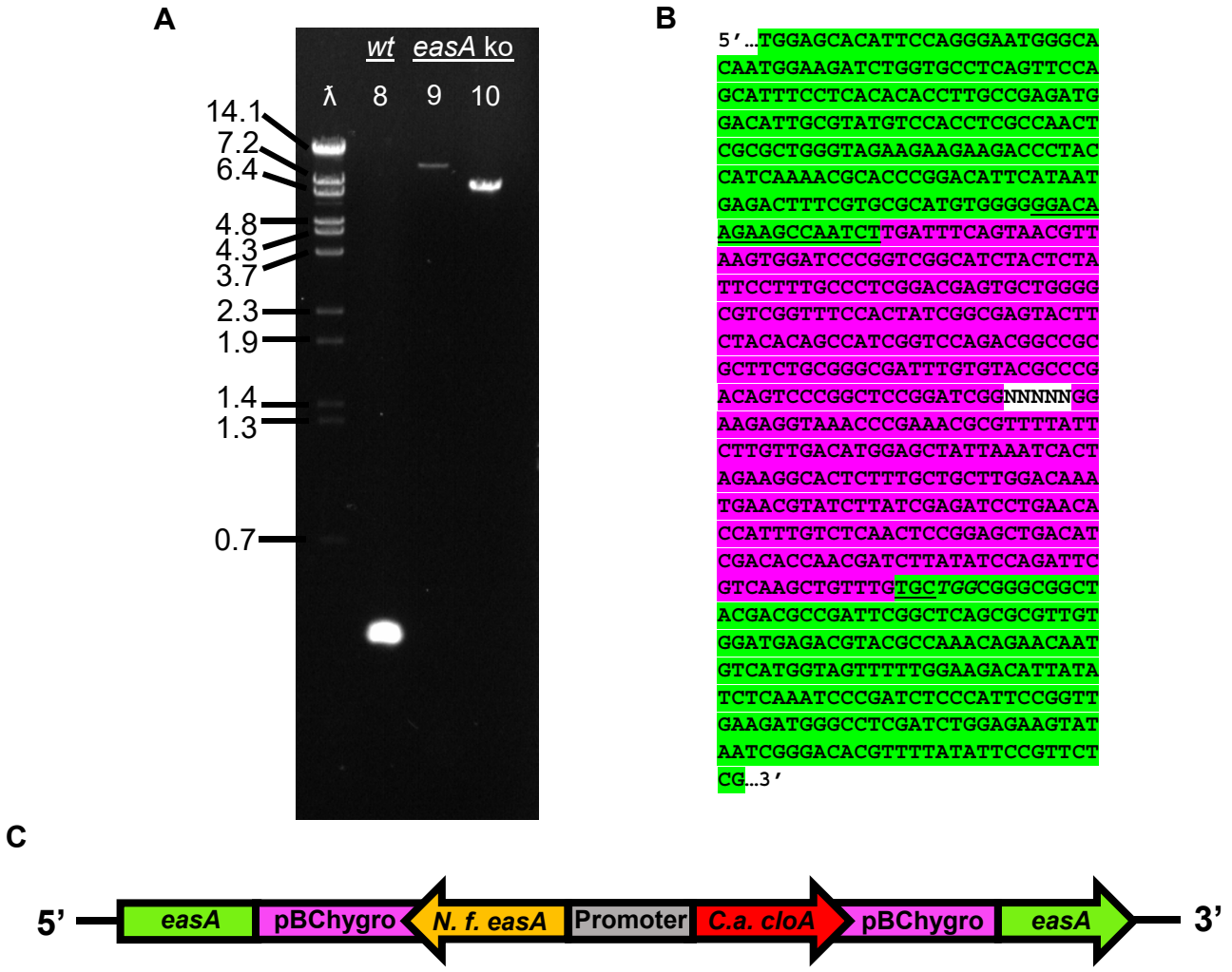

Fig S5. PCR analysis of *M. brunneum easA* locus following gene knockout and transformation with dihydro expression construct. (A) Products from primer combinations 8, 9, or 10 (Table 1) and genomic DNA isolated from wild type (wt) and dihydroLAH-producing strains (*easA* ko) of *M. brunneum*. Primer combination is listed in each lane below the corresponding strain. Sizes of relevant fragments from *Bst*EII-digested bacteriophage lambda DNA are indicated to the left. Gel was stained with ethidium bromide. **(B)** DNA sequence of *easA* locus after CRISPR mutagenesis concomitant with introduction of the dihydro construct. Green highlight indicates sequences flanking the site of CRIPSR-Cas9 recombination. Pink highlight represents sequence from dihydro expression construct with 11,090 nt omitted (represented by NNNNN) to simplify presentation. Target sequence is underlined and protospacer-adjacent motif (PAM) is italicized. **(C)** Graphic displaying the orientation and components integrated into cut sites for this mutant.