Supplemental Material

Genetic engineering activates biosynthesis of aromatic fumaric acid amides in the human pathogen *Aspergillus fumigatus*

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Running title: Aromatic fumaric acid amides in *Aspergillus fumigatus*

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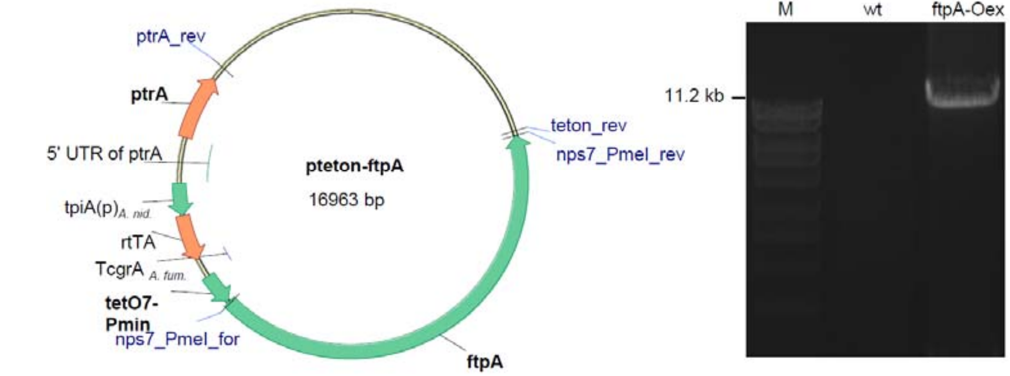

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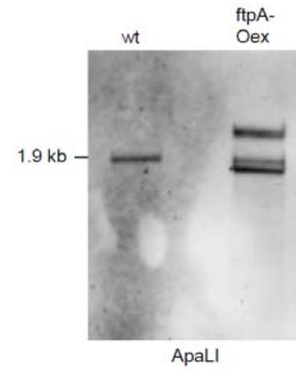

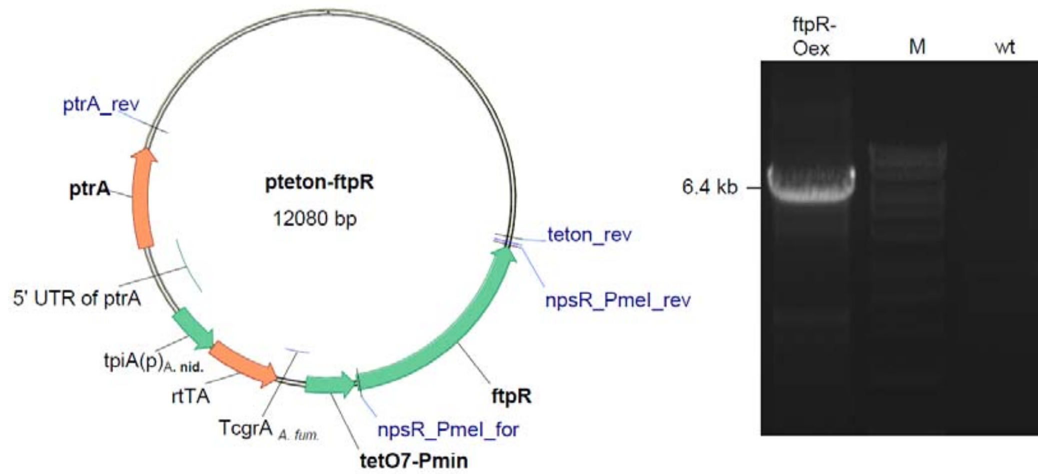

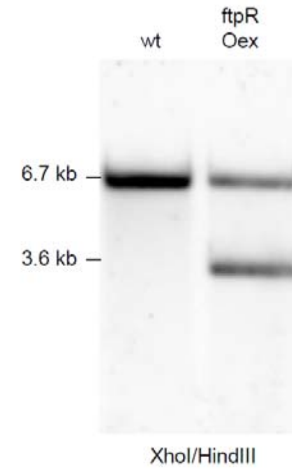

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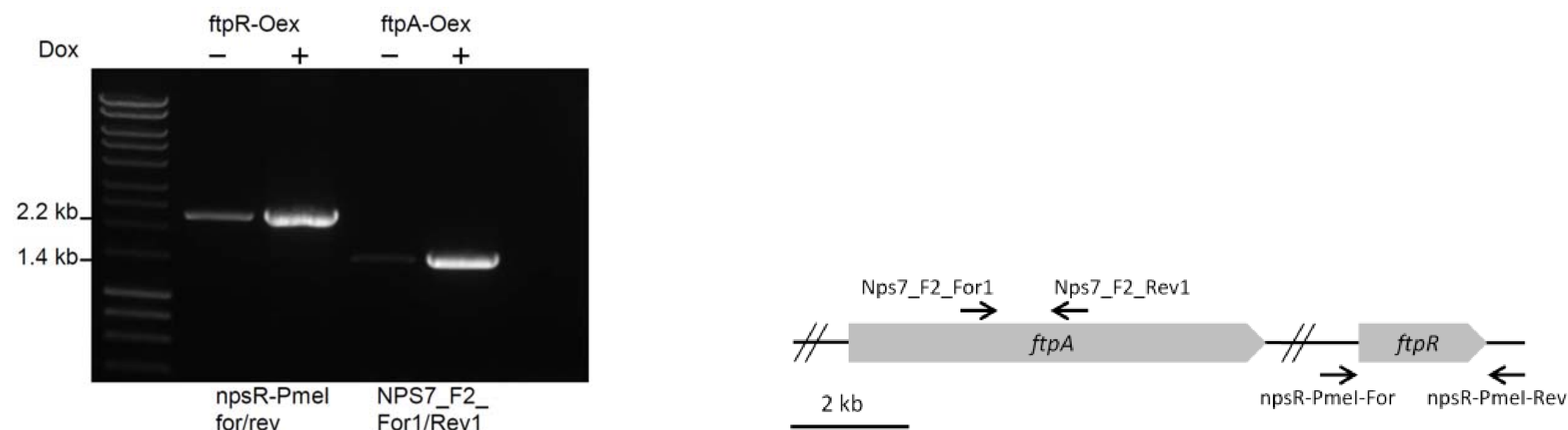

Figure S1: Genetic manipulation of *A. fumigatus.*

A) PCR-based verification that the teton-*ftpA* and teton-*ftpR* overexpression constructs (pteton-ftpA and pteton-ftpR) were successfully integrated into the *A. fumigatus* genome Integration of the ftpA-Oex and ftpR-Oex constructs was verified using primers ptrA-rev and teton-rev (Table S1). The expected 11.2 kb and 6.4 kb fragments, respectively, were amplified. PCR parameters were: initial denaturation step at 98°C for 30 s, 30 cycles of denaturation (10 s at 98°C), primer annealing (10 s at 62 °C) and elongation (3 min at 72 °C). For PCR analysis of the mutant strains, the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Germany) was used according to the manufacturer's instructions. M = DNA size marker.

B) Southern blot verification of teton-*ftpA* and teton-*ftpR* integration. Genomic DNA of the wild type and the ftpA-Oex strain was digested with ApaLI restriction endonuclease, yielding a 1.9 kb signal both in the wild type and in ftpA-Oex. The latter showed two additional signals caused by ectopic integrations of the plasmid. The probe was amplified from genomic *A. fumigatus* DNA using primers nps7_PmeI_for and nps7_int_rev. For analysis of the ftpR-Oex strain, DNA was digested with XhoI and HindIII, yielding a 6.7 kb band both in the wild type and in the the ftpR-Oex mutant. The latter showed an additional signal with a size of 3.6 kb caused by ectopic integration of the plasmid. The probe was amplified from genomic *A. fumigatus* DNA using primers ftpR_int_for and npsR_PmeI_rev.

C) Verification of doxycycline-mediated inducibility of *ftpA* and *ftpR* by RT-PCR and agarose gel electrophoresis. The *A. fumigatus* strains ftpR-Oex and ftpA-Oex were cultivated for 16 h in *Aspergillus* minimal medium. Overexpression of *ftpR* and *ftpA* was then induced by addition of doxycycline (10 µg/mL final concentration), followed by incubation for another 2 h. Non-induced cultures served as control. Mycelium was harvested by filtration and stored in liquid nitrogen. RNA isolation was performed with the SV Total RNA Isolation System (Promega), subsequent first strand cDNA synthesis was carried out using the Thermo

Scientific First Strand cDNA Synthesis Kit. PCR was performed using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) and 0.25 µl cDNA of the respective strains as template in a 30 µl reaction, and primer pairs NpsR_PmeI_for/NpsR_PmeI_rev and Nps7_F2_For1/ Nps7_F2_Rev1, respectively (Table S1). The positions of the PCR primers for *ftpR* and partial *ftpA* amplification are indicated in the graph on the right hand side. Thermal cycling conditions were: 30 s initial denaturation at 98°C, 30 cycles (10 s denaturation, 10 s annealing at 62°C, 40 s elongation at 72°C). Agarose gel electrophoresis showed the presence of the expected PCR products (2.2 and 1.4 kb, respectively). The weak DNA bands in the non-induced (doxycycline negative) samples indicate basal transcription due to an incomplete suppression under non-inducing conditions. Dox = doxycycline.

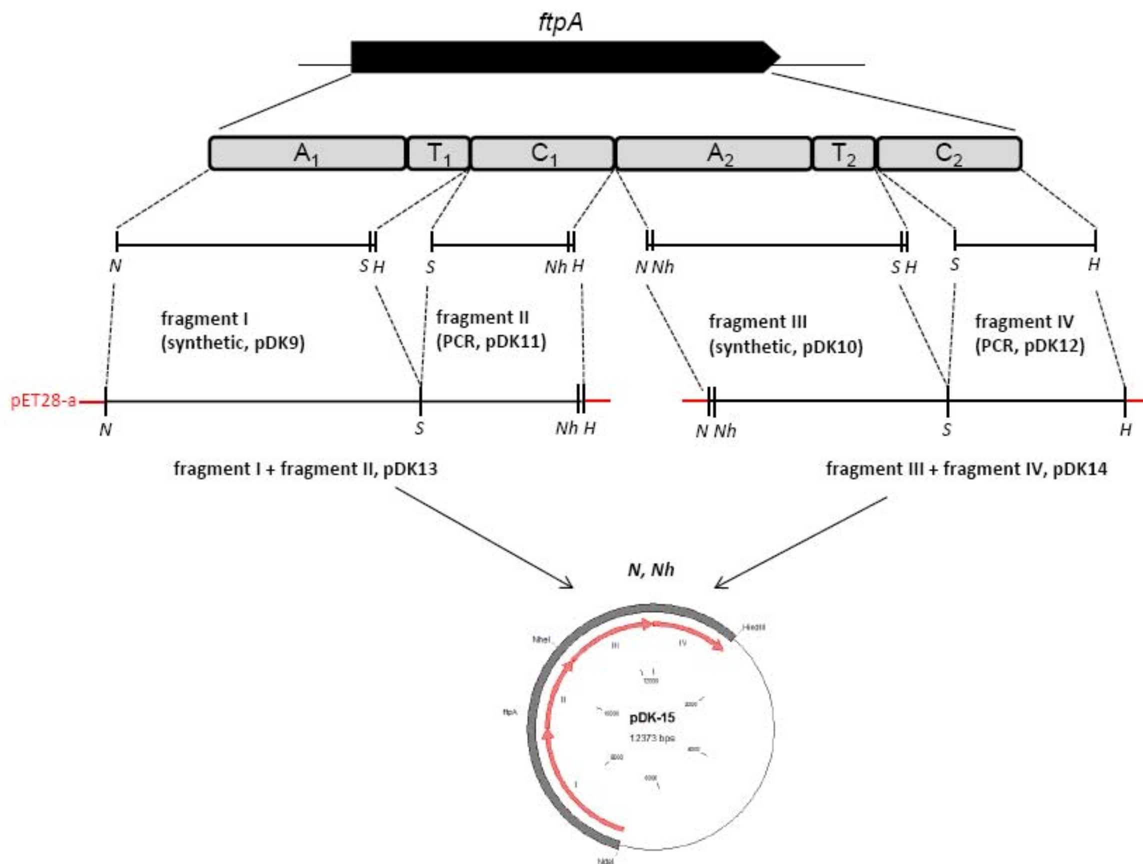

Figure S2: Reconstitution of the full length *ftpA* reading frame to create the pET28a-based gene expression construct pDK15. Fragment I (fragment III) was synthesized as codon-optimized gene and inserted via the NdeI and HindIII sites into pET28a to create plasmid pDK9 (pDK10). Fragment II (fragment IV) were created by PCR and cloned into pBluescript to yield plasmid pDK11 (pDK12). To assemble fragments I and II (III and IV), the latter was cloned between the SacII and HindIII sites of pDK9 (DK10), to yield plasmid pDK13 (pDK14). The insert of pDK13 was released by restriction with NdeI and NheI, and inserted into pDK14 via the same sites, to yield expression plasmid pDK15 that includes the reconstituted full-length *ftpA* gene. Restriction sites are abbreviated as follows: H: HindIII, N: NdeI, Nh: NheI, S: SacII.

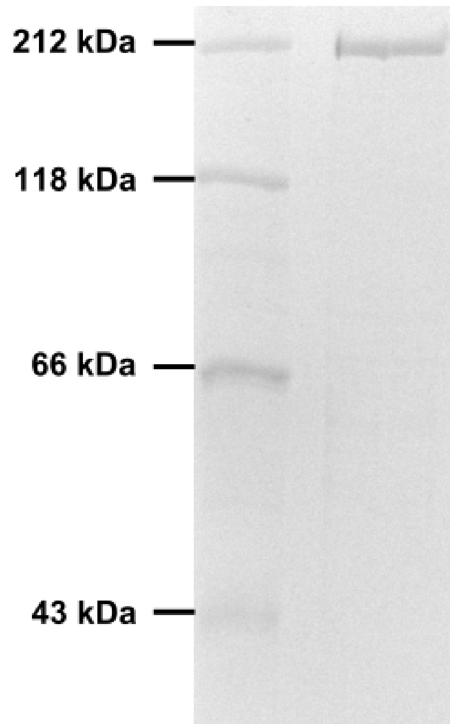

Figure S3: SDS-polyacrylamide gel of *Aspergillus fumigatus* full length FtpA after purification on Ni^{2+}-NTA.

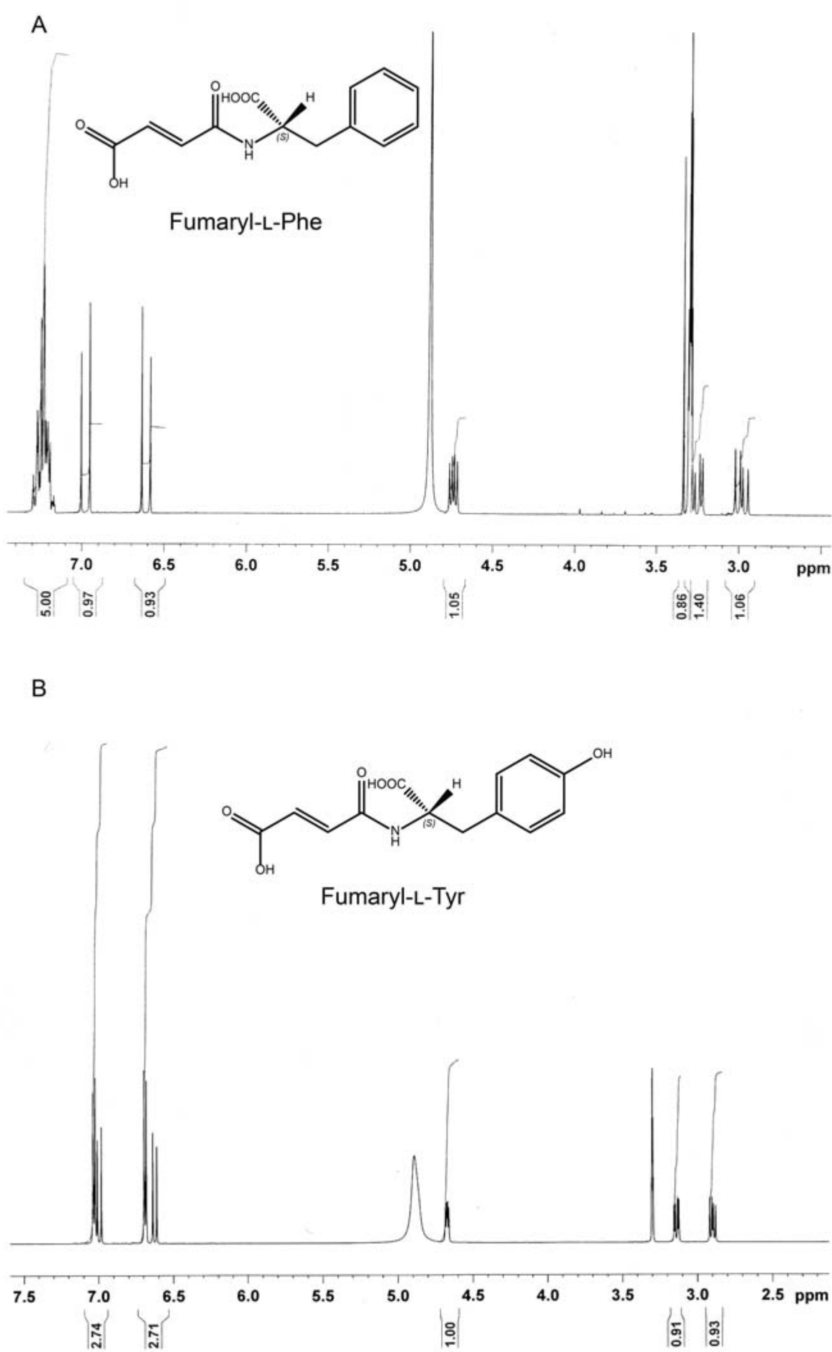

Figure S4: ^{1}H NMR spectra (CD_3OD, 600 MHz) of synthetic fumaryl-L-phenylalanine (panel A) and fumaryl-L-tyrosine (panel B) standards.

Table S1: Oligonucleotide primers used during this study.

Oligonucleotide	Sequence (5' - 3')	Restriction site[a]
NPS7_A1_f1	**GCTAGC**ACCCTGCAGCGTGTTACCGC	*Nhe*I
NPS7_A1_r1	**GCGGCCGC**TTAGCTACCAATACTGCTACGTTC	*Not*I
NPS7_F2_For1	GAGACGCTGTTCCAAGTTGC	-
NPS7_F2_Rev1	GCTAC**AAGCTT**AGAGGCGGCCATTCTCTG	*Hin*dIII
NPS7_F4_For1	CTGGGCAGTATGCTTCGTC	-
NPS7_F4_Rev2	GCTAC**AAGCTT**GTAGACCCCCAGCTCCCCAACC	*Hin*dIII
NpsR_PmeI_for	**GTTTAAAC**ATGATCTGCATGGATTCAGC	*Pme*I
NpsR_PmeI_rev	**GTTTAAAC**CGTTCTATCTGAGCAAAAC	*Pme*I
Nps7_PmeI_for	**GTTTAAAC**ATGGCCCCATACATCGGTAC	*Pme*I
Nps7_PmeI_rev	**GTTTAAAC**CTAGACCCCCAGCTCCCCAAC	*Pme*I
ptrA_rev	GCTTGATGGCCTAGATGGCCTC	
teton_rev	GTTTACGTCGCCGTCCAGC	
nps7_int_rev	CCTCAATCAGGGAATGAGCC	
ftpR_int_for	GCCAGTTTCGACCACACAAC	

[a]refers to artificial restriction sites (bold) introduced by PCR primers.

Table S2: Compounds used in substrate pools and/or individually during the ATP-[^{32}P]pyrophosphate radiolabel exchange assay to characterize the substrate preference of FtpA adenylation domains. Amino acids were L-configured.

Substrate pool #	Substrates
I	Gly, Ala, Val, Leu, Ile
II	Cys, Met, Ser, Thr, Pro
III	His, Phe, Tyr, Trp
IV	Asp, Asn, Glu, Gln
V	Lys, Arg, Orn, oxalic acid
VI	pyruvic acid, α-ketoglutaric acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, indolyl-3-pyruvic acid
VII	malonic acid, succinic acid, fumaric acid, maleic acid, malic acid, tartaric acid, citric acid
VIII	benzoic acid, 4-hydroxybenzoic acid, salicylic acid, 2,3-dihydroxybenzoic acid.

Table S3: Adenylation activity of the FtpA A_1 and A_2 domains as a function of pH and temperature, determined by the ATP-[^{32}P]pyrophosphate radioisotope exchange assay. Optimal turnover was set to 100%.

pH	A_1 [%]	A_2 [%]
5.5	< 20	< 20
6.0	< 20	< 20
6.5	< 20	56.1
7.0	71.1	86.8
7.5	100.0	100.0
8.0	72.1	77.0
8.5	64.5	67.4
9.0	58.2	51.2

temperature [°C]	A1 [%]	A2 [%]
5	33.6	55.1
10	62.7	62.6
15	66.7	76.2
20	72.9	80.7
25	76.6	84.8
30	81.1	88.3
37	100.0	100.0
45	22.1	71.5