Supporting Information for

Genomic mushroom hunting decrypts coprinoferrin, a siderophore secondary metabolite vital to fungal cell development

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1. Supporting Methods and Results

1.1 Reagents, strains and general techniques for DNA manipulation.

All chemicals were purchased from Merck KGaA, Tokyo Chemical Industry Co. Ltd. and Wako Pure Chemical Industries Ltd., unless otherwise specified. Purchased chemicals were of reagent grade and used without further purification. *Coprinopsis cinerea* ku3-24 was established previously[1]. *Escherichia coli* XL1-Blue (Agilent Technologies) was used for plasmid propagation by standard procedures. DNA restriction enzymes were used as recommended by the manufacturer (Thermo Fisher Scientific Inc.). PCR was carried out using PrimeSTAR GXL DNA polymerase (TAKARA Bio Inc.) as recommended by the manufacturer. Sequences of PCR products were confirmed through DNA sequencing (Macrogen Japan Corporation). *Saccharomyces cerevisiae* BY4705[2] was used for plasmid assembly.

1.2 Construction of mutated strains of *C. cinerea*

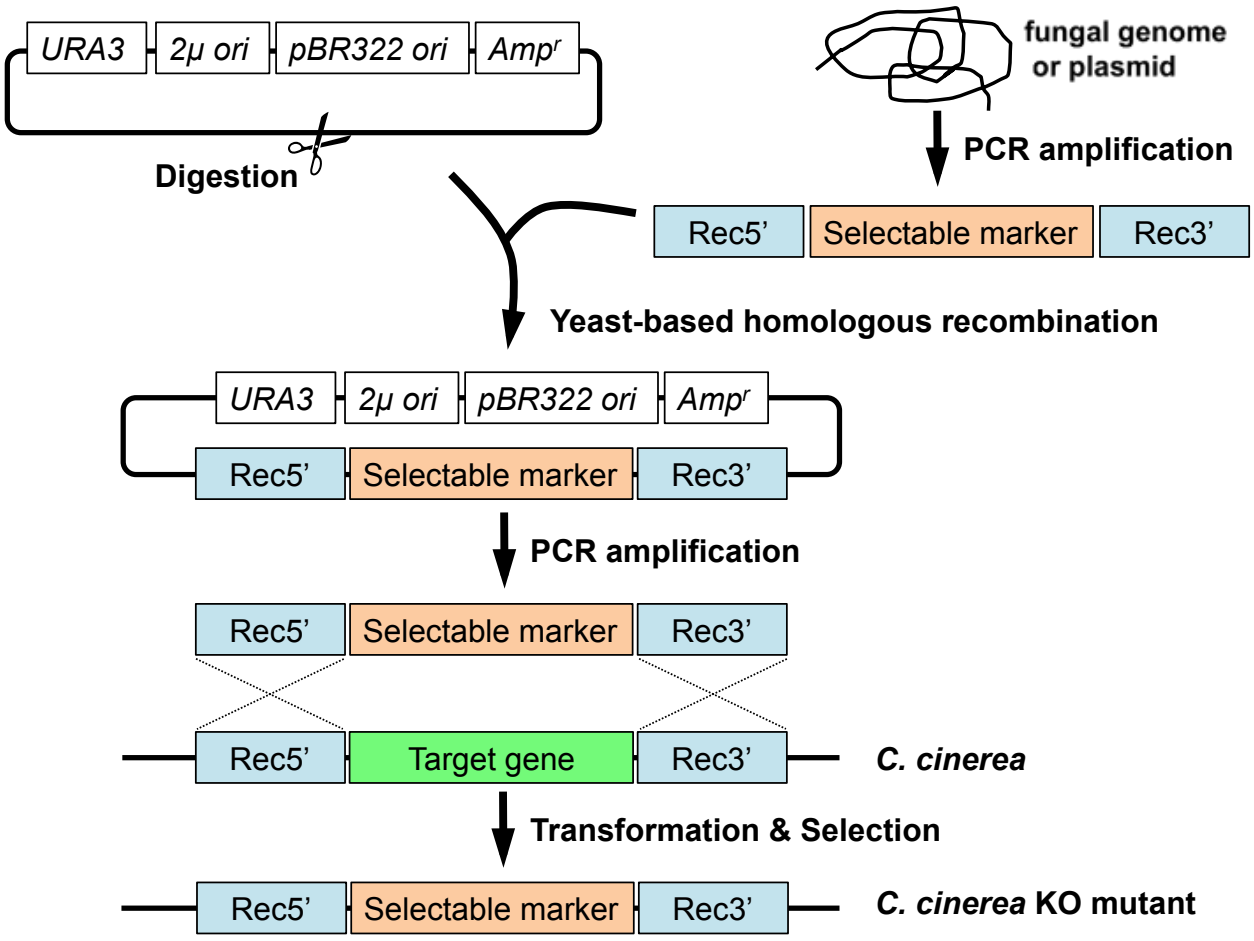
Table S1 *Coprinopsis cinerea* strains used in this study.

Strain name		Genotype	Parent strain	Source
C. cinerea	ku3-24	*A43mut B43mut pab1-1 Cc.ku70* (FltR)	—	[1]
	Δ*laeA*	*A43mut B43mut pab1-1 Cc.ku70* (FltR) *laeA*(CC1G_00498)::*hph*	ku3-24	This study
	Δ*laeA*Δ*cpf1*	*A43mut B43mut pab1-1 Cc.ku70* (FltR) *laeA*(CC1G_00498)::*hph* *cpf1*(CC1G_04210)::*pab1*	Δ*laeA*	This study

1.2.1 General procedure for the construction of the disruption cassette.

The disruption cassette is comprised of a selection marker sandwiched in between a 5' side- and a 3' side-flanking fragment (Rec5' and Rec3', respectively) as shown in **Figure S1**. Rec5' and Rec3' are 1,500-base pair fragments that are homologous to the site of recombination at or near the 5' and 3' end of the target gene in the *C. cinerea* ku3-24 genome. The primer sets given in Tables S2–S4 were used to prepare the required flanking homologous regions for each of the target genes. The sequences of the primer sets are listed in Table S4. PCR amplification of Rec5', Rec3' and the selectable marker fragments were carried out using PrimeSTAR GXL DNA polymerase as recommended by the manufacturer (TAKARA Bio Inc.). For plasmid construction, the three fragments (Rec5', selection marker, and Rec3'), each at 50 to 150 ng in a total volume of 45 μL, were mixed with 2 μg of a suitable pre-digested delivery vector. For the preparation of the Δ*laeA* strain, pRS426[3] was used as a delivery vector (**Table S2**). The mixture was transformed into *S. cerevisiae* BY4705 for constructing the plasmid possessing deletion cassette through *in vivo* homologous recombination. These four DNA fragments were joined *in situ* by the endogenous homologous recombination activity of *S. cerevisiae* through the 25-bp homologous sequences present at the ends of those DNA fragments. The desired transformants were selected for the presence of the selection marker. For the preparation of the Δ*laeA* strain, transformants were selected on an uracil-deficient plate, because pRS426 carried an *URA3* selection marker. The resulting plasmid pKW22056 was recovered from the yeast transformant and transferred to *E. coli*. The plasmid was amplified in *E. coli* for subsequent characterization by restriction enzyme digestion and DNA sequencing to confirm its identity. For targeted homologous recombination, a PCR product was amplified from the plasmid carrying a desired cassette using the corresponding Rec5' forward and Rec3' reverse primers (**Figure S1**). Subsequently, the PCR product was used to transform *C. cinerea*.

Figure S1. A schematic diagram showing the construction of a disruption cassette-containing plasmid using a yeast homologous recombination method for preparation of a deletion mutant of *C. cinerea*.



1.2.2 Procedure for *C. cinerea* transformation.

The *C. cinerea* ku3-24 strain was initially cultured on an MYG agar medium (10 g/L malt extract, 4 g/L D-glucose and 4 g/L yeast extract medium with 15 g/L agar) at 30 °C for 5 days.[4] Homogenized mycelia collected from a single plate were used to inoculate 200 mL of MYG liquid medium at 30 °C for 16 h with shaking at 180 r.p.m. Grown cells were collected by filtration and washed with 0.8 M sodium chloride. The cells were incubated with 1 mL of MM buffer pH 5.5 (0.5 M mannitol and 0.05 M maleic acid) containing 1 mg/mL chitinase and 50 mg/mL lysing enzyme (Sigma-Aldrich) at 30 °C for 4 h. The resulting protoplasts were filtered and subsequently centrifuged at 2,500 × g for 5 min at room temperature. The collected protoplasts were washed with 0.8 M sodium chloride and centrifuged to remove the wash solution. Approximately 0.5×10^8 to 1×10^8 of protoplasts were suspended in 200 μL of MMC buffer at pH 5.5 (0.5 M mannitol, 0.05 M maleic acid and 0.05 M calcium chloride). Then 40 μL of PEG solution at pH 8.0 (400 mg/mL polyethylene glycol 8,000, 50 mM calcium chloride and 50 mM Tris-HCl) was added to the protoplast suspension. The mixture was subsequently combined with 5 μg of the DNA fragment with which the cells were to be transformed. The mixture was incubated on ice for 30 min to allow the transformation to proceed. After incubation on ice, 1 mL of the PEG solution was added to the reaction mixture, and the mixture was incubated at room temperature for additional 15 min. In the case of the Δ*laeA* strain construction, *hph* was used for the selection marker. The cells were plated on RM agar (RM medium: 2.0 g/L L-asparagine, 0.12 g/L $MgSO_4$, 5.0 g/L D-glucose, 171.2 g/L sucrose, 1.0 mg/L thiamine, 5.0 g soluble starch, 1.5 g/L NH_4Cl, 1.25 g/L KH_2PO_4, 0.3 g/L Na_2SO_4, 1.45 g/L Na_2HPO_4; and 15.0 g/L agar was added) plates containing 1 mM *p*-aminobenzoic acid in order to recover the transformed *C. cinerea* ku3-24. After 24 h at 30 °C incubation, the plate was overlaid with RM top agar (RM medium with 0.5 g/L agar) containing 0.6 mg/mL hygromycin B and incubated at 30 °C for 7 days to select for desired mutant strains of *C. cinerea* ku3-24. Second selection was carried out by using an RM agar plate containing 1 mM *p*-aminobenzoic acid and 0.15 mg/mL hygromycin B at 30 °C for 7 days. In the case of the Δ*laeA*Δ*cpf1* strain construction, *pab1* marker was used for the selection. The cells were plated on RM agar plates and immediately overlaid with RM top agar. After a period of cultivation, the emerged colonies were transferred to a new RM agar plate and incubated for 1 week for the second selection. In both cases, replacement of the target region was confirmed by diagnostic PCR (**Figure S3**).

1.2.3 Confirmation of the targeted gene replacement in the Δ*laeA* and Δ*laeA*Δ*cpf1* strains.

To verify that the target gene was replaced with the cassette, the genomic DNA isolated from the transformants was analyzed by diagnostic PCR. Three sets of PCR primers were designed for this verification (**Figure S2** and

). For the first set, one primer that anneals to the selection marker and another primer that anneals at the 3' side of the Rec3' region were designed (the "right arm" set in

). With this primer set, gDNA from the recipient strain will not produce any PCR product with this primer set. However, a PCR product around 1.5–3.0 kb in size will be formed from the gDNA of a strain containing the desired gene deletion. For the second set, one primer that anneals to the selection marker and another primer that anneals at the 5' side of the Rec5' region were designed (the "left arm" set in

). It works basically in an identical manner to the right arm set. For the third primer set, one primer that anneals near the 3' end of the target gene and another primer that anneals approximately 500 bp inside of the target gene (**Figure S2**, the "negative PCR" set in

). With this primer set, PCR on the gDNA of an unaltered parent strain, such as *C. cinerea* ku3-24, will yield a PCR product approximately 1.0 kb in size. PCR on the gDNA of strains having the target gene replaced by a selection marker will not yield the 1.0 kb PCR product. Combination of the results from those three separate PCR reactions ensured us that we had the targeted gene replaced by a selection marker. Results of the PCR analyses are given in **Figure S3** in the main text.

Figure S2. A schematic diagram showing the primer annealing locations for the PCR reactions and the expected products formed that are used to confirm the desired knockout event in the genomic DNA of the modified strains. For the construction of the Δ*laeA* (**A**) and Δ*laeA*Δ*cpf1* (**B**) strains are represented.

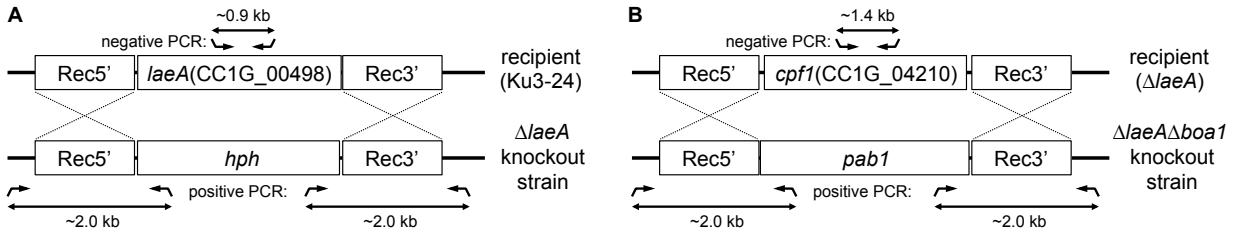

A
~0.9 kb
negative PCR:
Rec5'
laeA(CC1G_00498)
Rec3'
recipient
(Ku3-24)
Rec5'
hph
Rec3'
ΔlaeA
knockout
strain
positive PCR:
~2.0 kb
~2.0 kb
B
~1.4 kb
negative PCR:
Rec5'
cpf1(CC1G_04210)
Rec3'
recipient
(ΔlaeA)
Rec5'
pab1
Rec3'
ΔlaeAΔboa1
knockout
strain
positive PCR:
~2.0 kb
~2.0 kb

Table S2. The list of primer sets used to generate the *C. cinerea* mutant strains, Δ*laeA* and Δ*laeA*Δ*cpf1*.

Plasmid name	Target gene	Marker	Primer set for Rec5'	Primer set for marker	Primer set for Rec3'	Digested original vector
pKW22056	*laeA* (CC1G_00498)	*hph*	pKW22056-LF	Ma13F	pKW22056-RF	pRS426/*Bam*HI, *Eco*RI
			pKW22056-LR	Ma13R	pKW22056-RR	
pKW22091	*cpf1* (CC1G_04210)	*pab1*	pKW22091-LF	c.cinerea_pab1_F	pKW22091-RF	pRS426/*Bam*HI, *Eco*RI
			pKW22091-LR	c.cinerea_pab1_R	pKW22091-RR	

Figure S3. PCR analyses for the confirmation of the deletion of target genes. (**A**) PCR analysis for confirming the deletion of *laeA* (CC1G_00498) using the genomic DNA of the Δ*laeA* strain and the parent strain ku3-24 as templates. (**B**) PCR analysis for confirming the deletion of *cpf1* (CC1G_04210) using the genomic DNA of the Δ*laeA*Δ*cpf1* strain and the parent strain Δ*laeA* as templates. The primer sets used in this study are shown in
. The sequences of the primer sets are listed in **Table S4**.

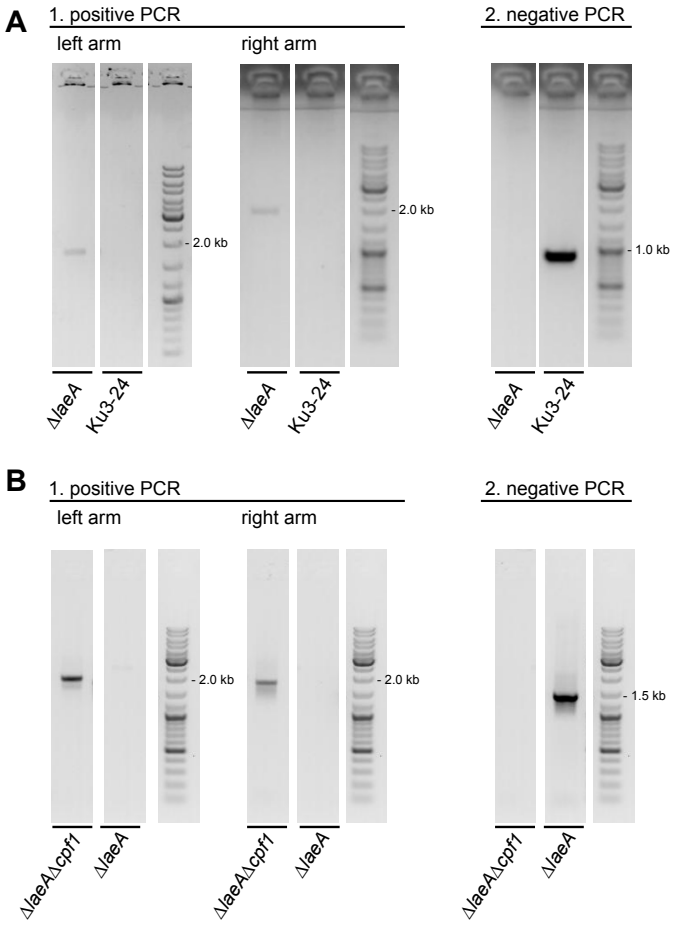


Table S3. The list of primer sets used to confirm successful preparation of the *C. cinerea* mutant strains, Δ*laeA* and Δ*laeA*Δ*cpf1*.

Plasmid name	Target gene	Marker	Primer set for positive PCR (left arm)	Primer set for positive PCR (right arm)	Primer set for negative PCR
pKW22056	*laeA* (CC1G_00498)	*hph*	pKW22056-pos-LF	Cchph-pos-RF	CC1G_00498-neg-F
			Cchph-pos-LR	pKW22056-pos-RR	CC1G_00498-neg-R
pKW22091	*cpf1* (CC1G_04210)	*pab1*	pKW22091-pos-LF	Ccpab1-pos-RF	CC1G_04210-neg-F
			Ccpab1-pos-LR	pKW22091-pos-RR	CC1G_04210-neg-R

1.3 List of primer sequences

Table S4. Oligonucleotide primer sequences. DNA primers were designed on the basis of sequence data obtained from the *C. cinerea* sequence database.

Primer name	Sequence, 5'-3'
pKW22056-LF	gacggtatcgataagcttgatatcgcaatgaccgccccctagtacacg
pKW22056-LR	tactagtggatccgagctcggtaccgacatcggtgctcgagttcagtcg
Ma13F	ggtaccgagctcggatccactagtaacg
Ma13R	gtaatacgactcactatagggcgaattgggc
pKW22056-RF	ttcgccctatagtgagtcgtattactacataattgtgttcaggatttgccttgttatacgc
pKW22056-RR	ggtggcggccgctctagaactagtgtccattgagatgtgtctacagaaaatgttccg
pKW22091-LF	gacggtatcgataagcttgatatcgcctcttgtgttcggacgttgcg
pKW22091-LR	acgctcggaaagccgtttaaatgaaccaacagaatgtcagaggcgcc
c.cinerea_pab1_F	ttcatttaaacggctttccgagcgtc
c.cinerea_pab1_R	caatattcatctctcaactgaaggagcgttgag
pKW22091-RF	tccttcagttgagagatgaatattgggagggtcgctggtggagg
pKW22091-RR	ggtggcggccgctctagaactagtgcggcgtttgcgacggc
pKW22056-pos-LF	ttcacattgaattgttggaactcttggagc
Cchph-pos-LR	aagtcagcttcattttccgtgtggc
Cchph-pos-RF	ctctccacctacctccaccacctacg
pKW22056-pos-RR	cggataaactggtgttatcgatctccacg
CC1G_00498-neg-F	gcggatgaagaggagtgggatcg
CC1G_00498-neg-R	gacttgctcgaaacctggatgcg
pKW22091-pos-LF	aaggggttcctccaggtgcg
Ccpab1-pos-LR	gcgttaccccacttctccatggtagg
Ccpab1-pos-RF	gctgtctgcgttcctatccttggatca
pKW22091-pos-RR	cctcttccaattgttcctcttcctcgc
CC1G_04210-neg-F	gatcgtgtggctgtctgtctaagtcgc
CC1G_04210-neg-R	ccttgatctgggtgtcgatgcg

1.4 Cell culture and metabolite analysis

1.4.1 Determination of the production of coprinoferrin.

The *C. cinerea* ku3-24*,* Δ*laeA* and Δ*laeA*Δ*cpf1* strains were initially cultured on MYG agar plates at 30 °C for 5 days. Approximately one square centimeter of the fungal mycelia collected from a plate and was used to inoculate 25 mL of MYG medium. This culture was shaken at 30 °C with 180 r.p.m. For the analysis of metabolites throughout the cultivation period, a portion of the cultured broth (0.5 mL) was sampled from the culture and directly extracted with EtOAc (0.4 mL). The resultant organic layer (0.2 mL) was concentrated *in vacuo*, dissolved in 50 μL of DMF containing 2.0 μg/mL anthraquinone as an internal standard, and analyzed by LC–MS. LC–MS analysis was performed with a Thermo SCIENTIFIC Q Exactive Focus mass spectrometer coupled with liquid chromatography system UltiMate 3000 using both positive and negative electrospray ionization. The HPLC traces were monitored at λ = 280 nm. Metabolites were separated for analysis on an AQUITY UPLC 1.8 μm, 2.1 × 50 mm C18 reverse-phase column (Waters) using a linear gradient of 10–50% (v/v) CH_3CN in H_2O supplemented with 0.05% (v/v) formic acid at a flow rate of 500 μL/min. The production titer of coprinoferrin was determined based on the area of *m*/*z* 744.487 in the selected ion monitoring mode (SIM).

Figure S4. Time-course plots of the production of (**A**) coprinoferrin or (**B**) the Fe-bound form of coprinoferrin by the *C. cinerea* strains, ku3-24 and Δ*laeA*. Production titers (mg/L culture) of two individual clones of the Δ*laeA* strain (closed and opened squares) and a single strain of the ku3-24 strain (closed triangle) are presented.

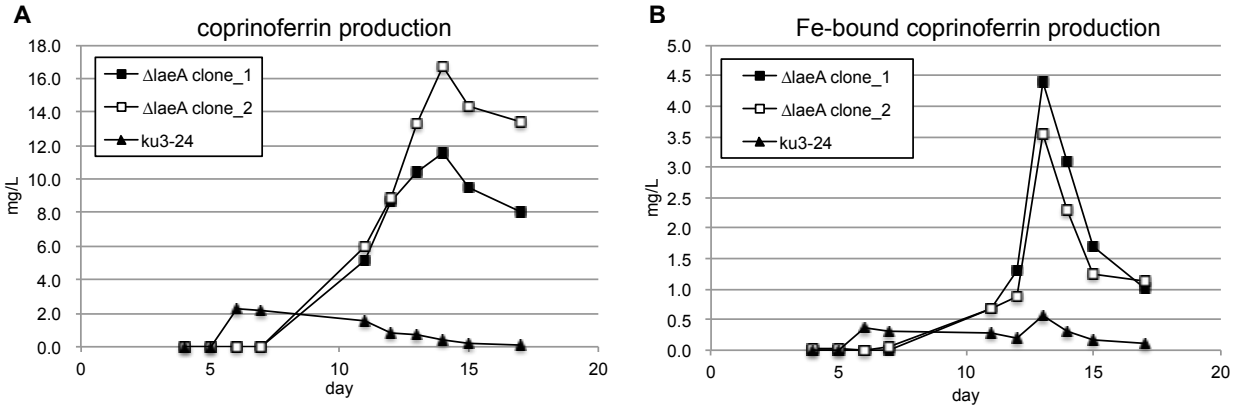
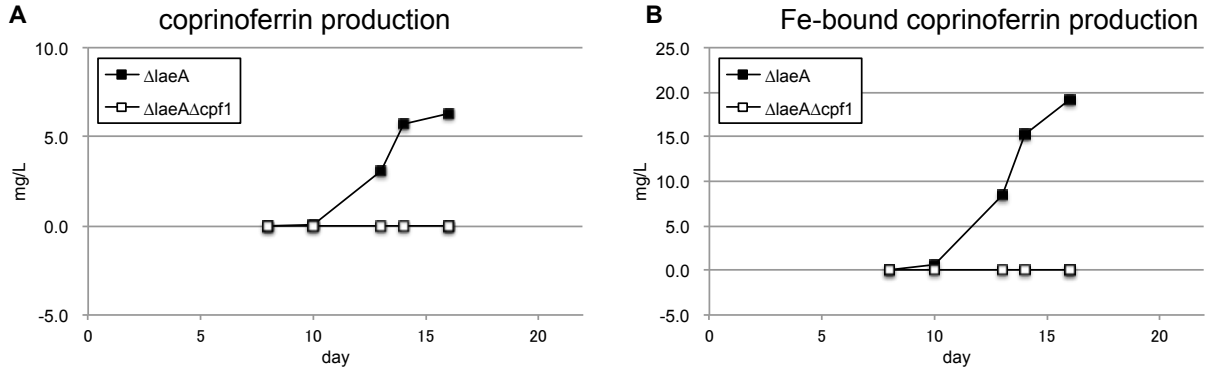


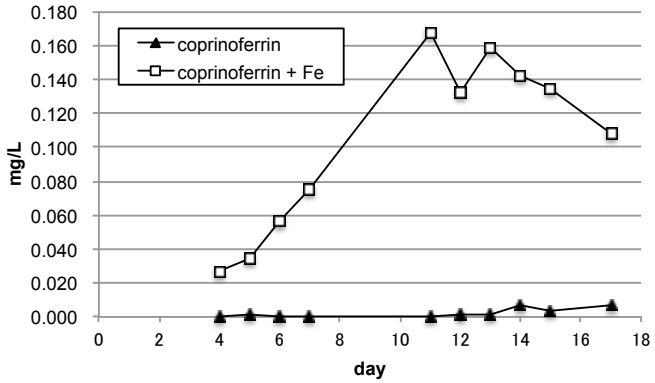
Figure S5. Time-course plots of the production of (**A**) coprinoferrin or (**B**) the Fe-bound form of coprinoferrin by the *C. cinerea* strains, Δ*laeA* and Δ*laeA*Δ*cpf1*. Production titers of a single strain each of the Δ*laeA* (closed square) and Δ*laeA*Δ*cpf1* strains are presented.



1.4.2 Determination of the coprinoferrin production in the iron-limited condition.

To investigate whether the coprinoferrin production is induced in a low iron medium, *C. cinerea* ku3-24 strain was inoculated in 25 mL of MM medium (2.0 g/L L-asparagine, 0.12 g/L $MgSO_4$, 5.0 g/L D-glucose, 1.0 mg/L thiamine, 5.0 g soluble starch, 1.5 g/L NH_4Cl, 1.25 g/L KH_2PO_4, 0.3 g/L Na_2SO_4, 1.45 g/L Na_2HPO_4) supplemented with 1 mM *p*-aminobenzoic acid. This culture was shaken at 30 °C with 180 r.p.m. Procedure of the production analysis is essentially same as described in the previous section.

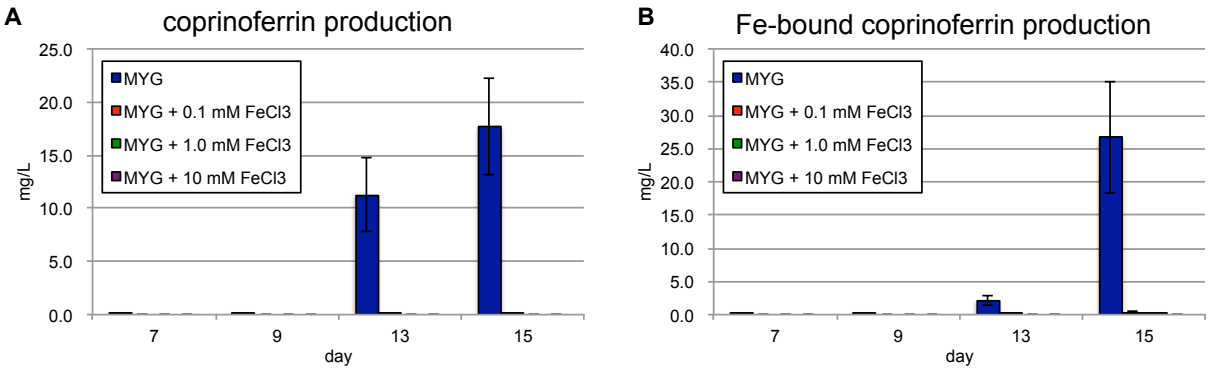
Figure S6



1.4.3 Effect of Fe(III) against the production of coprinoferrins in the Δ*laeA* strain

To test whether environmental Fe(III) affects the production of coprinoferrins, the *C. cinerea* Δ*laeA* strain was cultured in MYG liquid medium supplemented with various concentrations of $FeCl_3$. Procedure of this experiment is essentially same as described in the previous section.

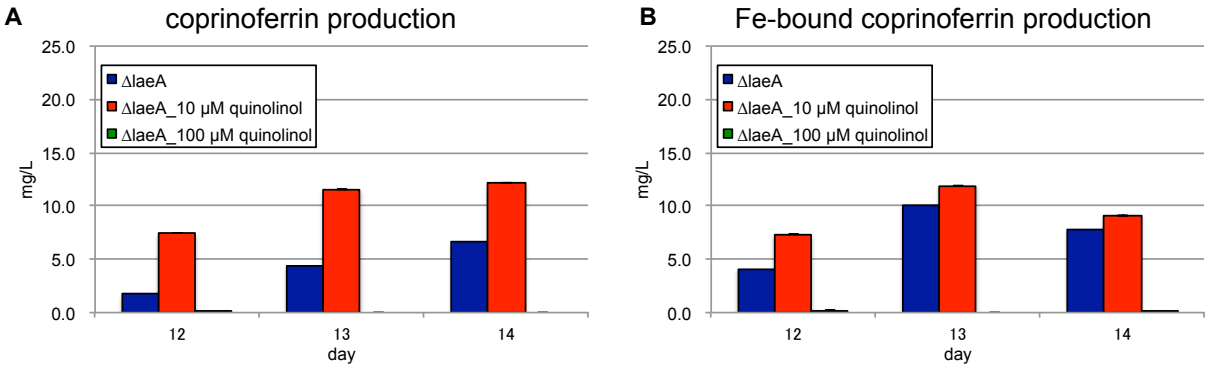
Figure S7. Time-course plots of the production of (**A**) coprinoferrin or (**B**) the Fe-bound form of coprinoferrin production by the *C. cinerea* Δ*laeA* strain. MYG medium (blue) and MYG medium supplemented with 0.1 mM (red), 1.0 mM (green) or 10 mM (purple) of $FeCl_3$ were used for this assay. Quantification was carried out at 7, 9, 13, 15 days after inoculation.



1.4.4 Effect of Fe(III) against the production of coprinoferrins in Δ*laeA* strain.

To test whether Fe(III) chelator affects the production of coprinoferrins, *C. cinerea* Δ*laeA* strain was cultured in MYG liquid medium supplemented with various concentrations of 8-quinolinol. Procedures of this experiment are essentially same as described in the previous section.

Figure S8 Time-course of coprinoferrin(**A**) or Fe-bound form of coprinoferrin(**B**) production by the *C. cinerea* Δ*laeA* strain. MYG medium (blue) and MYG medium supplemented with 10 μM (red) or 100 μM (green) of 8-quinolinol were used for this assay. It should be noted that growth defect was observed in the MYG medium containing 100 μM 8-quinolinol.



1.5 Chemical characterization

1.5.1 Instrumental measurements.

Optical rotations were measured with JASCO P-2020 polarimeter, and infrared (IR) spectra were obtained on JASCO FT/IR-4100 FT-IR spectrometer with JASCO ATR PRO450-S. NMR spectra were recorded using Bruker AVANCE III HD 500 MHz spectrometer, and chemical shifts δ (ppm) were calibrated by solvent residual hydrogen (^{1}H) or carbon (^{13}C) peaks of DMSO-d_6 (δ_H: 2.50 ppm, δ_C: 39.52 ppm).

1.5.2 Culturing of the *C. cinerea* strains and purification of 1 and 2.

The *C. cinerea* Δ*laeA* strain was initially cultured on MYG agar plates at 30 °C for 5 days. Approximately 1 square centimeter of the mycelia collected from a single plate were used to inoculate 25 mL of MYG medium. This culture was shaken at 180 r.p.m. for 24 h at 30 °C. Grown cells were transferred to 7 x 1.0 L of MYG liquid media, and the incubation was continued under the same condition. After 13 days, the mycelia and the broth were separated by filtration. The mycelia are extracted twice with 1.0 L of acetone. After the acetone solution was concentrated under the reduced pressure, it was then dissolved in 0.5 L of H_2O and extracted twice with 0.5 L of EtOAc. On the other hand, the cultured broth was directly extracted with EtOAc (7.0 L x 2 times). Each EtOAc layer was concentrated, and the extracted metabolites were analyzed on LC–MS. Since the desired compound was detected in the EtOAc layers from both the mycelia and the broth, they were combined and subjected to silica gel column chromatography with a combination of $CHCl_3$ and MeOH as eluents. One of the fractions containing **1** (52.8 mg, $CHCl_3$/MeOH = 100/20 elution) was further purified by reversed phase HPLC with a gradient solvent system (MeCN/H_2O = 20/80 to 100:0 in 30 min., Column: Cosmosil 5C_{18} MS-II, ϕ20 x 250 mm, Nacalai tesque Inc.) to give 3.3 mg of nearly purified **1**. Final purification was carried out by isocratic HPLC (MeCN/H_2O = 40/60, Column: Cosmosil 5C_{18} AR-II, ϕ10 x 250 mm, Nacalai tesque Inc.) to afford 2.1 mg of **1** as a colorless powder.

For the purification of **2**, a part of the fractions (1.4 mg) previously described was subjected to isocratic HPLC (MeCN/H_2O = 50/50, Column: Cosmosil 5C_{18} AR-II, ϕ10 x 250 mm, Nacalai tesque Inc.) to provide 0.3 mg of **2** as a brown powder.

1.5.3 Structure elucidation of 1.

The molecular formula of **1** ($C_{35}H_{65}N_7O_{10}$) was established by the mass data [HRESIMS: *m/z* 744. 4866$[M+H]^+$, calcd. for $C_{35}H_{66}N_7O_{10}^+$ to be 744. 4866, Δ = 0.0 mmu]. $[\alpha]_D^{22}$ –10.5 (*c* 0.17, MeOH), IR (ATR) 1620, 3330 cm^{-1}.

Figure S9 The chemical structure of coprinoferrin (1).

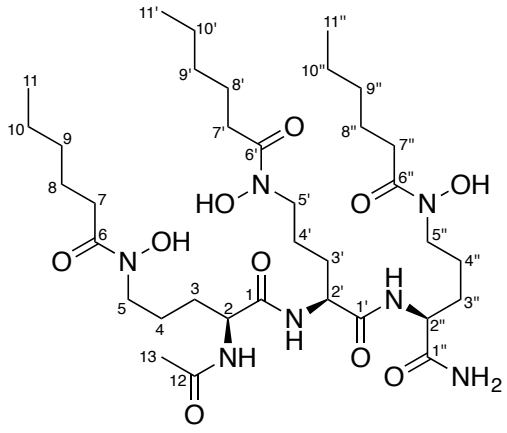


Table S5. NMR data of compound **1** in DMSO-d_6.

Position	δ_H[a]		mult. (*J* in Hz)	HMBC	δ_C[b]
1					171.9
2	4.21	1H	m		52.4
2-NH	8.07	1H	d (7.6)	2, 12	
3	1.46	1H	m		29.4[c]
	1.59	1H	m		
4	1.48	1H	m		23.2[d]
	1.52	1H	m		
5	3.44	1H	m		46.9[e]
	3.48	1H	m		
5-N-OH	9.61	1H	br s		
6					172.8
7	2.32	2H	t (6.9)	6, 8, 9	31.7
8	1.47	2H	m		23.9
9	1.23	2H	m	10, 11	31.1
10	1.26	2H	m	9, 11	22.0
11	0.85	3H	t (7.0)	9, 10	13.9
12					169.6
13	1.84	3H	s	12	22.5
1'					171.3
2'	4.20	1H	m		52.5
2'-NH	7.96	1H	d (7.6)	1, 1'	
3'	1.46	1H	m		29.1[c]
	1.59	1H	m		
4'	1.48	1H	m		23.2[d]
	1.52	1H	m		
5'	3.44	1H	m		46.9[e]
	3.48	1H	m		
5'-N-OH	9.61	1H	br s		
6'					172.8
7'	2.32	2H	t (6.9)	6', 8', 9'	31.7
8'	1.47	2H	m		23.9
9'	1.23	2H	m	10', 11'	31.1
10'	1.26	2H	m	9', 11'	22.0
11'	0.85	3H	t (7.0)	9', 10'	13.9
1"					173.4
1"-NH2	7.05	1H	br s	1", 2"	
	7.23	1H	br s	2"	
2"	4.12	1H	m		52.1
2"-NH	7.73	1H	d (7.9)	1'	
3"	1.46	1H	m		28.9[c]
	1.59	1H	m		
4"	1.48	1H	m		23.0[d]
	1.52	1H	m		
5"	3.44	1H	m		46.8[e]
	3.48	1H	m		
5"-N-OH	9.61	1H	br s		
6"					172.8
7"	2.32	2H	t (6.9)	6", 8", 9"	31.7
8"	1.47	2H	m		23.9
9"	1.23	2H	m	10", 11"	31.1
10"	1.26	2H	m	9", 11"	22.0
11"	0.85	3H	t (7.0)	9", 10"	13.9

a) Recorded at 500 MHz. b) Recorded at 125 MHz. c) interchangeable. d) interchangeable. e) interchangeable.

Figure S10. ^{1}H NMR spectrum of **1** in DMSO-d_6 (500 MHz).

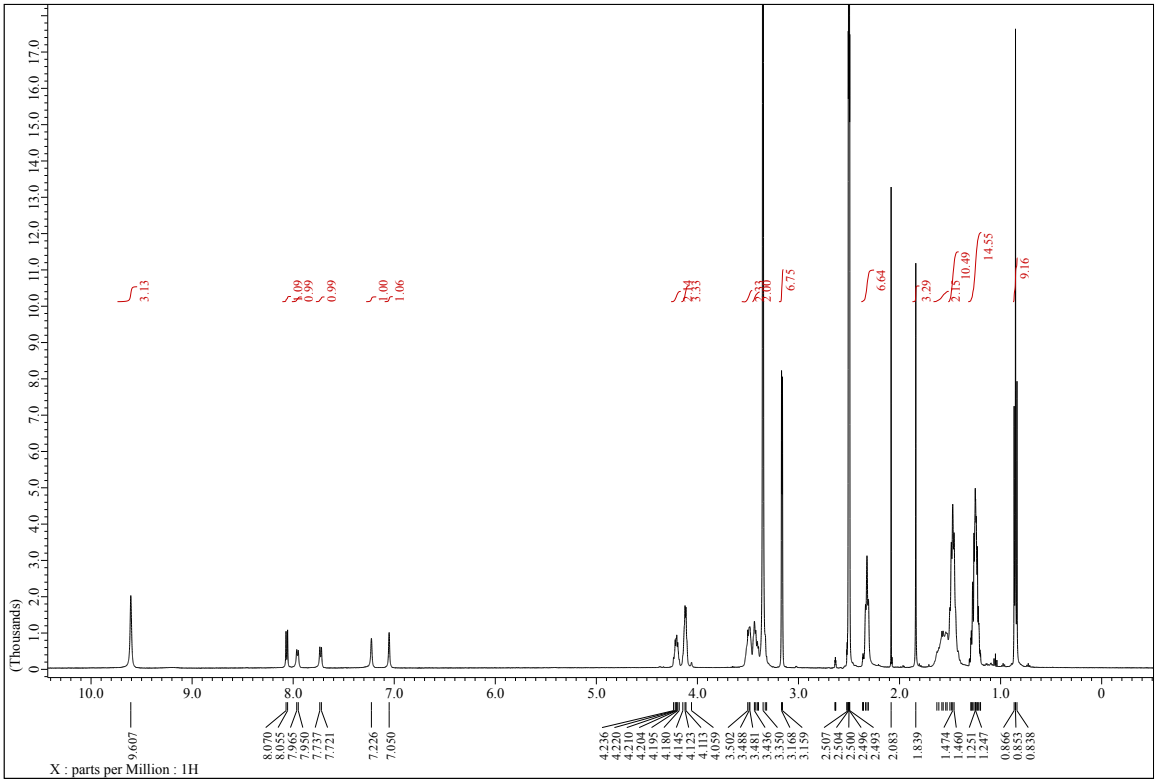


Figure S11. ^{13}C NMR spectrum of **1** in DMSO-d_6 (125 MHz).

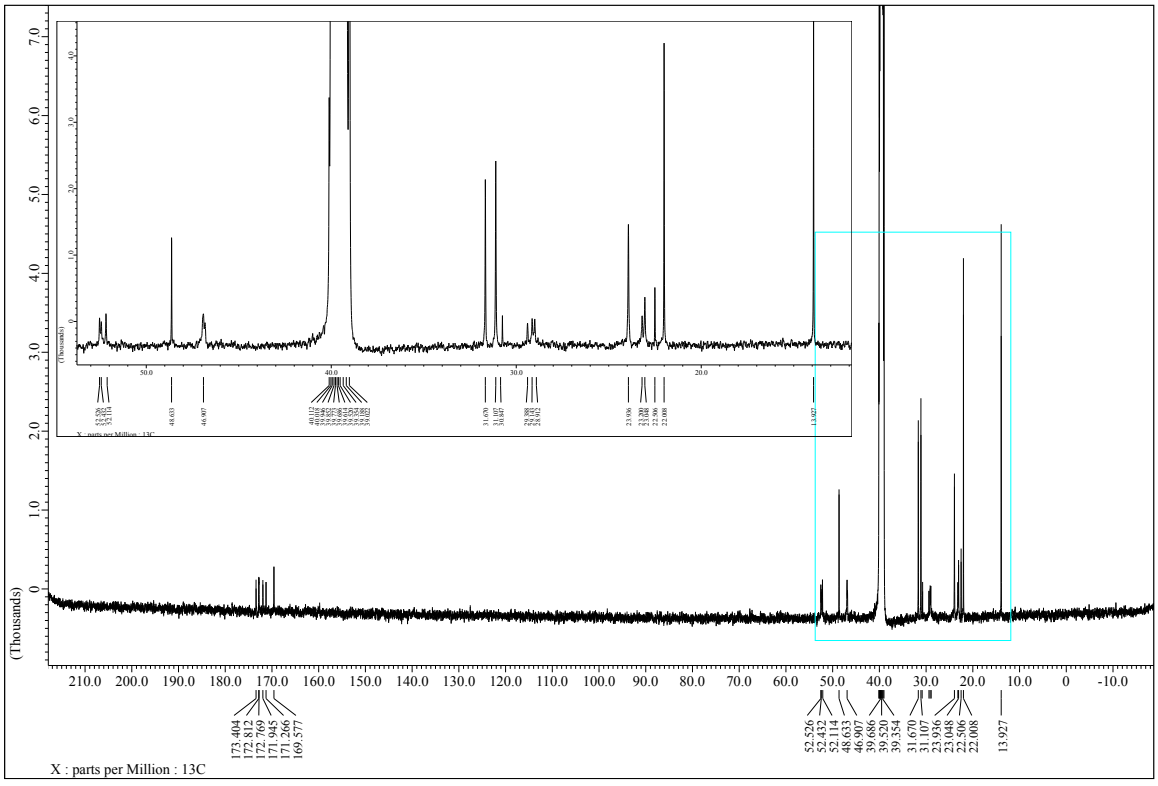


Figure S12. DQF–COSY spectrum of **1** in DMSO-d_6 (500 MHz).

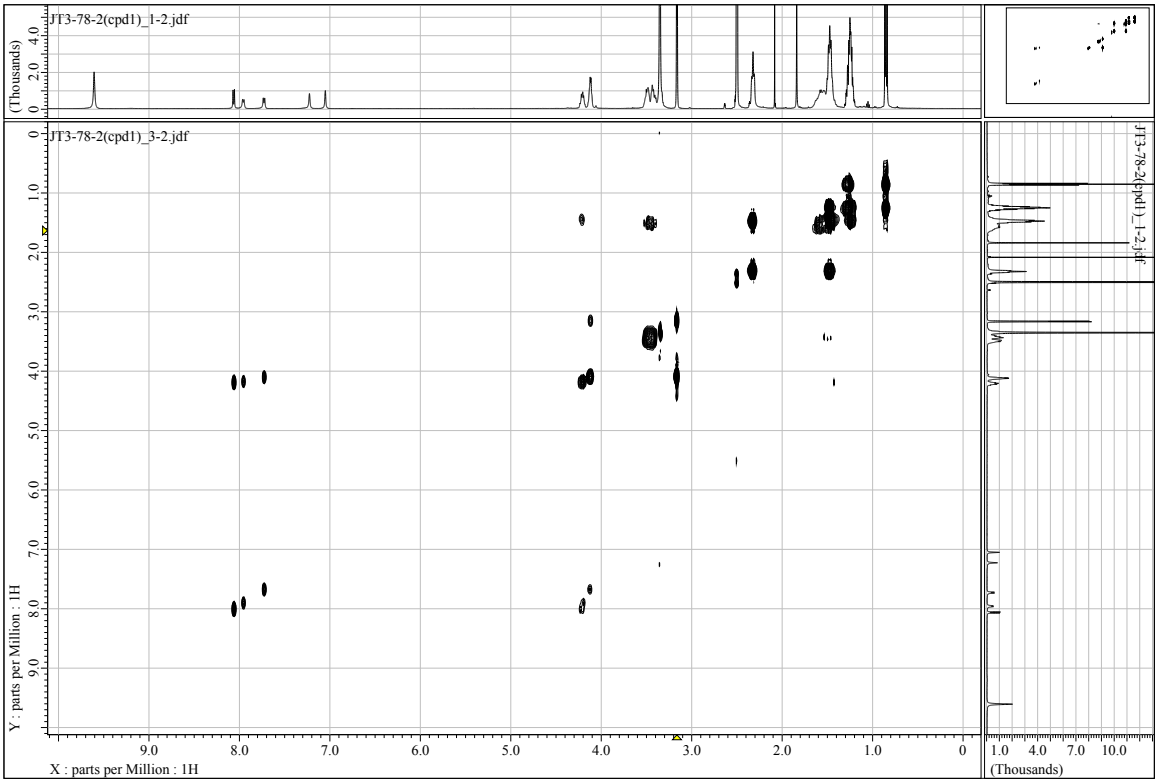


Figure S13. TOCSY spectrum of **1** in DMSO-d_6 (500 MHz).

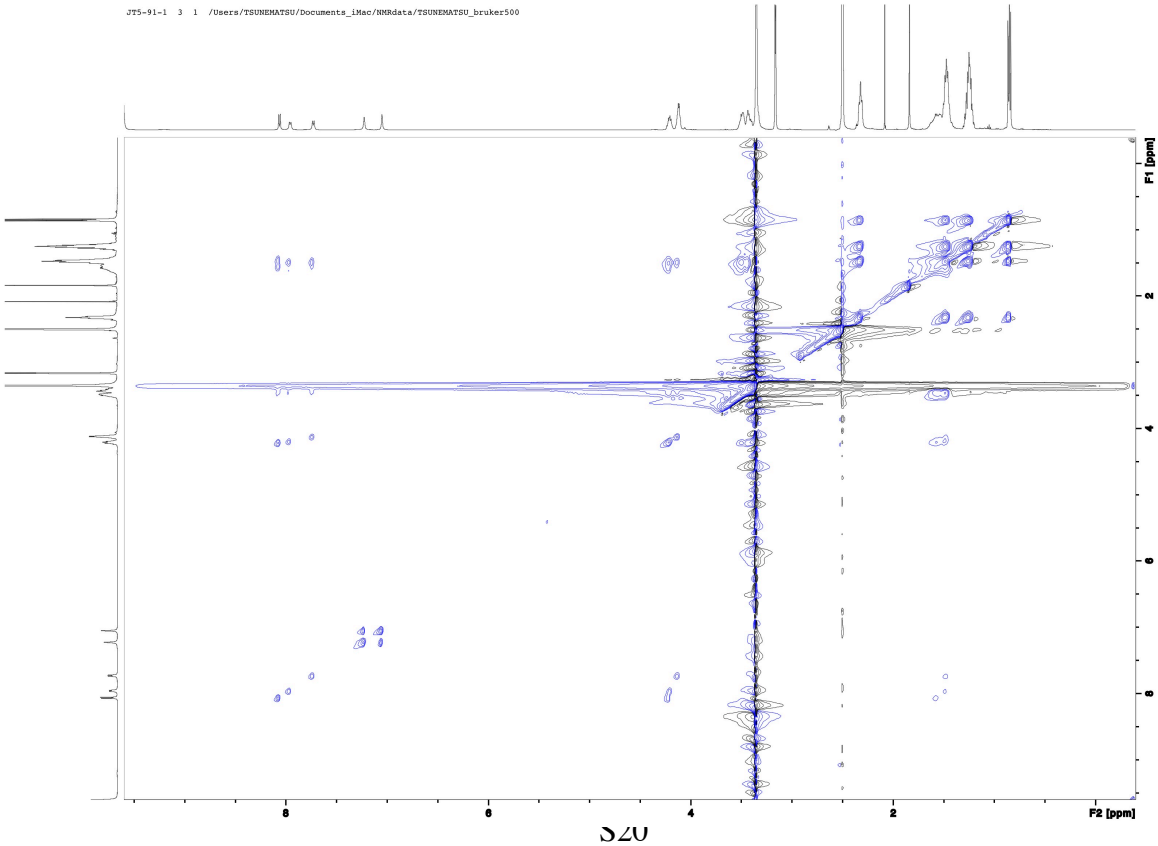


Figure S14. Edited-HSQC spectrum of **1** in DMSO-d_6 (500 MHz).

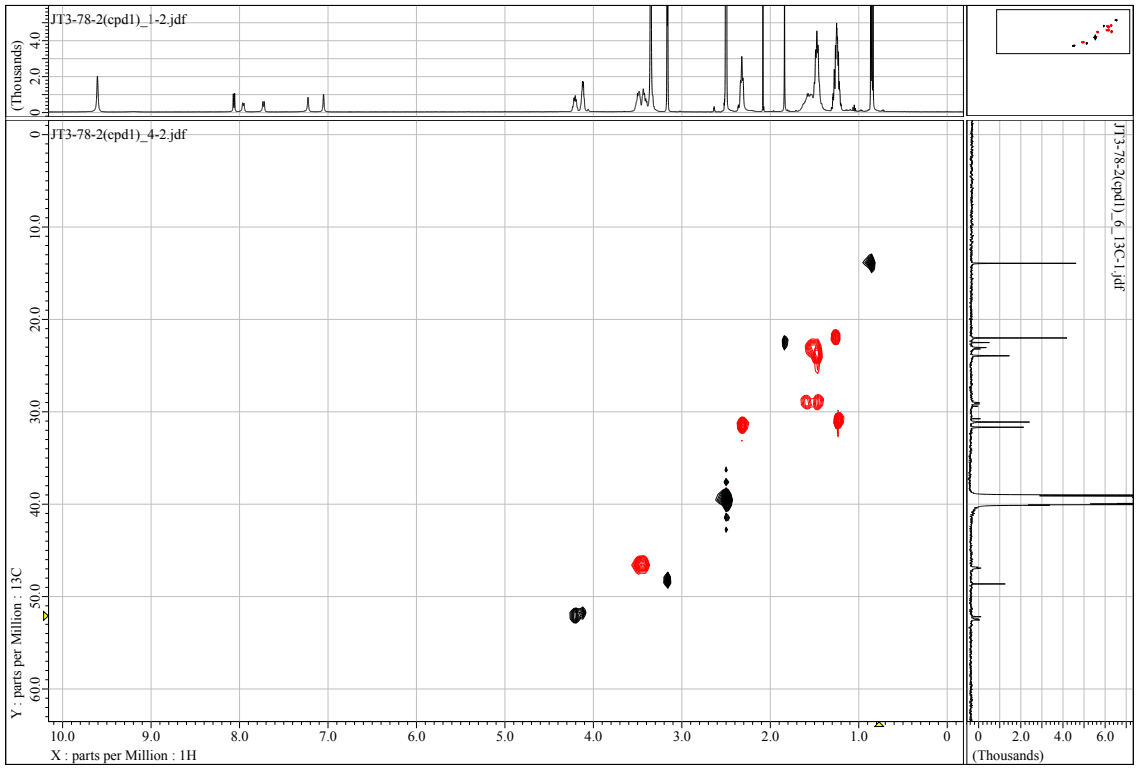
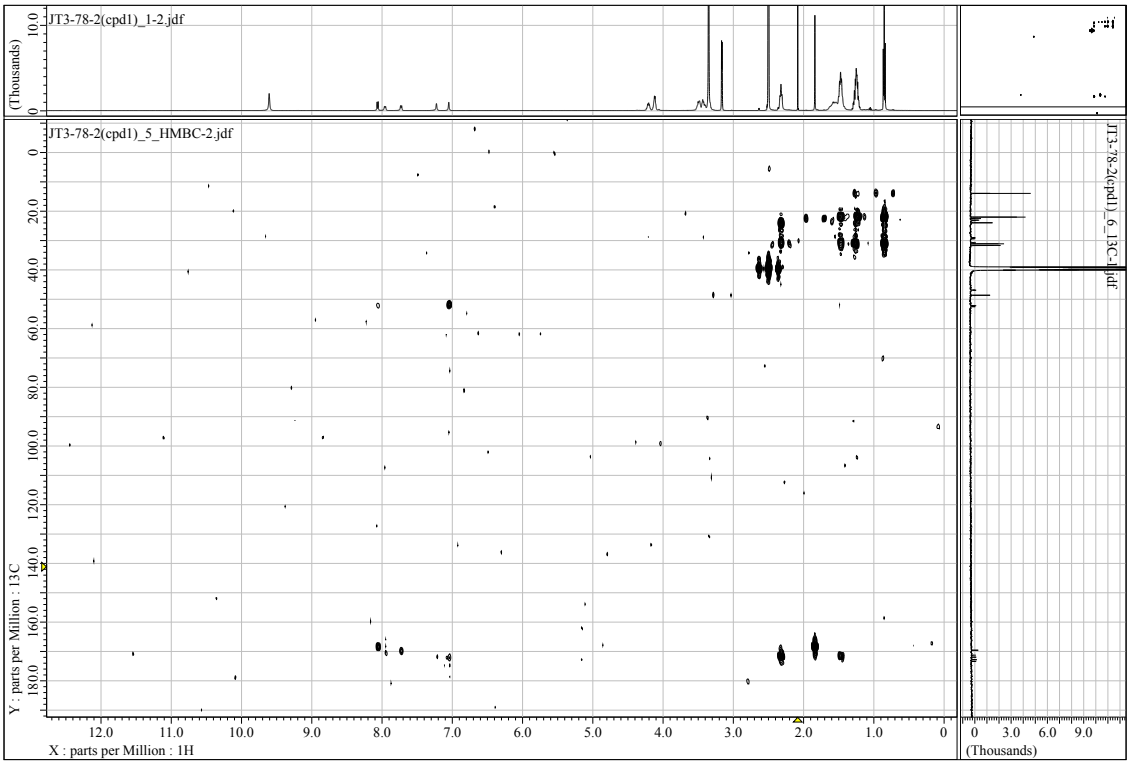


Figure S15. HMBC spectrum of **1** in DMSO-d_6 (500 MHz).



1.5.4 Determination of the absolute configuration of 1 by the advanced Marfey's method.

Purified **1** (0.1 mg) was dissolved in 100 µL of 1 N HCl and hydrolyzed at 100 °C for 12 h. The solution was added 100 µL of H_2O and 50 µL of 1M $NaHCO_3$. After the solution was divided into two portions, one portion was mixed with 50 µL of 1% w/v L-FDLA (N^{α}-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide) in acetone, and the other portion was mixed with 50 µL of 1% w/v D-FDLA in acetone. These solutions were incubated at 50 °C for 1 h and were neutralized by 0.1 N HCl. Commercially available L- and D-ornithine were also derivatized by L- and D-FDLA in the same procedure and used as the references. All FDLA-derivatives were analyzed on LC–MS for determination of the absolute configuration of **1**. The results are given in **Figure 1C** in the main text.

1.5.5 Fe(III)-binding assay of 1.

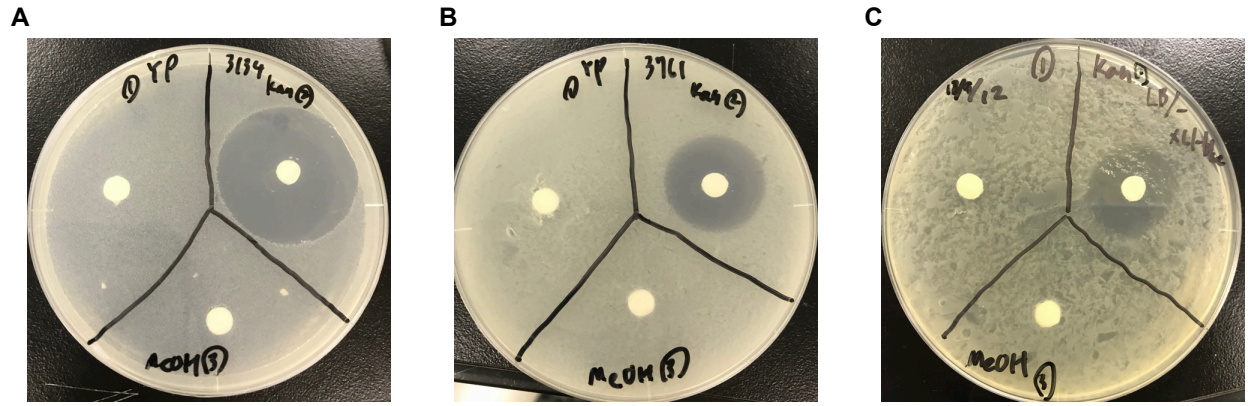
Purified **1** (0.1 mg, 0.13 µmol) in 50 µL MeOH was added with 1.3 µL of 100 nM $FeCl_3$ solution (0.13 µmol, 1.0 eq.) and mixed gently. The mixture was analyzed by LC–MS according to the method previously described. Anthraquinone was used as an internal standard. The result is given in **Figure 1D** in the main text.

1.6 Biological activity of 1 and 2

1.6.1 Antimicrobial tests against *Bacillus subtilis, Staphylococcus aureus and E. coli.*

Bacillus subtilis (NBRC 3134) and *Staphylococcus aureus* (NBRC 3761) were purchased from National Institute of Technology and Evaluation, Japan. Each bacterium was cultured in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) at 30°C for 12 h with shaking at 180 r.p.m. Three paper disks respectively containing 50 µg of **1**, MeOH as a negative control, or 50 µg of kanamycin as a positive control were plated on a YPD agar plate (YPD medium with 2% agar). The resultant plate was overlaid with 10 mL of YPD soft agar (YPD with 0.5% agar) containing 1.0 mL of the culture of a single bacterium prepared earlier, and incubated at 30 °C for 12 h. Antimicrobial test for *E. coli* XL1-blue was conducted in essentially similar procedure as described above.

Figure S16. Antimicrobial tests against *B. subtilis* (A), *S. aureus* (B) and *E. coli* (C). The assay used paper disk containing 50 µg of **1** (left upper), 50 µg of kanamycin (right upper), and MeOH (lower center), respectively.



1.6.2 Growth defect complementation assay.

The *C. cinerea* Δ*laeA*Δ*cpf1* strain was initially cultured on an MYG agar medium at 30 °C for 5 days. Approximately 1 square millimeter of the fungal mycelia collected from the plate was inoculated at the center of an RM plate. Two paper disks respectively containing 74.4 µg of **1** (100 nmol) or DMSO were plated at the position depicted in **Figure 4C(i)** in the main text. The plate was incubated at 30 °C for 20 days. The result is given in **Figure 4C(ii)**.

1.6.3 Fruiting body formation assay.

Each of the *C. cinerea* strains, Δ*laeA*Δ*cpf1*, Δ*laeA* and ku3-24, was initially cultured on an MYG agar medium at 30 °C for 5 days. Approximately 1 square millimeter of the fungal mycelia collected from each plate was inoculated at the center of an MYG plate. Two paper disks respectively containing the test compound (either **1** at 22.3 μg, **2** at 23.9 μg or $FeCl_3$ at 243 μg), or the negative control (DMSO or H_2O) were plated at the position depicted in **Figure 5A** in the main text. The plate was incubated at 30 °C for 22 days. Fruiting body formation was confirmed visually. The result is given in **Figure 5B–D** in the main text.

1.7 Bioinformatic analysis

Figure S17. Conserved domains of LaeA (CC1G_00498) as determined by a BLASTP search against the Swiss-Prot database.[5]

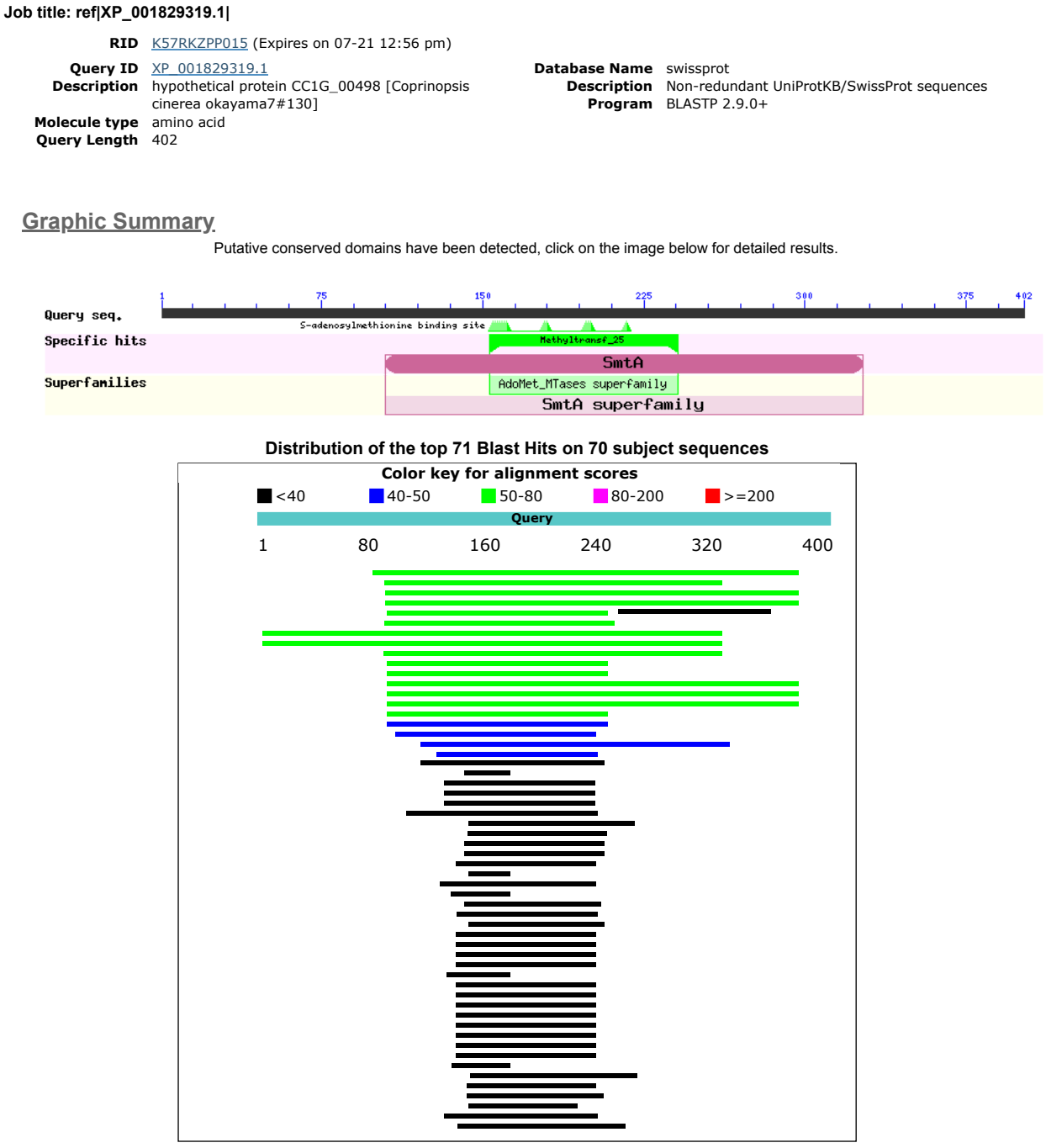


Table S6. List of homologous proteins of LaeA (CC1G_00498) determined by a BLASTP search against the Swiss-Prot database.[5]

Description		Total Score	Query Cover	E value	Per. Ident	Accession
Secondary metabolism regulator laeA	*Penicillium rubens* Wisconsin 54-1255	79.7	75%	5.00E-15	25.63%	B6H9U8.1
Secondary metabolism regulator laeA	*Aspergillus niger* ATCC 1015	77.4	59%	2.00E-14	28.05%	G3XRG4.1
Secondary metabolism regulator laeA	*Aspergillus fumisynnematus*	76.6	73%	4.00E-14	25.71%	A0A0H4LJX8.1
Secondary metabolism regulator laeA	*Aspergillus fumigatus* Af293	75.9	73%	6.00E-14	25.71%	Q4WRY5.1
Secondary metabolism regulator LAE1	*Bipolaris maydis* C5	74.7	39%	9.00E-14	30.62%	M2SNN6.1
Secondary metabolism regulator laeA	*Aspergillus nidulans* FGSC A4	73.2	40%	5.00E-13	30.98%	C8VQG9.1
Secondary metabolism regulator laeA	*Aspergillus flavus* NRRL3357	72.4	81%	9.00E-13	25.89%	B8N406.2
Secondary metabolism regulator laeA	*Aspergillus oryzae* RIB40	134	81%	2.00E-12	25.89%	Q2ULA2.2
Secondary metabolism regulator laeA	*Monascus pilosus*	69.3	59%	9.00E-12	28.34%	A2SUH3.1
Secondary metabolism regulator LAE1	*Botrytis cinerea* B05.10	67.8	39%	3.00E-11	32.93%	A0A0B5L7R4.1
Secondary metabolism regulator LAE1	*Trichoderma atroviride* IMI 206040	63.5	39%	6.00E-10	31.93%	G9P9X3.1
Secondary metabolism regulator LAE1	*Fusarium verticillioides* 7600	58.5	73%	2.00E-08	23.92%	W7LAD1.2
Secondary metabolism regulator laeA	*Fusarium oxysporum* f. sp. lycopersici 4287	58.5	73%	2.00E-08	23.92%	A0A0J9UBD6.1
Secondary metabolism regulator LAE1	*Fusarium fujikuroi* IMI 58289	57	73%	8.00E-08	23.59%	S0DQI7.2
Secondary metabolism regulator laeA	*Fusarium graminearum* PH-1	52.8	39%	2.00E-06	27.95%	I1RAW4.2
Secondary metabolism regulator LAE1	*Trichoderma reesei* QM6a	48.9	39%	3.00E-05	27.71%	G0RNN3.2
Malonyl-[acyl-carrier protein] O-methyltransferase	*Calditerrivibrio nitroreducens* DSM 19672	41.2	35%	0.007	22.07%	E4TI44.1
Phosphoethanolamine N-methyltransferase 3	*Arabidopsis thaliana*	41.2	54%	0.011	24.27%	Q9C6B9.2
Uncharacterized protein RP028	*Rickettsia prowazekii* str. Madrid E	40.4	28%	0.014	26.15%	O05972.1
Phosphoethanolamine N-methyltransferase 1	*Arabidopsis thaliana*	39.3	32%	0.049	28.47%	Q9FR44.1
Malonyl-[acyl-carrier protein] O-methyltransferase	*Legionella pneumophila* subsp. pneumophila str. Philadelphia 1	37.7	7%	0.12	51.61%	Q5ZT34.2
Ubiquinone biosynthesis O-methyltransferase	*Coxiella burnetii* Dugway 5J108-111	37.4	26%	0.13	24.32%	A9KGL7.1
Ubiquinone biosynthesis O-methyltransferase	*Coxiella burnetii* CbuK_Q154	37.4	26%	0.13	24.32%	B6J5Y2.1
Ubiquinone biosynthesis O-methyltransferase	*Coxiella burnetii* RSA 331	37.4	26%	0.14	24.32%	A9NBI0.1
Ubiquinone biosynthesis O-methyltransferase	*Yersinia pestis* Pestoides F	37	33%	0.16	24.83%	A4TNI8.1
Demethylmenaquinone methyltransferase	*Lactococcus lactis* subsp. lactis Il1403	36.6	29%	0.27	27.42%	P49016.1
2-methyl-6-phytyl-1,4-hydroquinone methyltransferase	*Synechocystis* sp. PCC 6803 substr. Kazusa	36.2	24%	0.4	24.51%	P74388.1
Phosphoethanolamine N-methyltransferase	*Spinacia oleracea*	36.2	24%	0.41	26.92%	Q9M571.1
Phosphomethylethanolamine N-methyltransferase	*Arabidopsis thaliana*	36.2	24%	0.43	27.88%	Q944H0.2
Ubiquinone biosynthesis O-methyltransferase	*Escherichia coli* O127:H6 str. E2348/69	35.8	24%	0.47	22.32%	B7UFP4.1
tRNA (guanine-N(7)-)-methyltransferase	*Rubrobacter xylanophilus* DSM 9941	35.4	6%	0.53	57.14%	Q1AUF5.1

Figure S18. Multiple alignment of amino acid sequence of *C. cinerea* LaeA with homologous proteins. Catalytically important GXGXG motif is highlighted in yellow. The data was generated using ClustalW.[6]

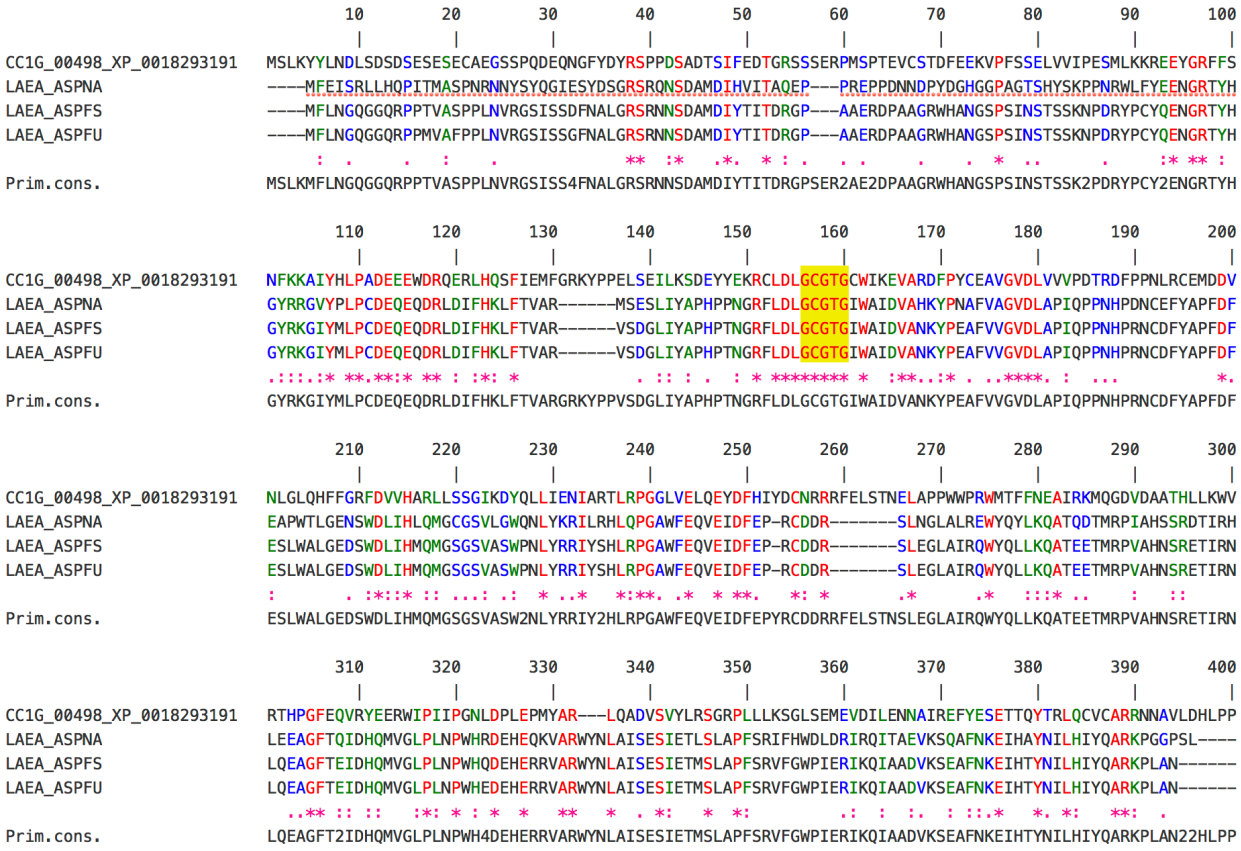
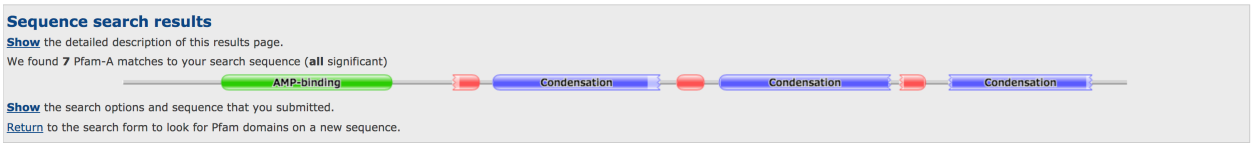


Figure S19. Domain organization of *C. cinerea* Cpf1, predicted by Pfam 32.0.



Sequence search results

Show the detailed description of this results page.

We found **7** Pfam-A matches to your search sequence (**all** significant)

Show the search options and sequence that you submitted.

Return to the search form to look for Pfam domains on a new sequence.

Significant Pfam-A Matches

Show or hide all alignments.

Family	Description	Entry type	Clan	Envelope		Alignment		HMM		HMM length	Bit score	E-value	Predicted active sites	Show/hide alignment
				Start	End	Start	End	From	To					
AMP-binding	AMP-binding enzyme	Family	CL0378	237	646	237	646	1	423	423	283.2	2.8e-84	n/a	Show
PP-binding	Phosphopantetheine attachment site	Domain	CL0314	793	856	808	856	**19**	67	67	25.6	1.2e-05	n/a	Show
Condensation	Condensation domain	Family	CL0149	889	1290	891	1260	**3**	**401**	457	108.8	2.6e-31	n/a	Show
PP-binding	Phosphopantetheine attachment site	Domain	CL0314	1331	1396	1332	1395	**2**	**66**	67	32.8	6.5e-08	n/a	Show
Condensation	Condensation domain	Family	CL0149	1433	1844	1436	1839	**4**	**450**	457	113.5	1e-32	n/a	Show
PP-binding	Phosphopantetheine attachment site	Domain	CL0314	1866	1927	1876	1925	**14**	**64**	67	25.5	1.3e-05	n/a	Show
Condensation	Condensation domain	Family	CL0149	1985	2328	1992	2315	**27**	**358**	457	90.8	7.4e-26	n/a	Show

2. Supporting References

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