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Molecular genetic analysis reveals that a nonribosomal peptide synthetase-like (NRPS-like) gene in *Aspergillus nidulans* is responsible for microperfuranone biosynthesis

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Abstract

Genome sequencing of *Aspergillus* species including *A. nidulans* has revealed that there are far more secondary metabolite biosynthetic gene clusters than secondary metabolites isolated from

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these organisms. This implies that these organisms can produce additional secondary metabolites have not yet been elucidated. The *A. nidulans* genome contains twelve nonribosomal peptide synthetase (NRPS), one hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS), and fourteen NRPS-like genes. The only NRPS-like gene in *A. nidulans* with a known product is *tdiA* which is involved in terrequinone A biosynthesis. To attempt to identify the products of these NRPS-like genes, we replaced the native promoters of the NRPS-like genes with the inducible alcohol dehydrogenase (*alcA*) promoter. Our results demonstrated that induction of the single NRPS-like gene AN3396.4 led to the enhanced production of microperfuranone. Furthermore, heterologous expression of AN3396.4 in *A. niger* confirmed that only one NRPS-like gene, AN3396.4, is necessary for the production of microperfuranone.

Keywords

Aspergillus nidulans; nonribosomal peptide synthetase-like; microperfuranone; biosynthesis

Introduction

Aspergillus species are known to produce medicinally important natural products such as lovastatin as well as toxins such as aflatoxin (Kennedy et al., 1999; Minto and Townsend, 1997). Genome sequencing of *Aspergillus* species has revealed that there are far more secondary metabolism genes than secondary metabolites that have been ever isolated from these organisms (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005). This implies that more secondary metabolites await discovery. The availability of genome sequencing information has facilitated secondary metabolite discovery in a strategy often termed “genome mining” (Chiang et al., 2011a; Winter et al., 2011). This approach involves the use of bioinformatic analysis of genomic data for the identification of putative biosynthesis genes followed by genetic knock out or heterologous expression for the verification of gene function. Genome mining of secondary metabolism genes in *A. nidulans* has been greatly facilitated by the continuous refinement of the genome annotation and the creation, development and refinement of the community databases, Aspergillus Genome database (AspGD), Broad *Aspergillus* Comparative Database, Central Aspergillus Data Repository (CADRE), Secondary Metabolite Unique Regions Finder (SMURF) (Khaldi et al., 2010), and Department of Energy Joint Genome Institute Fungal Genomics Program (JGI). We and others have initiated programs to identify the products of the secondary metabolism genes in *A. nidulans* (Bergmann et al., 2010; Bergmann et al., 2007; Bok et al., 2009; Bok et al., 2006; Chiang et al., 2010; Chiang et al., 2009; Chiang et al., 2008; Scherlach et al., 2010; Schroeckh et al., 2009; Szewczyk et al., 2008). With the rapid development of next generation sequencing whole fungal genome sequencing is now within the budget of individual labs (Nowrousian et al., 2010) and *A. nidulans* is an excellent model organism for the development of strategies and tools that can be translated to many genome-sequenced fungal species.

The initial genome analysis of *A. nidulans* identified twenty-seven polyketide synthases (PKSs) and twelve nonribosomal peptide synthetases (NRPSs). Several single module NRPS-related genes were not identified in this initial genome annotation effort (Galagan et al., 2005). A recent comprehensive review by von Dohren reevaluated the NRPS genes in *A. nidulans* and grouped them into twelve NRPS, one hybrid PKS/NRPS, and fourteen NRPS-like genes (von Dohren, 2009). Monomodular NRPS-like genes in *A. nidulans* are not well characterized either genetically or biochemically. NRPS-like genes share the catalytic domains found in NRPS but are missing the critical condensation domain necessary for peptide formation. The only NRPS-like gene in *A. nidulans* the product of which is known is *tdiA* (AN8513.4 using the CADRE gene designation), which is involved in terrequinone A (compound **2**, Fig. 1) biosynthesis (Balibar et al., 2007; Bok et al., 2006; Schneider et al., 2008). The *tdiA* gene contains three domains found in a typical NRPS gene. They are an adenylation (A) domain which loads a specific amino acid, a thiolation (T) domain, and a thioesterase (TE) domain but the condensation domain is missing. Terrequinone A is a secondary metabolite derived from amino acids but does not have peptide bonds in its structure. Examination of the fourteen NRPS-like genes in *A. nidulans* revealed that one additional NRPS-like gene (AN3396.4) contains the A-T-TE domain structures found in TdiA (von Dohren, 2009). The remaining twelve NRPS-like genes contain either a NAD-binding domain in place of the TE domain or are missing both domains. We cultivated *A. nidulans* in a variety of growth conditions and media in the hopes of identifying conditions that would enable production of a metabolite from the thirteen NRPS-like genes that have not yet been characterized at a level detectable by LC/MS (Sanchez et al., 2010). However, despite numerous attempts, we were unable to detect new metabolites that correspond to biosynthetic pathways that include the thirteen NRPS-like genes. This suggests that these genes are silent or expressed in very low amounts in the conditions we examined. Since it was difficult to obtain conditions to activate the native promoters we initiated a strategy to replace the native promoters with inducible promoters to turn on expression of these genes. We first replaced the native promoters of the thirteen NRPS-like genes with the alcohol dehydrogenase promoter [*alcA*(p)] that can be induced at very high levels of expression using cyclopentanone. We observed that induction of the single NRPS-like gene AN3396.4 led to enhanced production of microperfuranone (compound **1**, Fig. 1). Microperfuranone was first isolated from the fungus *Anixiella micropertusa* and also isolated from a marine strain of *Emericella nidulans* (Fujimoto et al., 1998; Kralj et al., 2006). We named the gene AN3396.4 as *micA* for microperfuranone synthase. To verify that indeed only one NRPS-like protein is necessary to produce microperfuranone we heterologously expressed *micA* in *A. niger*, which has a well characterized secondary metabolome and is known to not produce microperfuranone or similar compounds (Nielsen et al., 2009).

Materials and methods

Generation of fusion PCR fragments, A. nidulans protoplasting and transformation

We generated fusion PCR fragments to replace the native promoters of the thirteen NRPS-like genes with an *alcA* promoter. For example, to obtain strains overexpressing *micA*, an 100 bp fragment immediately upstream of the *micA* start codon was replaced with a fragment containing the *A. fumigatus pyroA* gene (AfpyroA) followed by a 404-bp fragment containing the *A. nidulans alcA* promoter such that the coding sequence of *micA* was placed under the control of the *alcA* promoter. Construction of fusion PCR products, protoplast production and transformation were carried out as described (Szewczyk et al., 2006). For construction of fusion PCR fragments, two ~1,000-bp fragments, one upstream and one downstream of the targeted endogenous promoter, were amplified from genomic *A. nidulans* DNA by PCR. Using two nested primers a fusion PCR reaction attached the two 1000 bp fragments to flank the *A. fumigatus pyroA* selective marker. The primers for fusion PCR are listed in Table 1. An *A. nidulans* strain, LO2026, carrying a deletion of the *stcJΔ* that prevents sterigmatocystin production was used as a recipient strain for transformation (Bok et al., 2009). The transformants with correct promoter replacement were further verified with diagnostic PCR using the external primers used in the first round of PCR (Table 1 and Fig. S1). In each case at least two transformants carrying the correct promoter replacement were used for further study. *A. nidulans* strains used in this study are listed in Table 2. Deletions of ten genes, designated AN3391.4-AN3395.4 and AN3397.4-AN3401.4 were generated by replacing each gene with the *A. fumigatus pyrG* gene in the *A. nidulans* strain CW3023 (*stcJ*Δ, *alcA(*p*)-micA*). The primers for gene deletion and complete genotypes are listed in Table S1 and Table S2, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The total RNA of *A. nidulans* parental and mutant strains were extracted using the Qiagen RNeasy plant mini kit according to the manufacturer's instructions. The first strand cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) following the supplied protocols. The cDNA was then used as template for PCR amplification with the following specific primer sets that flank the intron except AN3397.4 which do not have a predicted intron: AN3391.4, ACCTATACCAGTGCGGAAC and GAGCCACGCACTCAATATTC; AN3392.4, GAGGCACGGTTAGCTCTAC and CCCAAACGCAATAGGCATG; AN3393.4, ATCAGGACCAGCACCACTG and CAGCTCGTTGGAGGTGTAG; AN3394.4, CTGCGTCACAATTCAGTGC and GCTTGTAAGTCAAGGCTGC; AN3395.4, GTCTTCGCCTTGTCAACAC and GCGATACTAGTATGGCCAC; AN3396.4, GACCACGTTGCTAGTTTGAC and AATCACTTCGGCTTGGACAC; AN3397.4, CCACGTCGAGGTGATCAAG and GGCAGTGAAGTCGACGTTC; AN3398.4, GACTCGCAAAGACCTATGC and GCATTCTAAGCTGGCGCTG; AN3399.4, CTGCACTGTGACGAGAGTC and GAACCACTCCTCGATTGCAC; AN3400.4, TGCGTGAGCTCTCCTCTAG and GTGAACGAGTCCACCATCC; AN3401.4, GCGTCAAAATTCGGCTGAG and CATTCGGGCCTCCTGTAAC; β-Tubulin, CATGATGACAGCTGCCAAC and GAGCAGTTTGGACGTTGTTG. Amplification products were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Heterologous expression of micA

Expression of *micA* was achieved in *A. niger* by fusing the coding sequence of the gene to a promoter sequence taken from the *A. oryzae amyB* gene. The *amyB* gene from *A. oryzae* (Locus ID, AO090120000196) has been used for heterologous expression in other systems and is known to be responsive to growth on different carbon sources (Kanemori et al., 1999). The construct was built using the yeast gap repair method and plasmid described in a previous study for creating fungal gene fusions (Bourett et al., 2002). In brief, the *amyB* promoter was amplified from *A. oryzae* strain RIB40 genomic DNA using primers AN3396.amyF and AN3396.amyR. The *micA* coding region was amplified using primers AN3396.ATG and AN3396.TAA. The five prime ends of AN3396.amyF and AN3396.TAA have homology to regions within pSM565 (Genbank, *AY142483.1*) that can repair an *XhoI* digestion of this plasmid when transformed into *Saccharomyces cerevisiae*. Sequences on primers AN3396.amyR and AN3396.ATG directly fuse the start codon from the amylase promoter to the *micA* coding sequence. The plasmid created was isolated from yeast colonies, amplified in *E. coli* cultures and subsequently transformed into *A. niger* strain KB1001 (Chiang et al., 2011b). Positive transformants are resistant to hygromycin due to the presence of the *hph* gene on the plasmid. Spores were collected from the *OE:micA* strains by cultivating 1.0×10^7 spores per 10-cm plate for 5 days at 30°C on YAG medium (5 g of yeast extract/liter, 15 g of agar/liter, and 20 g of D-glucose/liter supplemented with a 1 ml/liter trace element solution) containing 100 μg/ml hygromycin B. To test for expression of the micA gene and production of microperfuranone, a 30 ml liquid YG culture supplemented with 100 μg/ml hygromycin B was inoculated with 3.0×10^7 spores and grown at 30°C with shaking at 170 rpm for 18 h. The hyphae were collected with miracloth and put into medium to induce the amylase promoter of *OE:micA* using GMM medium with 2% (w/v) maltose as the carbon source. After 2 days of induction, the medium were collected by filtration and then extracted as described below.

Fermentation and Purification

For the fermentation, 3.0×10^7 spores of *A. nidulans* were grown in 125 ml of flask containing 30 ml liquid LMM medium (15 g/l lactose, 6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4 \cdot 7H_2O$, 1.52 g/l KH_2PO_4, 1 ml/l trace elements) supplemented when necessary with uracil (1 mg/ml) and uridine (10 mM) at 37°C with shaking at 200 rpm (Chiang et al., 2008). For *alcA* promoter induction, cyclopentanone at a final concentration of 10 mM was added to the medium after 18 h of incubation. Culture medium was collected 48 h after cyclopentanone induction by filtration and extracted with the same volume of EtOAc two times. The combined EtOAc layers were evaporated *in vacuo*, re-dissolved in 0.75 ml of 1:4 DMSO/MeOH and 10 μl was injected for LC-DAD-MS analysis. Conditions for MS included a capillary voltage 5.0 kV, a sheath gas flow rate at 60 arbitrary units, an auxiliary gas flow rate at 10 arbitrary units, and the ion transfer capillary temperature at 350°C. HPLC-MS was carried out in positive mode using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with an RP C_{18} column (Alltech Prevail C18; particle size, 3 μm; column, 2.1 by 100 mm) at a flow rate of 125 μl/min. Microperfuranone was eluted at 26.5 min.

For structure elucidation, a strain carrying *alcA*(p)-*micA* was cultivated in 2 liter LMM medium. After 2 days of induction, the medium was collected by filtration and then extracted with equal amount of EtOAc twice. The combined EtOAc extracts were evaporated *in vacuo*. The crude extract in EtOAc layer (448 mg) was coated on 6720 mg of C_{18} reverse phase gel (Cosmosil 75C_{18}-OPN, Nacalai USA) which was then suspended in 10% of MeOH/ddH_2O and applied to a C_{18} reverse phase column (30 × 60 mm). This column was then eluted with MeOH/ddH_2O mixtures of decreasing polarity (fraction A, 10% MeOH, 300 ml; fraction B, 30% MeOH, 300 ml; fraction C, 70% MeOH, 300 ml; and fraction D, 100% MeOH, 300 ml). All fractions were analyzed by HPLC-DAD-MS. Fraction C containing microperfuranone was further subjected to semi-preparative reverse phase HPLC (Phenomenex Luna 5 μm C_{18}, 250 × 21.2 mm) with a flow rate of 5.0 ml/min and measured by a UV detector at 254 nm. The solvent gradient for HPLC was 100% MeCN (solvent B) in 5% MeCN/H_2O (solvent A), 20% B from 0 to 20 min, 60 to 100% B from 20 to 22 min, maintained at 100% B from 22 to 25 min, 100 to 20% B from 25 to 26 min, and re-equilibration with 20% B from 26 to 30 min. Microperfuranone (20 mg) was eluted at 20.0 min.

Compound identification

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were collected on a Varian Mercury Plus 400 spectrometer, whereas HRESIMS spectra were obtained on an Agilent Technologies 1200 series high-resolution mass spectrometer. Microperfuranone was isolated as colorless plates and its molecular formula was deduced to be $C_{17}H_{14}O_3$ ($[M + H]^+$ *m/z* found 267.1022; calcd. for $C_{17}H_{15}O_3$: 267.1021, Fig. S2). The ^{1}H (Fig. S3 and S4) and ^{13}C (Fig. S5 and S6) NMR data in $CDCl_3$ or acetone-d_6 were in good agreement with the published data (Fujimoto et al., 2006; Fujimoto et al., 1998).

Results

Induction of the micA gene using an inducible promoter stimulates the production of microperfuranone

Previous data have shown that *A. nidulans* secondary metabolite production is heavily dependent on culture conditions (Sanchez et al., 2010; Scherlach and Hertweck, 2006; Scherlach et al., 2010). Attempts to grow *A. nidulans* under twenty different conditions failed to produce any additional metabolites that could be produced by NRPS-like genes. Analysis by von Dohren identified fourteen NRPS-like genes in *A. nidulans*. Since one of the NRPS-like is *tdiA* (AN8513.4), we focused on the other thirteen genes for promoter replacements (AN1680.4, AN2064.4, AN2924.4, AN3396.4, AN3495.4, AN4827.4, AN5318.4, AN6444.4, AN8105.4, AN8504.4, AN9291.4, AN10297.4, and AN10486.4) (von Dohren, 2009). We replaced the native promoter of the thirteen genes with the inducible alcohol dehydrogenase promoter. The replacement was accomplished using our previously reported strategy involving an *nkuA*Δ *A. nidulans* strain and fusion PCR (Chiang et al., 2008; Nayak et al., 2006; Szewczyk et al., 2006). The promoter replacements were carried out in an *A. nidulans* strain carrying a deletion of the *stcJ*Δ gene (*stcJ*Δ), which prevents the production of the major polyketide sterigmatocystin (Chiang et al., 2009). We cultivated three separate promoter exchanged strains per gene and a control strain LO2026 in lactose minimal media and used cyclopentanone as an inducer. All strains were verified by diagnostic PCR (Fig. S1). Metabolite profiles from each induced strain were analyzed by LC-DAD-MS and only the *alcA(*p*)-micA* strains were able to produce a new metabolite with the molecular weight of 266 (m/z = 267 $[M+H]^+$) (Fig. 2 and S2). The compound was isolated from large-scale cultivation of the *alcA(*p*)-micA* strain by purification initially from flash chromatography followed by preparative HPLC. Both ^{1}H and ^{13}C NMR analysis of the compound (Fig. S3 – S6) identified the product as microperfuranone which has been isolated from *Anixiella micropertusa* and *Emericella nidulans var. acristata* (Fujimoto et al., 2006; Fujimoto et al., 1998; Kralj et al., 2006). The twelve other strains failed to produce new metabolites suggesting that co-overexpression of additional genes in their pathways might be necessary to elicit detectable and isolatable products.

Heterologous expression of micA in the heterologous host A. niger demonstrates that only one gene is necessary for microperfuranone biosynthesis

Our data so far suggested that overexpression of a single gene, *micA*, was sufficient to elicit microperfuranone production. Since genes that are involved in secondary metabolite biosynthesis are generally clustered in *A. nidulans*, we wondered if genes proximal to *micA* participated in the biosynthesis of microperfuranone. RT-PCR analysis of the genes nearby *micA* confirmed that *micA* was actively transcribed in the induction condition (Fig. S7). Other genes nearby *micA*, such as AN3394.4 and AN3395.3, were also detected in the RT-PCR analyses. To determine if genes nearby *micA* were necessary for the biosynthesis microperfuranone, we created deletions of ten additional genes (AN3391.4-AN3395.4 and AN3397.4-AN3401.4) flanking *micA*. The deletions were accomplished using our previously reported strategy involving a *nkuA*Δ strain and fusion PCR (Chiang et al., 2008). The targeted genes were replaced with *the A. fumigatus pyrG* gene (Nayak et al., 2006). Metabolite analysis of the extracts from the ten deletion mutant strains revealed that all ten mutant strains continue to produce microperfuranone (data not shown). To confirm that only one single gene *micA* without any other accessory genes is sufficient for microperfuranone biosynthesis, we expressed *micA* in a heterologous host *A. niger*. We selected *A. niger* as a heterologous host because the *A. niger* secondary metabolome has been extensively studied and is not known to produce microperfuranone (Chiang et al., 2011b; Nielsen et al., 2009). We fused *micA* with the amylase promoter and transformed into *A. niger* (Kanemori et al., 1999). The amylase promoter can be induced by using maltose as a carbon source in the media and is inhibited by using fructose. We cultivated the *OE:micA A. niger* using maltose as a carbon source and as a control we also cultivated wild type *A. niger* as control. Using LC/MS we detected microperfuranone in *OE:micA A. niger* and not in the *A. niger* wild type control strain demonstrating that only *micA* is necessary for microperfuranone biosynthesis (Fig. 3).

Discussion

There are fourteen NRPS-like genes in the *A. nidulans* genome only the product of *tdiA* from the terrequinone A pathway has been characterized either genetically or biochemically. By replacing each of the promoters of the thirteen NRPS-like genes in *A. nidulans* with an inducible promoter we show that apparently one of the NRPS-like genes *micA* (AN3396.4) is sufficient for the biosynthesis of the metabolite microperfuranone. Replacement of the promoter of the other twelve NRPS-like genes in *A. nidulans* with the inducible promoter failed to produce metabolites suggesting that activation of additional genes in the genome are necessary for their production. Creation of double and other multiple promoter replacements strains in *A. nidulans* are currently underway and their results will be reported in due course.

From our genetic analysis and heterologous overexpression experiments, we propose a speculative but plausible mechanism for microperfuranone biosynthesis (Fig. 4). These steps include the activation of phenylpyruvic acid (PPA), a precursor available in both *A. nidulans* and *A. niger*, by the MicA A domain to AMP-phenylpyruvic acid followed by loading of the PPA unit to the T domain and eventually transferring to the TE domain. After loading another PPA unit onto the T domain, aldol condensation establishes the carbon-carbon bond between the α- and β-carbon of the two PPA units. The carbon-carbon bond formation by the TE domain is not unprecedented and has been demonstrated biochemically in terrequinone A biosynthesis by the Walsh group (Schneider et al., 2007). Sulfur assisted furan ring formation, TE domain mediated hydrolysis, decarboxylation, and keto-enol tautomerization, would generate microperfuranone attached to the T domain of MicA. Finally, microperfuranone is released by the TE domain and the catalytic cycle continues. Recently the biosynthesis pathway for furanone from the gram-negative bacterium *Ralstonia solanacearum*, a secondary metabolite sharing structural similarities to microperfuranone was characterized genetically and biochemically (Wackler et al., 2011). Our data suggested that microperfuranone is biosynthesized in *A. nidulans* using a similar pathway.

In summary, our studies demonstrated that we induced the expression of *micA*, a NRPS-like gene, stimulates the production of microperfuranone. To verify that indeed *micA* is sufficient, we overexpressed the gene in a heterologous host *A. niger* and enabled the production of microperfuranone. Our results confirm that only *micA* is necessary for microperfuranone biosynthesis in *A. nidulans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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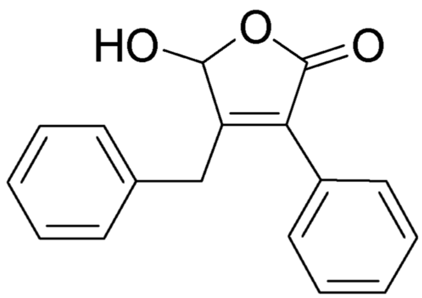

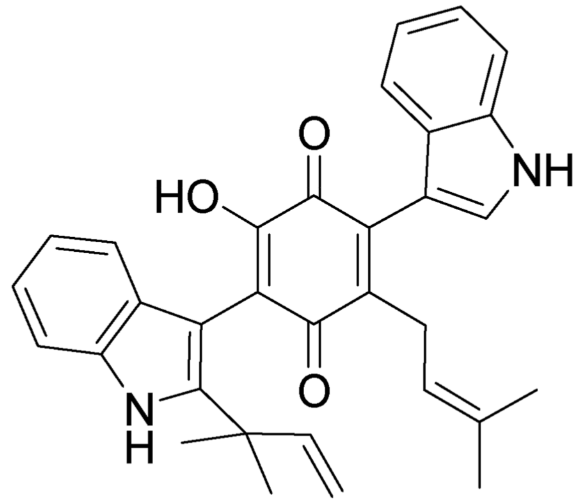

Microperfuranone (**1**)

Terrequinone A (**2**)

Fig. 1.
Chemical structures of microperfuranone and terrequinone A.

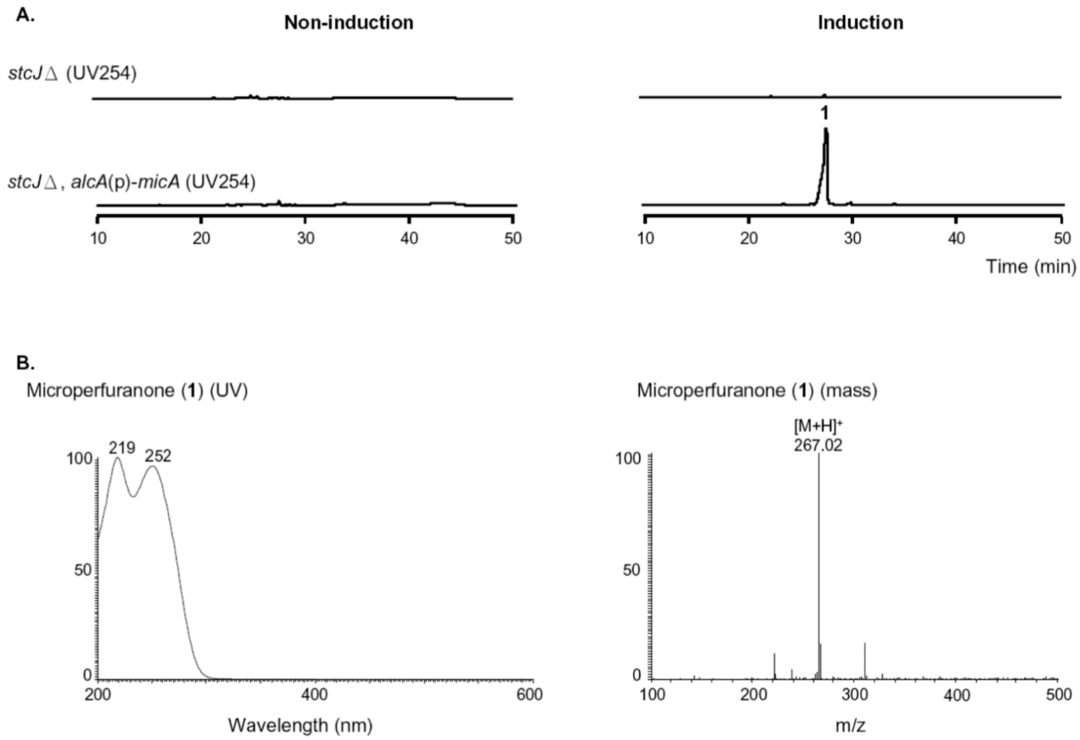

Fig. 2.
LC-DAD-MS Analysis of wild-Type *A. nidulans* and *alcA*(p)-*micA* mutant metabolites. (**a**) HPLC profiles of extracts as detected by UV absorption. (**b**) UV-Vis and ESIMS spectra (positive mode) of microperfuranone.

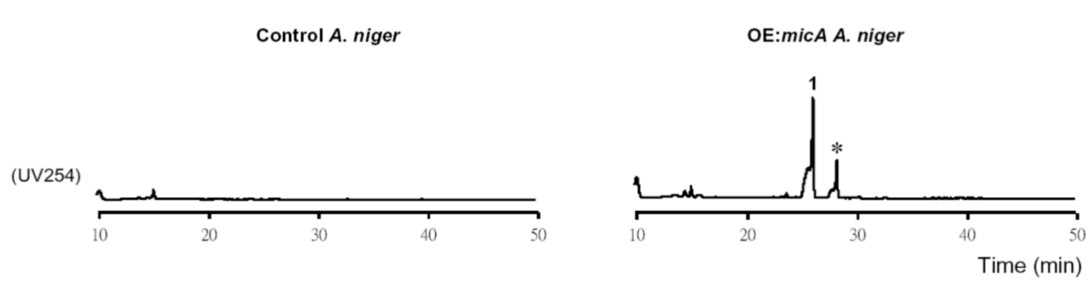

Fig. 3.
LC-DAD-MS Analysis of wild-Type *A. niger* and *amylase*(p)-*micA* (OE:*micA*) mutant metabolites. *A related compound that has similar MS/MS fragmentation with microperfuranone.

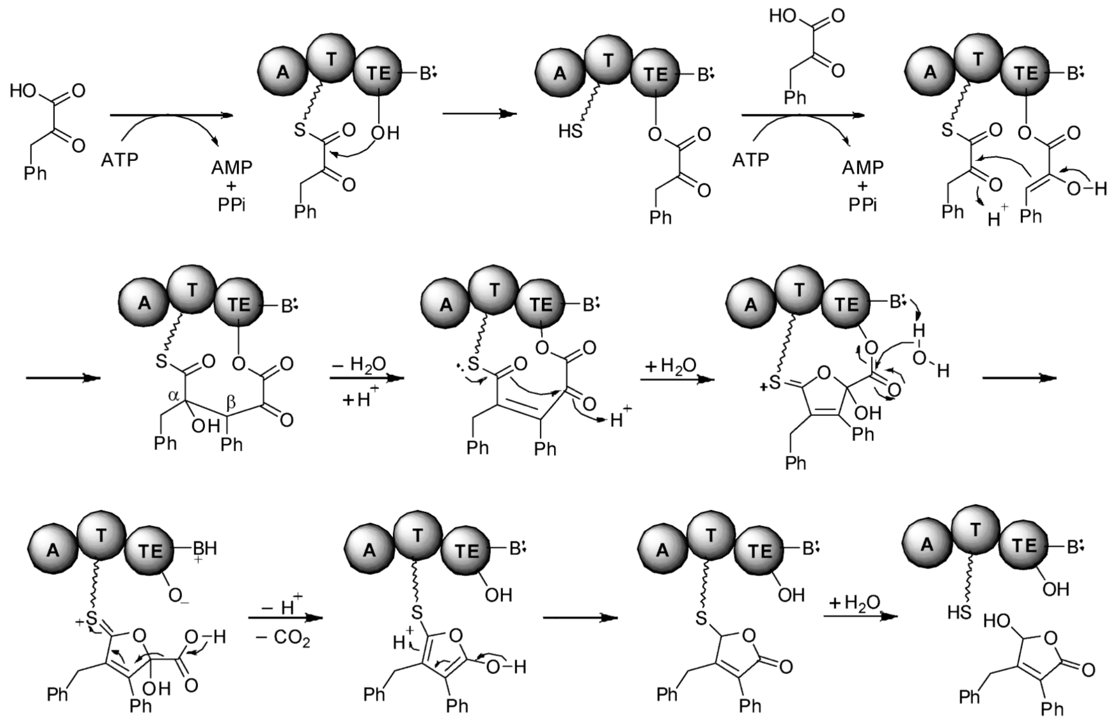

Fig. 4.
Proposed model of microperfuranone biosynthesis.

Table 1

Primers used in this study

Primer	Sequence (5′→3′)
alcA_AN1680.4P1	ATC TTA TGC ACT GGC CTT GG
alcA_AN1680.4P2	TTA AGC AAG GTC TCC GTC GTC
alcA_AN1680.4P3	CGA AGA GGG TGA AGA GCA TTG ACT GCC GTA ACG GCT CGG AG
alcA_AN1680.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG ATG GCG TCA CCA GCT G
alcA_AN1680.4P5	GCG TCG TGT GCA AGT AGA AAC
alcA_AN1680.4P6	TCC CGA AAC GAG GTC ATA AG
alcA_AN2064.4P1	CGC TTA CCT GCG TTC ACT TTC
alcA_AN2064.4P2	TAA TAG TGC CAC AGC GCA TC
alcA_AN2064.4P3	CGA AGA GGG TGA AGA GCA TTG CAG TTC GCC CCA CTG GGA TTC
alcA_AN2064.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG ACT TCT CCT GTG GGA AAC C
alcA_AN2064.4P5	TTC TCC TCG GCG GAT AAC TA
alcA_AN2064.4P6	GGG ATT ATC TGG ATG CTG GAC
alcA_AN2924.4P1	AAC TGC AAA CCA GCG AGA CT
alcA_AN2924.4P2	AGA GAC GCC TTT CCT TGT GA
alcA_AN2924.4P3	CGA AGA GGG TGA AGA GCA TTG TAT CGA GTT ACT GTG GCG TC
alcA_AN2924.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG TCT CGC CTC AAG GAA TC
alcA_AN2924.4P5	TGA CGC AGC CAC TAA ATA CG
alcA_AN2924.4P6	CAA GCC TTA CCA CCT CCG TA
alcA_AN3396.4P1	TAC ATC CAT AGC GGT GGT CAG
alcA_AN3396.4P2	GAC GAT GAG GCG TAT CTG G
alcA_AN3396.4P3	CGA AGA GGG TGA AGA GCA TTG CAG CAG CAG CAT CAG CAG G
alcA_AN3396.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG GTA GGC TCA GTG GTT GA
alcA_AN3396.4P5	AAT CAC TTC GGC TTG GAC AC
alcA_AN3396.4P6	AGT TAT GAA CCA GCC ATC CG
alcA_AN3495.4P1	TTG AAT AGC GGT ATC CTG GG
alcA_AN3495.4P2	CAT CGA ATA CAG CGA CTC CA
alcA_AN3495.4P3	CGA AGA GGG TGA AGA GCA TTG TCG CCT GTC GGC AGG TAT AC
alcA_AN3495.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG TCT CAC TCA ATG TCA TC
alcA_AN3495.4P5	CTC AAG TTC TGC AGC CCA AT
alcA_AN3495.4P6	AGA AGG CAG CTT CGA CTT TG
alcA_AN4827.4P1	CCT GTT CAG CTA TGC TGG GA
alcA_AN4827.4P2	CAA TAG CTG GCA ATC CCA GT
alcA_AN4827.4P3	CGA AGA GGG TGA AGA GCA TTG GCA CTA TTC TCA TAT GGT CCG
alcA_AN4827.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG CTG GCA CAA ATC GGC AG
alcA_AN4827.4P5	TCC ACT CCA CCT GGA ACT TC
alcA_AN4827.4P6	CAC AAG GTA ATC GCC CAA CT

Primer	Sequence (5′→3′)
alcA_AN5318.4P1	ATT GTG GCG ACA GGG ATT AG
alcA_AN5318.4P2	CGA TTT ACG GCC AGT TCA CG
alcA_AN5318.4P3	CGA AGA GGG TGA AGA GCA TTG AGA CGA GGA AGT TGC GAA AG
alcA_AN5318.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG GCC ATC ATT GAC ACC AC
alcA_AN5318.4P5	CAA CGC AGA GTT CAC CAG AA
alcA_AN5318.4P6	CAG TGC GGT ACA TGA CAG CT
alcA_AN6444.4P1	GAG GTG GTA GGT CAT CAG GT
alcA_AN6444.4P2	GGA CAG AGG CAT TGT TCC AT
alcA_AN6444.4P3	CGA AGA GGG TGA AGA GCA TTG CGT TCA GCT TGC GTC TAG CA
alcA_AN6444.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG GCT CGA AAC CAG CAA CT
alcA_AN6444.4P5	GAG GGA ACG GTC ATG AAA GA
alcA_AN6444.4P6	TCT TGA GGG ACG AAG ATC GG
alcA_AN8105.4P1	GGC CAG CAA ACT TTC AGT GT
alcA_AN8105.4P2	GAA AAG GAA GCA CAG CGT TC
alcA_AN8105.4P3	CGA AGA GGG TGA AGA GCA TTG GCG AAA CGA CTA GAA GAG AC
alcA_AN8105.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG CCT TGG AAA CCT CCA CC
alcA_AN8105.4P5	GGA GGT CTG AAT CGA CAA CG
alcA_AN8105.4P6	GCC TGG AAT GCC CAA ATG TG
alcA_AN8504.4P1	GTA CAA TGA TCG ACG GCC T
alcA_AN8504.4P2	CCC TAT TCT GCC TGG ATC A
alcA_AN8504.4P3	CGA AGA GGG TGA AGA GCA TTG AGA TCA TCG TAC CAT AGG CG
alcA_AN8504.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG ATT GTT GGT GAA AAG CC
alcA_AN8504.4P5	GGT AAC CAG TTG TCG ACG G
alcA_AN8504.4P6	GGT AAA GAT GGG AGT GCG A
alcA_AN9291.4P1	TCC TCC TGT CCA ACT CGA C
alcA_AN9291.4P2	CCA GAA TTC CTT TCG CTC TC
alcA_AN9291.4P3	CGA AGA GGG TGA AGA GCA TTG TGT GAT AGC CCA TCT GGA T
alcA_AN9291.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG ACT CCA GTA TCG CTC CG
alcA_AN9291.4P5	CTA AAC GGA TCT CGC GGT AA
alcA_AN9291.4P6	AGG TGG AAA GGG AGT CAG GT
alcA_AN10297.4P1	GGT CAG GAG TGG ATG TGT C
alcA_AN10297.4P2	CGC CAG TAT ACC CGA CAT TT
alcA_AN10297.4P3	CGA AGA GGG TGA AGA GCA TTG ATG GCA CGT CAT AAA GCG
alcA_AN10297.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG GGG AAC TCT CAG GAT AC
alcA_AN10297.4P5	GAC TGA CTC CGG CTT AGC A
alcA_AN10297.4P6	CCT GAT CGA AGA AGC CCT G
alcA_AN10486.4P1	ACA CGC TAC GAG GTC ATT CC
alcA_AN10486.4P2	CAA AGC AAG GCA CCC TTA TC
alcA_AN10486.4P3	CGA AGA GGG TGA AGA GCA TTG ACG TGA TCA GGA ATC CGG AC

Primer	Sequence (5′→3′)
alcA_AN10486.4P4	CCA ATC CTA TCA CCT CGC CTC AAA ATG TTG TCT ACC ATT CGC CCT C
alcA_AN10486.4P5	CCC ATT CTC AAC CAG CAA G
alcA_AN10486.4P6	CCA TCT TTT ATC GCC AGG AG
AN3396.amyF	AAC AAT AAA CCC CAC AGA AGG CAT TTA TGG TAG GCT CAG TGG TTG A
AN3396.amyR	GAG GAG CCT GAA TGT TGA GTG GAA TGA TGC GTT GCT ACC TAC GAT GAC
AN3396.ATG	CCG ATC AAT AGA CAT CTT CCG CAA ACA TGG TAG GCT CAG TGG TTG A
AN3396.TAA	GGG GGT ACA ACA CCA GCA TTA GTG GAC GTT GCT ACC TAC GAT GAC

The underlined sequences are tails that anneal to the *A. fumigatus pyroA* (AfpyroA) or *alcA* promoter fragment during fusion PCR.

Table 2

Aspergillus nidulans strains used in this study

Strain	Secondary metabolite mutations	Genotype	References
L02026	Δ *stcJ*	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ::AfriboB*	Bok et al., 2009
CW3006, CW3008, CW3010	*stcJ*Δ, *alcA*(p)-AN1680.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ::AfriboB; AN1680.4::AfpyroA-alcA*(p)-AN1680.4	This study
CW3011, CW3013, CW3015	*stcJ*Δ, *alcA*(p)-AN2064.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN2064.4::AfpyroA-alcA*(p)-AN2064.4	This study
CW3016, CW3017, CW3018	*stcJ*Δ, *alcA*(p)-AN2924.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN2924.4::AfpyroA-alcA*(p)-AN2924.4	This study
CW3021, CW3023, CW3025	*stcJ*Δ, *alcA*(p)-AN3396.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN3396.4::AfpyroA-alcA*(p)-AN3396.4	This study
CW3026, CW3027, CW3028	*stcJ*Δ, *alcA*(p)-AN3495.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN3495.4::AfpyroA-alcA*(p)-AN3495.4	This study
CW3032, CW3033	*stcJ*Δ, *alcA*(p)-AN4827.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN4827.4::AfpyroA-alcA*(p)-AN4827.4	This study
CW3036, CW3038, CW3040	*stcJ*Δ, *alcA*(p)-AN5318.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN5318.4::AfpyroA-alcA*(p)-AN5318.4	This study
CW3041, CW3042, CW3043	*stcJ*Δ, *alcA*(p)-AN6444.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN6444.4::AfpyroA-alcA*(p)-AN6444.4	This study
CW3046, CW3047, CW3048	*stcJ*Δ, *alcA*(p)-AN8105.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN8105.4::AfpyroA-alcA*(p)-AN8105.4	This study
CW3231, CW3232, CW3233	*stcJ*Δ, *alcA*(p)-AN8504.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN8504.4::AfpyroA-alcA*(p)- AN8504.4	This study
CW3066, CW3068	*stcJ*Δ, *alcA*(p)-AN9291.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN9291.4::AfpyroA-alcA*(p)- AN9291.4	This study
CW3056, CW3057, CW3058	*stcJ*Δ, *alcA*(p)-AN10297.4	*pyrG89;pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN10297.4::AfpyroA-alcA*(p)- AN10297.4	This study
CW3001, CW3003, CW3005	*stcJ*Δ, *alcA*(p)-AN10486.4	*pyrG89; pyroA4, nkuA::argB; riboB2, stcJ:: AfriboB; AN10486.4::AfpyroA-alcA*(p)- AN10486.4	This study