Deletion of the epigenetic regulator GcnE in *Aspergillus niger* FGSC A1279 activates the production of multiple polyketide metabolites

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ABSTRACT

Epigenetic modification is an important regulatory mechanism in the biosynthesis of secondary metabolites in *Aspergillus* species, which have been considered to be the treasure trove of new bioactive secondary metabolites. In this study, we reported that deletion of the epigenetic regulator *gcnE*, a histone acetyltransferase in the SAGA/ADA complex, resulted in the production of 12 polyketide secondary metabolites in *A. niger* FGSC A1279, which was previously not known to produce toxins or secondary metabolites. Chemical workup and structural elucidation by 1D/2D NMR and high resolution electrospray ionization mass (HR-ESIMS) yielded the novel compound nigerpyrone (**1**) and five known compounds: carbonarone A (**2**), pestalamide A (**3**), funalenone (**4**), aurasperone E (**5**), and aurasperone A (**6**). Based on chemical information and the literature, the biosynthetic gene clusters of funalenone (**4**), aurasperone E (**5**), and aurasperone A (**6**) were located on chromosomes of *A. niger* FGSC A1279. This study found that inactivation of GcnE activated the production of secondary metabolites in *A. niger*. The biosynthetic pathway for nigerpyrone and its derivatives was identified and characterized via gene knockout and complementation experiments. A biosynthetic model of this group of pyran-based fungal metabolites was proposed.

Keywords

Histone acetyltransferase GcnE, Secondary metabolite, Epigenetic regulator, Polyketide, Nigerpyrone

1. Introduction

The genus *Aspergillus* is a well-known producer of secondary metabolites (SMs) including polyketides, non-ribosomal peptides, indole terpenes and terpenes (Varga et al., 2003; Bok et al., 2006; Sanchez et al., 2012), which are important resources for new drug discovery (Rossano et al., 1999; Fisch et al., 2009). For example, bioinformatic analysis of four

available genomes of *A. niger* species (NRRL3, ATCC 9029) (Baker, 2006), ATCC1015 (Andersen et al., 2011), CBS513.88 (Pel et al., 2007), and SH2 (Yin et al., 2014), indicates that *A. niger* encodes at least 81 putative SM biosynthetic gene clusters (Inglis et al., 2013) for 42 polyketides (PKS), 33 non-ribosomal peptides (NRPS), four PKS-NRPS hybrids and two indole alkaloids. The large number of putative SM biosynthetic gene clusters suggests that *A. niger* has the potential to produce abundant SM products. However, most of these putative gene clusters are silent in standard laboratory cultivation conditions (Fisch et al., 2009), and only limited SM products have been characterized from *A. niger* (Tanaka et al., 1966; Hiort et al., 2004; Serra et al., 2005; Nielsen et al., 2009; Sorensen et al., 2009; Chiang et al., 2011).

Epigenetic modification is an important regulatory mechanism in the biosynthesis of SM products in *Aspergilli* (Fisch et al., 2009), which could activate SM gene clusters and induce new SM products by changing the chromosomal region of SM gene clusters from a heterochromatic to a euchromatic state via histone demethylation and acetylation (Bayram et al., 2008; Fisch et al., 2009). For example, 10 novel SM compounds are induced by adding the epigenetic modification reagent 5-azacytidine (5-AZA) to *A. niger* ATCC1015 cultures (Fisch et al., 2009).

Spt-Ada-Gcn5-acetyltransferase (SAGA/ADA) complex is an epigenetic regulator in fungal secondary metabolism (Wu and Yu, 2015). GcnE (the Gcn5 homolog in *Aspergilli*) is a histone acetyltransferase (HAT) in SAGA/ADA complex that might regulate secondary metabolism by histone modification (Baker and Grant, 2007; Brakhage, 2013). Previous work illustrated that GcnE participates in increasing the acetylation level of histone H3 lysine K9 in *Aspergilli* (Reyes-Dominguez et al., 2008), and affects the biosynthesis of secondary metabolites. For example, *A. flavus* Δ*gcnE* mutant does not produce aflatoxin (Lan et al.,

2016). Deletion of the *gcnE* gene in *A. nidulans* decreases the production of orsellinic acid, sterigmatocystin, penicillin and terrequinone (Nutzmann et al., 2011).

A. niger strain FGSC A1279 (*kusA*::DR-*amdS*-DR, *pyrG*$^{-}$), a derivative of industrial strain NRRL3 (ATCC9029) that is used for gluconic acid production (Baker, 2006; Yuan et al., 2008; Carvalho et al., 2010; Arentshorst et al., 2015), is an SM-silent strain and has zero level of SM production. The low background of SM production in FGSC A1279 makes it ideal for investigating the role of epigenetic regulators on SM production. Moreover, genome sequences of NRRL3 (ATCC 9029) (Baker, 2006) and FGSC A1279 (Wang et al., 2017) are available for detailed gene information of the host strain.

In this study, we reported that deletion of the epigenetic regulator *gcnE*, a histone acetyltransferase in the SAGA/ADA complex, resulted in the production of 12 polyketide secondary metabolites in *A. niger* FGSC A1279. Chemical workup and structural elucidation by 1D/2D NMR and high resolution electrospray ionization mass (HR-ESIMS) yielded the novel compound nigerpyrone (**1**) and five known compounds: carbonarone A (**2**), pestalamide A (**3**), funalenone (**4**), aurasperone E (**5**), and aurasperone A (**6**). Based on chemical information and literature searches, the biosynthetic gene clusters of funalenone (**4**), aurasperone E (**5**), and aurasperone A (**6**) were located on the chromosomes of *A. niger* FGSC A1279. This study showed that inactivation of GcnE activated the production of toxins and metabolites in *A. niger*. This is distinct from the role of GcnE in previous studies, suggesting that the role of GcnE may have diverse effects on regulating SMs among different *Aspergilli*. The biosynthetic pathway of nigerpyrone and its derivatives was identified via gene knockout and complementation experiments, and a biosynthetic model of this group of pyran-based fungal metabolites was proposed.

2. Material and methods

2.1. Strains and culture conditions

All strains used in this study are in Table S1. Strains were maintained on potato dextrose agar (PDA) medium (20 g dextrose, 15 g agar, infusion from 200 g potatoes per 1 L medium, pH6.0) at 30 °C for spore harvest. For genetic transformation, CD medium (0.3% $NaNO_3$, 0.2% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 2.0% glucose, pH5.5) was used. WATM, YES, and CYA media were used for SM profile analysis. WATM medium was composed of 2.0 g/l yeast extract, 3.0 g/l peptone, 2.0 g/l dextrose, 30.0 g/l sucrose, 5.0 g/l corn steep solids, 2.0 g/l $NaNO_3$, 1.0 g/l $K_2HPO_4 \cdot 3H_2O$, 0.5 g/l $MgSO_4$, 0.2 g/l KCl, 0.01 g/l $FeSO_4 \cdot 7H_2O$, pH7.0. YES medium was composed of 20 g/l yeast extract, 150 g/l sucrose, pH 6.0. CYA medium was prepared on the basis of CD medium by adding 5 g/l yeast extract and 1 ml/l trace metal solution (1% $ZnSO_4 \cdot 7H_2O$, 0.5% $CuSO_4 \cdot 5H_2O$). For solid cultivation, 15 g/l agar was added.

An *A. niger gcnE* deletion mutant (Δ*gcnE*) was constructed according to the method developed by Szewczyk *et al.* (Szewczyk et al., 2006), based on homologous recombination using a PCR amplified deletion cassette containing the upstream flanking arm (1.4 kb) of *gcnE*, the selection marker *pyrG* (orotidine-5'-phosphate decarboxylase) and the downstream flanking arm of *gcnE*. The *pyrG* gene was used for auxotrophic selection. Uridine prototrophic transformants were selected and confirmed by PCR amplification (Fig. S1). Primers for deletion cassette construction and transformant identification are listed in Table S2. When required, 10 mM uridine was added to medium to maintain auxotrophy. The SM backbone gene *epaA* was knocked out using the same method (Table S3).

2.2. Cultivation of A. niger ΔgcnE mutant for secondary metabolite collection

To collect secondary metabolites, the *A. niger* Δ*gcnE* mutant was cultivated for 7 days at 25 °C in the dark on 10 Petri dishes with WATM medium. Cultures were extracted with ethyl acetate (EtOAc) plus 1% formic acid for 24 hours. The crude extract was filtered and dried on a rotary evaporator at 38 °C.

2.3. SPE separation of A. niger ΔgcnE crude SM extract

A C18 solid-phase extraction (SPE) column (SPE C18-E Giga Tube 20 g/60 ml, Strata, Phenomenex, USA) was used to separate *A. niger* Δ*gcnE* crude extracts. The SPE stationary phase was conditioned by sequential washing with 60 ml 100% methanol and 240 ml 100% deionized water. *A. niger* Δ*gcnE* crude extract (4.0 g) was chromatographed with four subfractions (480 ml each) generated stepwise from deionized water to methanol: 25% methanol, 50% methanol, 100% methanol, and 100% methanol plus 0.1% trifluoroacetic acid (TFA). Subfraction 2 (50% methanol, 50 mg) and 3 (100% methanol, 410 mg) were further purified by semi-preparative HPLC (semi-prep HPLC).

2.4. Semi-prep HPLC fractionation

Semi-prep HPLC was used to purify subfractions obtained by SPE separation. A semi-prep HPLC column ACE C18-HL (250 mm × 10 mm i.d., Advanced Chromatography Technologies, UK) was connected to an Agilent 1200 series binary pump and monitored by an Agilent photodiode array detector. Detection was at 230, 254, 280, and 410 nm. Flow rate was 1.5-2.0 ml/min. Solvent A consists of 95% H_2O, 5% methanol, and 0.05% TFA. Solvent B was 100% acetonitrile. Separations were done at room temperature (20-25°C). Optimal separation conditions were determined using analytical systems and then conditions were adapted to the semi-preparative scale.

2.5. LC/HR-ESIMS assay of A. niger secondary metabolites

For LC/HR-ESIMS analysis, each semi-prep HPLC subfraction was dissolved in MeOH:Milli-Q H_2O (9:1), and centrifuged at 10,000 rpm for 10 min. LC/HR-ESIMS analysis was conducted as follows. High resolution mass spectrometric data were obtained using an LTQ XL/LTQ Orbitrap Discovery MS system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump, C18 SunFire 150 × 4.6 mm Waters). Conditions used were:

capillary voltage 45 V, capillary temperature 320°C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, and mass range 100-2000 amu (maximum resolution 30,000). For LC/HR-ESIMS, a Waters SunFire C18 analytical HPLC column (5 µm, 4.6 × 150 mm) was used with mobile phase of 0-100% MeOH over 30 min at a flow rate of 1 ml/min. LC/HR-ESIMS data were analyzed by Xcalibur software followed by dereplication using Antibase2012 database.

2.6. NMR characterization of A. niger secondary metabolites

NMR spectra were used to elucidate the structure of *A. niger* secondary metabolites. ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC spectra were recorded on a Bruker Daltonics Advance III HD 400 NMR spectrometer, using deuterated solvent methanol-d_4 (Cambridge Isotopes Laboratories, USA). Chemical shift values were reported in parts per million (ppm) with tetramethylsilane (TMS) as the external standard. Coupling constants were recorded in Hertz (Hz). When higher field was needed, NMR spectra were recorded on a Bruker Daltonics Advance III HD 600 NMR. NMR data were analyzed using MestReNova 9.0.1 software and ACD/I-Lab (http://ilab.cds.rsc.org/?cdsrdr=1).

2.7. Characterization of putative biosynthetic gene clusters for A. niger secondary metabolites by gene knockout

Gene knockout of putative cluster genes for identified *A. niger* secondary metabolites was done based on homologous recombination using a PCR-amplified deletion cassette containing the upstream and downstream flanking arms of the target gene and the *ptrA* (pyrithiamine (PT) resistance) selection marker. Primers for constructing deletion cassettes are listed in Table S3. 0.5 µg/ml pyrithiamine (PT) was used to screen transformants. The host strain for gene knockout was the *A. niger* FGSC A1279 Δ*gcnE* mutant. Gene deletion transformants were confirmed by PCR amplification using internal primers (Table S3 and Fig. S1).

3. Results and discussion

3.1. Chemical diversity of A. niger FGSC A1279 ΔgcnE mutant

To investigate the effect of *gcnE* on *A. niger* secondary metabolism, we constructed a Δ*gcnE* mutant and a gcnE complementation (*gcnE*-com) strain using homologous recombination (Fig. S1). Three types of solid media (WATM, CYA and YES) commonly used for fungal secondary metabolite production were chosen (Frisvad and Thrane, 1987; Rank et al., 2012) to investigate metabolite changes in the Δ*gcnE* mutant and *gcnE*-com strain. Crude extract from cultures was subjected to LC/HR-ESIMS analysis. No compounds were produced in *A. niger* FGSC A1279 cultures. Metabolite profiling by LC/HR-ESIMS analysis demonstrated that multiple SMs were produced in all of three Δ*gcnE* mutant cultures and the SM profile of *gcnE*-com strain was similar with the wild strain (Fig. 1), suggesting that *gcnE* deletion activated the synthesis of SM products in *A. niger*. It has been reported that, in *Aspergilli*, histone acetylation usually correlates with transcriptional activation and chromatin rearrangement (Baker and Grant, 2007; Reyes-Dominguez et al., 2008). The *gcnE* gene is an epigenetic regulator that is a key histone acetyltransferase in the SAGA/ADA complex. In primary metabolism, GcnE increases the acetylation level of the proline utilization cluster, as shown by chromatin immunoprecipitation in *A. nidulans* (Reyes-Dominguez et al., 2008). However, the role of GcnE might vary for different secondary metabolite gene clusters in fungi. For example, inactivation of *gcnE* abolishes the production of aflatoxin in *A. flavus* (Lan et al., 2016) and reduces the production of four known metabolites (orsellinic acid, sterigmatocystin, penicillin and terrequinone) in *A. nidulans*, suggesting that *gcnE* is required for transcription activation in these two *Aspergillus* strains (Nutzmann et al., 2011). However, recent genome-wide ChIP-seq experiments revealed that most SM cluster genes in *A. nidulans* are poorly decorated with activating heterochromatic marks (histone demethylation and acetylation), even under inducing conditions (Connolly et

al., 2013; Gacek-Matthews et al., 2016), implying that *gcnE* may not be required for transcriptional activation of certain SM gene clusters. Therefore, GcnE may have diverse effects on regulating SMs among different *Aspergilli*. This result was in accordance with the effects of histone deacetylase HdaA in *A. nidulans*. Deletion of HdaA induces the biosynthesis of sterigmatocystin, penicillin, and norsolorinic acid in *A. nidulans*, but does not change the expression of terraquinone A (Shwab et al., 2007). A similar phenomenon was found in the plant pathogen *Fusarium graminaerum*. Deletion of heterochromatin protein 1 (*hep1*) in *F. graminaerum* leads to the activation of the aurofusarin gene cluster while repressed the deoxynivalenol cluster (Gacek and Strauss, 2012).

3.2. Dereplication of newly produced compounds in A. niger ΔgcnE mutant

To further analyze the chemical diversity in the Δ*gcnE* mutant, we used WATM medium since more SM products are likely to be produced on this medium (Fig. 1). Dereplication using the Antibase database (H, 2012) led to the identification of 11 SM products in the *A. niger* Δ*gcnE* mutant (Fig. 2 and Table S4), all of which were known fungal metabolites, including 9 compounds discovered in *Aspergilli*. The ion peak with t_R (7.84) min was likely to be carbonarone A, originally isolated from the fungal strain *A. carbonarius* WZ-4-11 (Zhang et al., 2007). Funalenone (t_R = 8.42 min) belongs to polyketide compound group of phenalenones which have diverse structures and biological activities (Gao et al., 2016). The biosynthetic gene cluster of funalenones has been fully characterized (Gao et al., 2016). Pestalamide A may have been eluted at t_R (10.10 min), which was isolated from fungal strain *Pestalotiopsis theae* W148 (Ding et al., 2008). The highly toxic metabolites fumonisin B2 (t_R = 10.94 min) and B4 (t_R = 11.92 min) were detected in the *A. niger* Δ*gcnE* mutant. *A. niger* NRRL3 (ATCC9029), the starting strain of FGSC A1279, is generally regarded as safe (GRAS) in the industry and should not produce any toxins or toxic compounds. This is particularly true for *A. niger* FGSC A1279 which is used for gluconic acid production (Baker,

2006). These results indicate that deletion of *gcnE* had an epigenetic impact on its SM production. Five polyketide derivatives (dimeric naphthopyrone family), aurasperone E (t_R = 13.70 min), fonsecinone B (t_R = 14.50 min), isoaurasperone A (t_R = 14.77 min), aurasperone A (t_R = 15.66 min) and asperpyrone C (t_R = 16.33 min) were also found in the extracts of the *A. niger* Δ*gcnE* mutant (Fig. 2). Taken together, these results indicated that the newly identified compounds were likely to be polyketide metabolites.

3.3. Structural elucidation by HR-ESIMS and 1D/2D NMR

To verify the identity of the newly emerged metabolites in the Δ*gcnE* mutant, the strain was cultivated on WATM medium at 2-L scale and crude extract was subjected to chemical workup and semi-prep HPLC purification, yielding six pure compounds **1** (3.7 mg), **2** (1.8 mg), **3** (7.8 mg), **4** (9.2 mg), **5** (1.5 mg), and **6** (3.0 mg).

The molecular formula of nigerpyrone (**1**) was established as $C_{12}H_{10}O_2$ by HR-ESIMS (187.07 $[M+H]^+$). Dereplication using the Antibase database suggested that this might be a new compound. The molecular formula suggested eight degrees of unsaturation. The ^{13}C and HSQC NMR data of **1** in CD_3OD (Fig. S2) revealed one benzene/aromatic ring (δ_C 128.38-136.30 ppm), one carbonyl (δ_C 178.14 ppm), and four olefinic carbons (δ_C 117.36-117.52 ppm and 158.45-169.48 ppm), consistent with the presence of five double bonds and one ring system. This analysis accounted for seven double bonds, suggesting the presence of an additional ring in the structure of **1**. Analysis of the ^1H-^1H COSY spectrum revealed two contiguous spin systems, one consisting of H-10 through H-14, and the other consisting of H-2 to H-3 (Fig. 3 and Fig. S2). The connection of these two spin systems at C-6 was evident by the HMBC correlation of H-5 and H-8 to C-6 (Fig. 3), which was further confirmed by the correlation of H-8 to C-5 in the HMBC spectrum. The connection of methylene to the benzene ring was confirmed by the HMBC correlation of H-8 to C-9 (Fig. 3). The structure was further confirmed by comparing ^{13}C experimental data with published data (Dai et al.,

2007) and predicted ^{13}C NMR data (Elyashberg et al., 2010) (Fig. S2). The resulting r^2 value of 0.9992 indicated that the proposed structure was correct (Bremser, 1978; Elyashberg et al., 2010). Inspection of the ^{1}H, ^{13}C, and HR-ESIMS data of compounds **2** and **3** (Fig. S3 and S4) indicated that both **2** and **3** were known compounds (Zhang et al., 2007; Ding et al., 2008): carbonarone A (**2**) was isolated from *A. carbonarius* WZ-4-11 (Zhang et al., 2007) and pestalamide A (**3**) was isolated from *Pestalotiopsis theae* W148 (Ding et al., 2008). Comparison of ^{1}H and ^{13}C NMR spectra of compounds **1**, **2** and **3** (Fig. S2, S3 and S4) confirmed the structure of the new compound **1**.

Inspection of the ^{1}H, ^{13}C and HR-ESIMS data of compounds **4** (Fig. S5), **5** (Fig. S6) and **6** (Fig. S7), indicated that they were known. Compound 4 is produced by *A. niger* FO-5904) (Inokoshi et al., 1999), 5 by *A. niger* CMI-IMI 205879 (Priestap, 1984), and 6 by *A. fonsecaeus* NRRL 67, O 16-1 (Priestap, 1984).

3.4. Putative biosynthetic gene clusters of compounds from the *ΔgcnE mutant*

Homologous BLAST search and comparison of the literature allowed the identification of the putative biosynthetic gene clusters of known compounds discovered from the Δ*gcnE* mutant (Table S5). Genes for synthesizing funalenone (**4**) were identified in *Penicillium herquei* (Gao et al., 2016). By homologous search against the annotated genome of *A. niger* CBS513.88, we identified five homologous genes in *A. niger* responsible for the biosynthesis of funalenone (Fig. S8 and Table S5). The literature (Chiang et al., 2010) indicated that monodictyphenone might be the precursor of aurasperone E (**5**), aurasperone A (**6**) and other derivatives such as fonsecinone B, isoaurasperone A, and asperpyrone C. In *A. nidulans*, the key biosynthetic enzyme of monodictyphenone is proposed to be a non-reducing polyketide synthase (NR-PKS) *mdpG* (Chiang et al., 2010). We therefore performed a homologous search of the *A. niger* genome using *mdpG* as the reference query, and identified an open reading frame (ORF) An11g07310 located in contig 11, annotated as a NR-PKS with

multiple domains of KS-AT-PT-PP (Table S5). Other proposed biosynthetic genes involved in the biosynthesis of **5** and **6** were not located in the close proximity of An11g07310 and were scattered in other genomic loci. However, genes for secondary metabolites are generally tightly clustered.

3.5. Characterization of key biosynthetic genes for pestalamide A

Inspection of the structures of **1**-**3** led to speculation that their precursors contain one phenylacetate and two acetate units, suggesting that compounds **1**-**3** may be synthesized via a polyketide biosynthetic pathway. The incorporation of phenylacetate in fungal secondary metabolism is also observed in the biosynthesis of the β-lactam antibiotic penicillin G in *Penicillium chrysogenum* (Koetsier et al., 2009). *In vitro* assays show that the gene product PhlB is a phenylacetyl-CoA ligase responsible for the activation of phenylacetate into phenylacetyl-SCoA (Koetsier et al., 2009). We performed a homolog search of PhlB in *A. niger*, and identified ORF An09g01820, annotated as a ferulate:CoA ligase (AMP-forming) (Fig. 4A and Table S6), with moderate sequence identity (35%) to PhlB.

Analysis of genes in proximity to An09g01820 (*epaB*) identified a candidate NR-PKS gene cluster (*epa*) spanning a 23.9-kb genomic region (Fig. 4A and Table S6). The *epa* cluster possesses six ORFs (Table S6). *In silico* analysis indicated that *epaA* (An09g01860) encodes a typical Clade III NR-PKS (2,617 amino acids in length) with a domain organization of SAT-KS-AT-PT-ACP-Met-R domains, which shares high amino acid sequence identity (97%) with AzaA polyketide synthase (2,599 amino acids in length) in the biosynthesis of azaphilones from *A. niger* ATCC 1015 (Zabala et al., 2012). The gene product EpaC is annotated as an acyl-CoA transferase. Adjacent to *epaA*, there are three genes, orf1-3, encoding oxidoreductase, 3-hydroxybenzoate 4-hydroxylase, and salicylate hydroxylase (Table S6).

To determine if the *epa* cluster was responsible for synthesizing pestalamide A (**3**), we knocked out the PKS gene (An09g01860, *epaA*) by homologous recombination (Table S3 and Fig. S1). Inactivation of the *epaA* gene abolished the production of **1**-**3** (Fig. 4B), and complementation strain (*epaA*-com) could produce compound **1**-**3** (Fig. S1 and Fig. 4B), suggesting that the identified biosynthetic genes were responsible for the synthesis of **1**-**3**. We also performed cell-free extract experiments, in which all biosynthetic enzymes and cofactors were available for the biosynthesis of **3**, to test if **1** and **2** were the biosynthetic precursors of **3** (data not shown). Feeding purified **1** and **2** into cell-free extracts of the Δ*gcnE* mutant did not produce **3**, suggesting that **1** and **2** were by-products in the biosynthesis of **3**. Based on the genetic and cell-free extract experiments, a biosynthetic model for **1**-**3** is proposed in Fig. 4C. We propose that the biosynthesis of **1**-**3** begins with the polyketide assembly by EpaA to form phenylacetyl triketide precursor from successive condensation of two malonyl-CoA, presumably with one phenylacetyl-CoA starter unit. For the nigerpyrone (**1**) biosynthesis, the reactive polyketide chain is released as an aldehyde (**8**) through the R-domain, in a manner similar to the previously characterized 3-methylorcinaldehyde synthase in *Acremonium strictum* (Bailey et al., 2007). The cyclization and dehydration of **8** may create nigerpyrone (**1)**. This nonenzymatic rearrangement to form pyrone moiety from aldehyde was also observed in the plant metabolites, arabidopyl derivatives (Weng et al., 2012). For the biosynthesis of **2** and **3**, an extra methyl group will be added through the C-methyltransferase domain, followed by the reduction to generate **8a**, which undergoes oxidation and transamination to produce the intermediate **9**. The candidate gene products for this series of biotransformation could be orf1-3 (Table S6). The cyclization of **9** produces carbonarone A (**2**). We propose that EpaC (An09g01800), an acyl-CoA transferase, could catalyse the transfer of 2-methylsuccinyl-CoA, a common intermediate in the ethylmalonyl-CoA pathway (Erb et al., 2009), to generate the final product pestalamide A (**3**).

4. Conclusions

In conclusion, we confirmed that deletion of *gcn*E resulted in the production of 12 polyketide metabolites in an *A. niger* FGSC A1279 Δ*gcnE* mutant. Chemical workup of organic extracts of the culture broth from the Δ*gcnE* mutant identified six pure compounds, including one new compound nigerpyrone (**1**) and five known fungal polyketide metabolites (**2**-**6**). The structures of these compounds were confirmed by HR-ESIMS and 1D/2D NMR. Finally, the biosynthetic gene cluster for **1**-**3** was verified via gene knockout and complementation, and a biosynthetic model of this group of pyran-based fungal metabolites was proposed. To our best knowledge, this was the first time to confirm that the inactivation of *gcn*E resulted in activating the biosynthesis of polyketide metabolites in *A. niger* FGSC A1279.

Declarations of interest

None.

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Figures

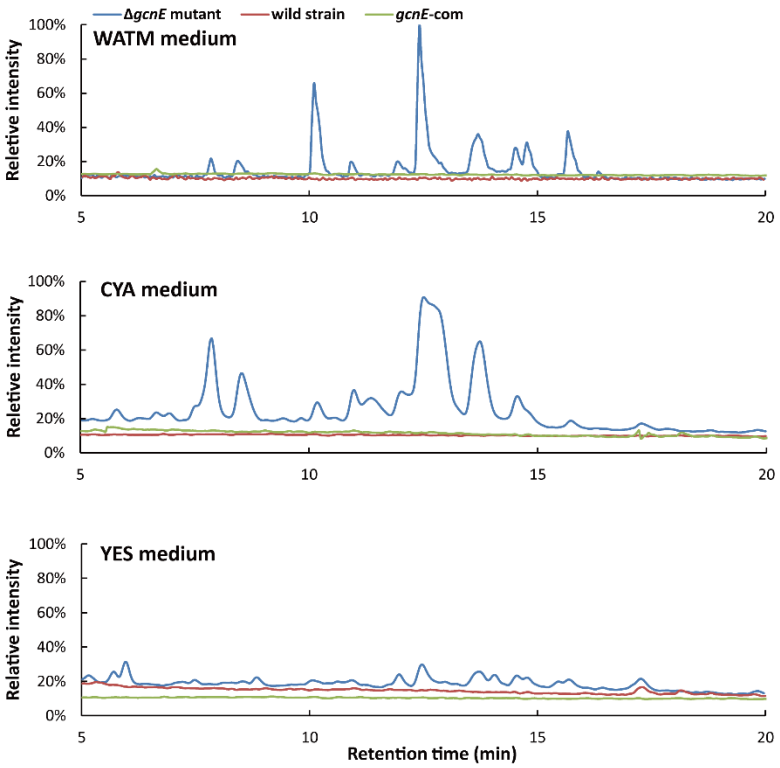

Figure 1. SM profile of *A. niger* FGSC A1279 Δ*gcnE* mutant cultivated on different media. Blue curve, Δ*gcnE* mutant; red curve, wild strain; green curve, *gcnE* complementation strain (*gcnE*-com). To collect secondary metabolites, *A. niger* strains were cultivated for 7 days at 25 °C in the dark using different media. The crude extract was dissolved in MeOH:Milli-Q H_2O (9:1) and centrifuged at 10,000 rpm for 10 min, before LC/MS analysis.

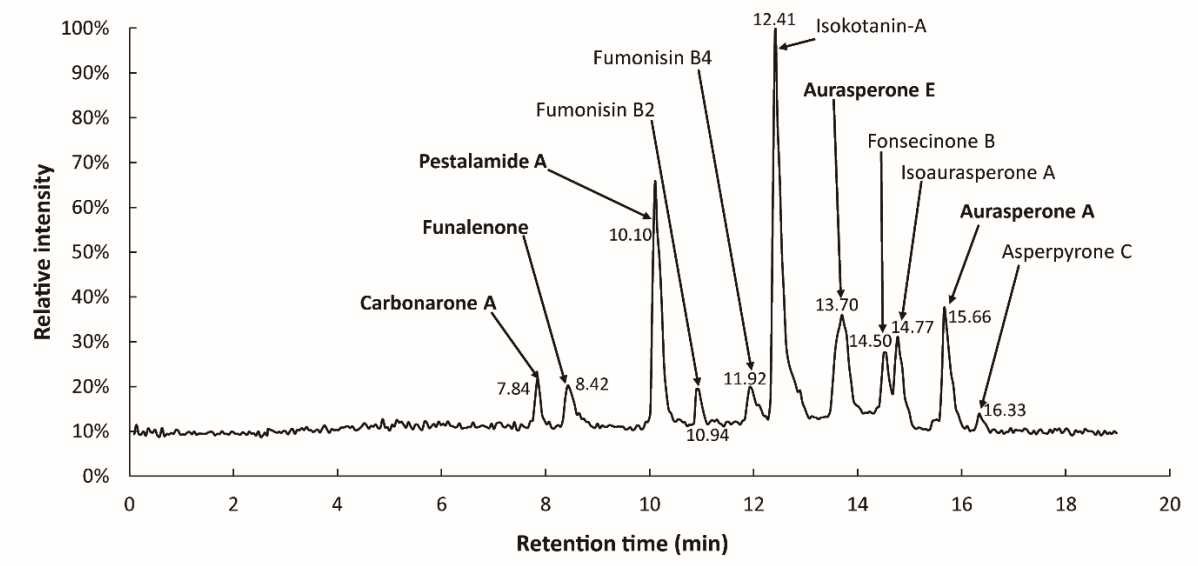

Figure 2. SM products detected in *A. niger* Δ*gcnE* mutant cultivated on WATM medium. *A. niger* ΔgcnE mutant was cultivated for 7 days at 25 °C in the dark on WATM medium. High resolution mass spectrometric data were obtained using an LTQ XL/LTQ Orbitrap Discovery MS system coupled to a Thermo Instruments HPLC system. LC/MS data were analyzed by Xcalibur software with Antibase2012 database. Compounds that were purified by chemical workup and characterized by spectroscopic analysis were marked in bold.

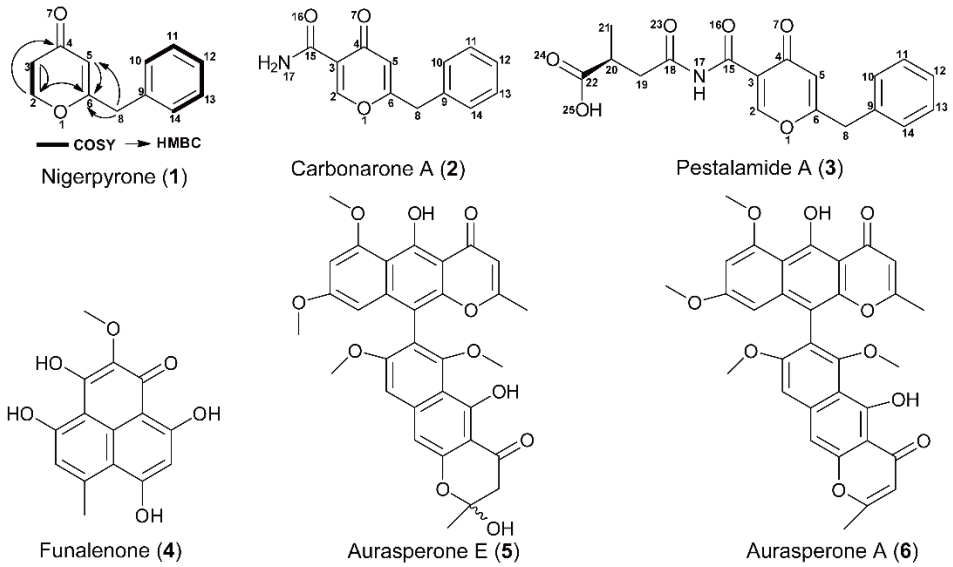

Figure 3. Secondary metabolites isolated and characterized by HR-ESIMS and 1D/2D NMR in *A. niger* Δ*gcnE* mutant. *A. niger* ΔgcnE mutant was cultivated on WATM medium. ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC spectra were recorded on a Bruker Daltonics

Advance III HD 400 NMR spectrometer, using deuterated solvent methanol-d_4. Chemical shift values were reported in parts per million (ppm) with tetramethylsilane (TMS) as the external standard. When higher field was needed, NMR spectra were recorded on a Bruker Daltonics Advance III HD 600 NMR. NMR data were analyzed using MestReNova 9.0.1 software and ACD/I-Lab (http://ilab.cds.rsc.org/?cdsrdr=1). Compound structures were drawn using ChemBioDraw software. Chemical formulas and exact masses are marked for each compound. Dereplication data are in online supplemental file (Fig. S2-S7).

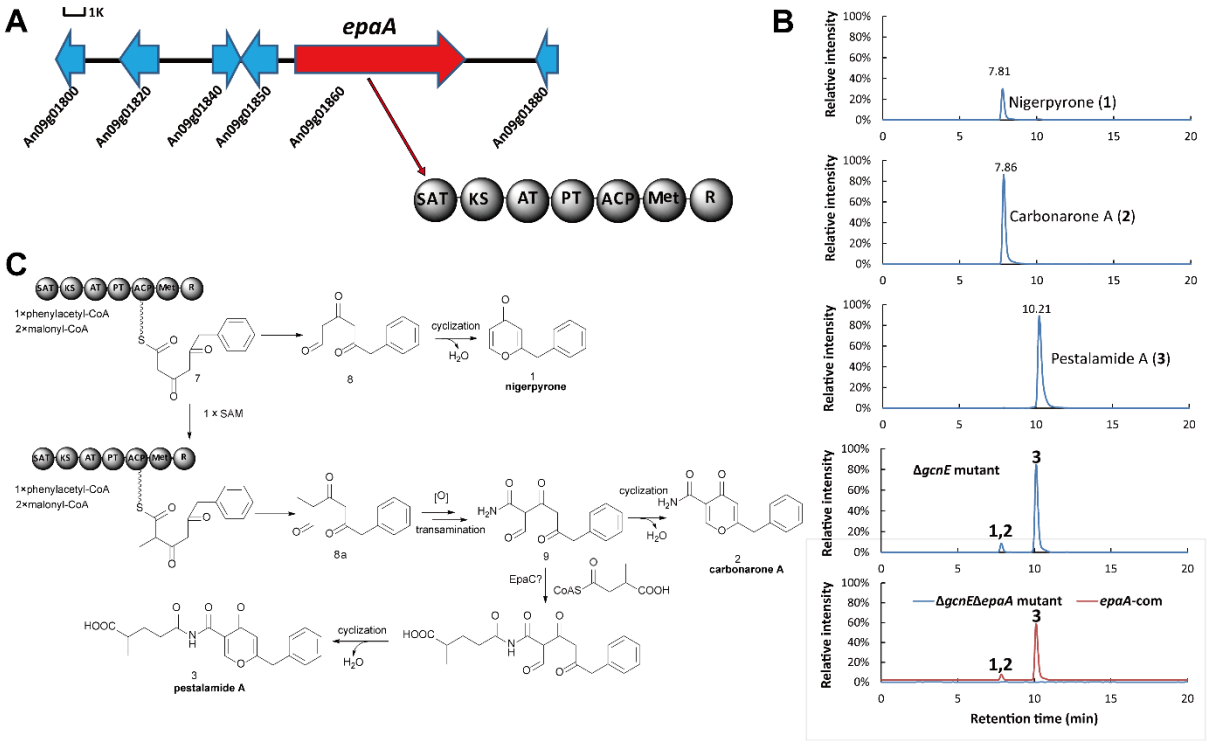

Figure 4. Characterization of the biosynthetic cluster for pestalamide A. (A) Putative gene cluster of compounds **1-3**, which was identified by the homologous BLAST search of *P. chrysogenum* PhlB gene. (B) LC/MS detection of compounds **1-3** in gene knockout and complementation strains. Retention time for purified compounds **1-3 was** determined by LC/MS. *A. niger* strains were cultivated on WATM medium. (C) Proposed model of the biosynthesis of nigerpyrone (**1**), carbonarone A (**2**) and pestalamide A (**3**).

Supplemental materials

Table S1. Strains and plasmids used in this study

Table S2. Primers used for *gcnE* deletion-cassette construction and transformant identification

Table S3. Primers used to knock out *epaA* (An09g01860) and verify mutants

Table S4. Putative compounds detected in *A. niger* FGSC A1279 Δ*gcnE* mutant based on LC/MS data and Antibase database

Table S5. Putative biosynthetic gene clusters for known compounds detected in the Δ*gcnE* mutant

Table S6. Putative biosynthetic gene cluster for pestalamide A.

Figure S1. Design of deletion cassettes, complementation cassettes, and PCR verification of *A. niger* mutants (Δ*gcnE*, Δ*gcnE*Δ*epaA*, *gcnE* complementation and *epaA* complementation).

Figure S2. LC/MS and NMR data for the novel compound nigerpyrone

Figure S3. LC/MS and NMR data of compound carbonarone A

Figure S4. LC/MS and NMR data of compound pestalamide A

Figure S5. LC/MS and NMR data of compound funalenone

Figure S6. LC/MS and NMR data of compound aurasperone E

Figure S7. LC/MS and NMR data of compound aurasperone A

Figure S8. Putative biosynthetic gene clusters of the known compounds in the Δ*gcnE* mutant