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# Targeted Disruption of Transcriptional Regulators in *Chaetomium globosum* Activates Biosynthetic Pathways and Reveals Transcriptional Regulator-Like Behavior of Aureonitol

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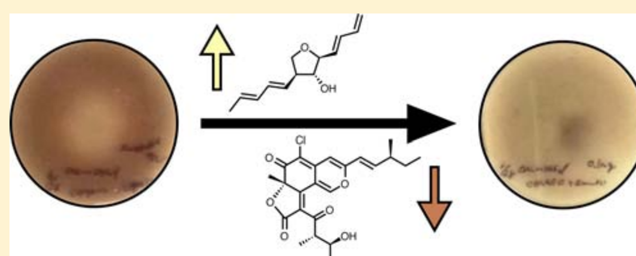
Ⓢ *Supporting Information*

**ABSTRACT:** Postgenomic analysis revealed that many microorganisms carry numerous secondary metabolite biosynthetic genes on their genome. However, activities of those putative genes are not clearly reflected in the metabolic profile of the microorganisms, especially in fungi. A recent genome mining effort is promising in discovering new natural products. However, many fungi and other organisms are not amenable to molecular genetics manipulations, making the study difficult. Here we report successful engineering of *Chaetomium globosum*, a known producer of various valuable natural products, that allows its genetic manipulation via targeted homologous recombination. This strain permitted us to abolish transcriptional regulators associated with epigenetic silencing of secondary metabolite biosynthetic pathways, leading to the identification of the products generated by different gene clusters and isolation of novel secondary metabolites. We were able to identify six gene clusters that are responsible for the biosynthesis of 11 natural products previously known to be produced by *C. globosum*, including one cytochalasan and six azaphilone-type compounds. In addition, we isolated two new compounds, mollipilin A and B, that were only recently identified in a related *Chaetomium* species. Furthermore, our investigation into the mechanism of biosynthesis of those natural products in *C. globosum* also led to the discovery of a secondary metabolite, aureonitol, that acts like a transcriptional regulator for the biosynthesis of other secondary metabolites. Similar approaches should facilitate exploration of the untapped potential of fungal biosynthetic capability and identification of various unique biological functions that those secondary metabolites possess.




## ■ INTRODUCTION

With the growing global threat of multidrug-resistant microbes and increasing economical and environmental concerns about synthetic production of natural products and their analogs, there is an increasing interest in developing alternate platforms for discovery, development, and production of pharmaceutically valuable compounds.[1−4] To date, many biosynthetic gene clusters encoding enzymes responsible for the biosynthesis of secondary metabolites, such as polyketides (PKs), nonribosomal peptides (NRPs), and mixed PK−NRPs, have been found in various different organisms, especially fungi,[5,6] through the recent genome and metagenome sequencing efforts.[7] However, many organisms are difficult to culture,[8] and others produce natural products at very low level or maintain their gene clusters silent under conventional culture conditions.[9] This results in a significant underrepresentation of the biosynthetic potential of those fungi in the panel of fungal PKs and NRPs that can be isolated from fungal culture grown under typical growth conditions.[10] One way to circumvent this problem is to use a fungal heterologous expression system where desired secondary metabolites are produced by transplanting the target gene cluster into a more easily culturable fungal strain, such as *Aspergillus nidulans*.[11] The difficulty of using this method lies in the frequent incompatibility in the transcriptional regulation and splicing control employed by different fungal species. Another way to exploit the potential of fungi and their genomic information for drug discovery and development is to establish a heterologous production system using a convenient nonfungal host organism capable of expressing exogenous genes and biosynthesizing desired compounds.[9] Our most recent studies successfully demonstrated the reconstitution of full-length PK synthase (PKS) and NRP synthetase (NRPS) cDNAs isolated from an important fungus, *Chaetomium globosum,* by using an overlap extension PCR-yeast homologous recombination (ExRec) method in *Saccharomyces cerevisiae* and biosynthesis of some PKs and NRPs in the engineered yeast.[5] However, these heterologous expression studies examined only a single PK synthase or NRP synthetase gene from a biosynthetic gene cluster, thereby

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precluding the production of mature natural products. Considering these drawbacks, it would be advantageous if mining for new natural products can be accomplished by activating silent biosynthetic gene clusters as a whole in the chromosomal DNA of the original fungal host. There are two approaches available for doing so using molecular biological techniques. One approach is to knock out or overexpress a transcriptional regulator (TR) associated with a silent biosynthetic gene cluster.[1,6,12] When a TR is located away from the gene cluster, such a TR can repress the production of its associated natural product. For example, An et al. reported that deletion of a siderophore biosynthetic gene cluster regulator *urbs1*, which is located remotely from its associated *sid1*−*sid2* siderophore biosynthetic gene cluster, upregulated the expression of the *sid1*−*sid2* genes.[13] On the other hand, overexpression of a TR located within a gene cluster can enhance the biosynthesis of its respective secondary metabolites. Lack of expression of this class of regulators is often the cause of low expression level of biosynthetic genes and hence less-than-desirable production of the corresponding natural product.[5,6] In our recent study, we demonstrated that overexpression of a TR encoded within silent putative polyketide biosynthetic gene clusters in *Aspergillus oryzae* and *C. globosum* could activate the gene clusters in a relatively straightforward fashion to obtain natural products and help broaden our knowledge of mechanisms of natural product biosynthesis.[5,6] However, these methods are not applicable when no putative TR that is associated with a biosynthetic gene cluster can be found. Even if a TR can be identified within a cluster, it may not regulate the transcription of all of the genes in the cluster for the biosynthesis of the corresponding mature natural product.

An alternate approach is to decrease or increase epigenetic regulation to alter the control exerted over the biosynthetic gene clusters. For secondary metabolites, decreasing or increasing epigenetic regulation can increase transcription from a biosynthetic gene cluster at the chromatin level by reducing or inducing modifications of histones. For example, histone methylation leads to heterochromatin formation and gene silencing, whereas histone acetylation activates gene transcription.[14−16] By artificially hindering the modification of histones through deletion of these histone-modifying enzymes or the use of chemical inducers or inhibitors, expression of silenced gene clusters in different fungi was enhanced to allow production of secondary metabolites.[2,14,17] The advantage of this approach is its simplicity and effectiveness in activating silent gene clusters in fungi, requiring as little as a single gene deletion. Based on *in silico* analysis of the genome sequence, *C. globosum* has a high potential to produce diverse secondary metabolites. However, because of the innate high non-homologous random recombination activity against foreign DNA molecules, *C. globosum* remained resilient to conventional molecular genetic methodology. The inability to modify *C. globosum* genetically made detailed studies of this fungus difficult thus far. In this study, we engineered a *C. globosum* strain that is amenable to genetic manipulation and looked for the chemical diversity of secondary metabolites generated by mutants of *C. globosum* carrying targeted disruption of epigenetic regulators. Through the study, we were able to not only assign known compounds to previously uncharacterized biosynthetic gene clusters but also isolate new compounds and discover an unexpected transcriptional regulatory function of a natural product.

## ■ MATERIALS AND METHODS

**Strains and General Techniques for DNA Manipulation.** The wild-type *C. globosum* was obtained from National Institution of Technology and Evaluation Biological Resource Center in Japan. *Escherichia coli* XL1-Blue (Stratagene) and *E. coli* TOP10 (Life Technologies) were used for plasmid propagation by standard procedures. Overproduction of recombinant proteins was carried out in *E. coli* BL21 (DE3) (Life Technologies). DNA restriction enzymes were used as recommended by the manufacturer (Fermentas). PCR was carried out using KOD-Plus-Neo (TOYOBO) and PrimeSTAR GXL DNA polymerase (TAKARA Bio Inc.) as recommended by the manufacturers. Sequences of PCR products were confirmed through DNA sequencing (Macrogen Japan Corporation). *Saccharomyces cerevisiae* BY4741 was used for homologous recombination-based molecular cloning of genes and plasmid assembly. PCR was performed with the *C. globosum* genomic DNA as a template to amplify the target genes using the listed primer sets (see Supporting Methods and Table S5, Supporting Information).

**Transformation and Cultivation for Production of Secondary Metabolites.** A mutant *C. globosum* strain was initially cultured on oatmeal agar plates at 30 °C for 14 days. Approximately (1−4) × $10^6$ sexual spores, or ascospores, collected from a single plate were used to inoculate 200 mL of MYG medium (10 g/L malt extract, 4 g/L glucose, and 4 g/L yeast extract), which was shaken for 16 h at 30 °C. Grown cells were collected by centrifugation and washed with 0.8 M sodium chloride. The cells were incubated with 1 mL of 10 mM sodium phosphate buffer (pH 8.0) containing 0.8 M sodium chloride, 50 mg/mL lysing enzyme (Sigma-Aldrich), and 1500 units of *β*-glucuronidase at room temperature for 3 h. The resulting protoplasts were filtered and subsequently centrifuged at 1500*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. The cells were suspended in 200 *μ*L of STC buffer at pH 8.0 (0.8 M sorbitol, 10 mM calcium chloride, and 10 mM Tris-HCl). Then 40 *μ*L of PEG solution at pH 8.0 (400 mg/mL poly (ethylene glycol) 8000, 50 mM calcium chloride, and 10 mM Tris-HCl) was added to the protoplast suspension. The mixture was subsequently combined with 4 *μ*g of the DNA fragment with which the cells were to be transformed. The mixture was incubated on ice for 20 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 5 min. The resulting cells were plated on MYG−sorbitol agar medium (MYG medium with 15 g/L agar and 0.8 M sorbitol) with a suitable selection agent. Specifically, to screen for carboxin resistance, the DNA−protoplast mixture was plated on an MYG−sorbitol agar medium supplemented with carboxin at a concentration of 25 *μ*g/mL and incubated at 30 °C. To select for hygromycin resistance, the DNA−protoplast mixture was plated initially on an MYG−sorbitol agar medium without marker antibiotics. After incubating the plate at 30 °C for 5 h, the cells were overlaid with an MYG−sorbitol agar medium supplemented with 1.2 mg/mL hygromycin to perform the selection. To prepare for a protoplast-mediated transformation of strains with Cg*pyrG* deficiency, cells were plated on RM agar medium (2 g/L aspartic acid, 1.5 g/L ammonium chloride, 0.12 g/L magnesium sulfate, 1.4 g/L potassium phosphate monobasic, 1.5 g/L sodium phosphate dibasic, 3 g/L sodium sulfate, 5 g/L glucose, 171.2 g/L sucrose, 5 g/L potato starch, 1 *μ*g/L thiamine, and 15 g/L agar). The transformation for complementation experiments using *CgpyrG* was performed by following the procedure described elsewhere.[18]

**Initial Identification of Natural Product Formation in the Mutant *C. globosum* Strains.** The culture of a mutant *C. globosum* strain grown on oatmeal agar plates overlaid with a sheet of sterilized cellophane (PT no. 300) and supplemented with 20 mM uridine and 0.18 mM uracil was terminated by extracting the mycelia and the agar media with ethyl acetate (250 *μ*L). The extract was dried *in vacuo*. The dried residue was dissolved in *N*,*N*-dimethylformamide (50 *μ*L). The resulting solution was subjected to LC−MS analysis. The LC−MS analysis was performed with a Thermo SCIENTIFIC LCQ Fleet and Exactive liquid chromatography mass spectrometer using both positive

and negative electrospray ionization. Samples were analyzed using a Prevail 3 μm, 2.1 × 100 $mm^2$ C18 reversed-phase column (Alltech) and separated on a 5−95% (v/v) $CH_3CN$ linear gradient in $H_2O$ supplemented with 0.05% (v/v) formic acid at a flow rate of 125 μL/min.

**Large-Scale Isolation of the Biosynthesized Compounds for Detailed Characterization.** A general method for large-scale isolation of compounds is given below. Specific modifications to the procedure for the isolation of **4**, **5**, and **6** are given in detail in the Supporting Information. Strains grown in oatmeal agar media were subjected to 5−7 day incubation at 30 °C. The acetone extracts of the mycelia and ethyl acetate extracts of the agar media were concentrated *in vacuo* to give an oily residue, which was fractionated by silica gel flash column chromatography with 0−100% $CH_3OH/CHCl_3$ gradient elution. The fractions containing the desired material were pooled and dried. The dried residue was dissolved in methanol and subjected to HPLC purification using Cosmosil MS-II column (20 × 250 $mm^2$, Nacalai Tesque) with $CH_3OH/H_2O$ isocratic elution. The presence of desired compounds in the HPLC fractions was confirmed by ESI−MS and NMR.

**Spectroscopic Analyses of the Isolated Compounds.** NMR spectra were obtained with a JEOL JNM-ECA 500 MHz spectrometer ($^1H$ 500 MHz, $^{13}C$ 125 MHz) and a Bruker BioSpin AVANCE 400 MHz spectrometer ($^1H$ 400 MHz, $^{13}C$ 100 MHz). $^1H$ NMR chemical shifts were reported in parts per million (ppm) using the following proton resonance of residual solvents as references: $CDCl_3$ δ 7.26 and $CD_3OD$ δ 3.30. $^{13}C$ NMR chemical shifts were reported relative to $CDCl_3$ δ 77.0 and $CD_3OD$ δ 49.9. Mass spectra were recorded with a Thermo SCIENTIFIC LCQ Fleet and Exactive liquid chromatography mass spectrometer by using both positive and negative ESI.

## RESULTS AND DISCUSSION

**Engineering *C. globosum* into a Homologous Recombination-Compatible Strain.** Use of homologous recombination allows efficient incorporation, expression control, and modification of endogenous as well as foreign genes. To take advantage of various tools available for genetically manipulating *C. globosum*, such as different plasmids with useful properties, we sought to inactivate a Lig4-equivalent DNA ligase that is known to be responsible for nonhomologous random recombination activity in other fungi.[19] A similar approach has been applied successfully in *A. oryzae* by deletion of its *ligD* gene.[20] CHGG_03097, a homologue of Lig4, was identified in the *C. globosum* genome by BLASTP analysis[21] using *A. nidulans* LigD as a query. CHGG_03097 showed a strong sequence similarity (47.4% identity, 62.5% similarity) to the *A. nidulans* LigD (UniProtKB accession number Q5BH83) and was therefore named CgLigD. To obtain a Δ*CgligD* mutant, we transformed the *C. globosum* protoplast with a linear deletion construct containing a hygromycin phosphotransferase gene *hph* sandwiched by a pair of 2.0-kilobase (kb) fragments that are homologous to the flanking regions of *CgligD*. The *hph* gene, which confers hygromycin resistance in *C. globosum*, was amplified from pPHT1 plasmid[22] as a selectable marker (Supporting Method 1.1.1 and Figures S1 and S2, Supporting Information). Thirty-four positive colonies thus obtained were subjected to PCR analysis using a combination of a marker-specific primer and a primer designed to anneal outside of the homologous region flanking the *hph* gene (Table S5, Supporting Information). Those that underwent a correct recombination event would produce an amplicon of a designated size. Further verification by Southern analysis provided us a desired mutant strain with stable Δ*CgligD* suitable for further experiments. This strain was designated as CGKW10. Similarly, another strain whose *CgligD* was replaced with $cbx^R$ was prepared, and it was designated as CGKW11. Lastly, a strain with a *CgpyrG* knockout was prepared to allow uridine/uracil selection. This strain was named CGKW14. This Δ*CgligD* strain achieved a 66.7% success rate for a targeted homologous recombination event, whereas only 5.8% underwent desired recombination in the wild type. We have confirmed that deletion of *CgligD* and *CgpyrG* had no apparent influence on the mycelial growth (Figures S5 and S6, Supporting Information) and deletion of *CgpyrG* did not alter the secondary metabolite production profile of *C. globosum* (Figure S50, Supporting Information).

**Targets in the *C. globosum* Genome for Activation of Biosynthetic Gene Clusters.** In fungi, production of secondary metabolites is often correlated with arrest of growth as well as sexual and asexual reproduction, and the genetic basis of regulating these processes is well documented.[23,24] More recent finding of a regulatory nuclear complex, including both the light-regulated developmental factor VeA[25,26] and the global regulator of secondary metabolites LaeA,[27] has established a mechanistic link between secondary metabolite production and morphological differentiation. However, much of how secondary metabolite biosynthesis is regulated in fungi still remains unclear. While *C. globosum* has been long known to produce chaetoglobosins and azaphilones,[28−31] no biosynthetic gene cluster responsible for those compounds has been identified in the genome until the very recent identification of the chaetoviridin/chaetomugilin biosynthetic gene cluster.[32] Therefore, only a very limited genetic or biochemical characterization of these biosynthetic pathways is currently available. Similarly, factors that regulate the gene clusters and hence biosynthesis of those natural products also remain unknown. Thus, we chose to target CgVeA (putative light-responsive protein, CHGG_10370),[25,33,34] CgLaeA (putative methyltransferase against histone, CHGG_01690),[16,27,35,36] and CgSptJ (putative acetyltransferase against histone, CHGG_09972)[16] for disrupting the epigenetic regulations present in *C. globosum* on its secondary metabolite biosynthetic pathways. For preparation of mutants with deletion of these three genes, deficiency of the orotidine-5′-phosphate decarboxylase gene *CgpyrG*[37] (Figures S3 and S4, Supporting Information) was picked as a selectable marker of choice, because it offered several advantages, such as nontoxic nutritional selection and ability to recycle the marker for complementation experiments. After preparing a *CgpyrG*-disrupted *C. globosum* strain CGKW14 from CGKW10 using homologous recombination with the knockout plasmid pKW3205 (Supporting Method 1.1.2 and Figures S3 and S4, Supporting Information), CGKW14 was used for deletion of each of the *CgveA*, *CglaeA*, and *CgsptJ* open reading frames (ORFs) (Figure 1) following essentially the same procedure as described in Supporting Method 1.1.2, Supporting Information.

**Modulation of Activities of Biosynthetic Gene Clusters upon VeA Deletion.** The culture extract of Δ*CgveA* strain presented an altered chemical landscape from that of the Δ*CgLigD*/Δ*CgpyrG* control strain CGKW14 as indicated by the shift in their UV traces from HPLC analyses (Figure 2A, I vs II). Previous work has shown that the major secondary metabolites produced by *C. globosum* are the polyketide chaetoviridins, which are categorized as azaphilone-type natural products. The deficiency of *CgveA* in the strain resulted in the increased productivity of cochliodinol (**1**)[38] and azaphilone-type secondary metabolites chaetoviridins (**2−4**)[28] (Figure 3). The fact that those compounds were formed indicates that the corresponding biosynthetic gene clusters were activated by the alteration in the heterochromatin


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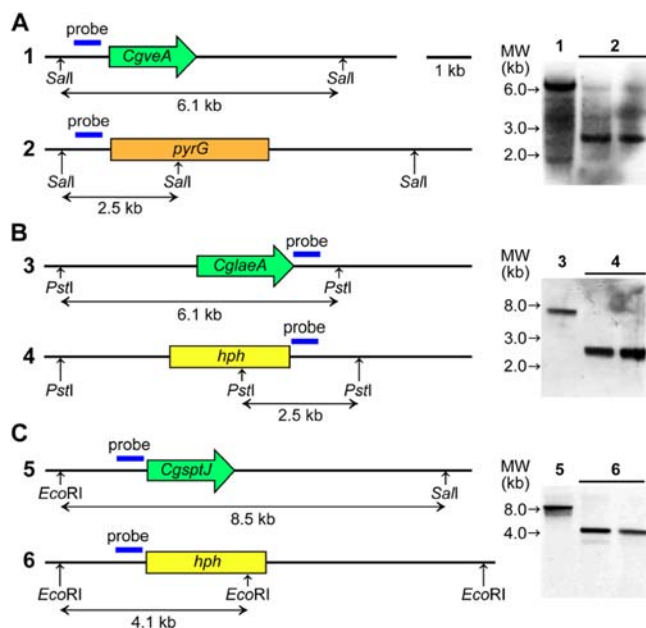

**Figure 1.** Design and Southern blotting confirmation of deletion of (A) *CgveA*, (B) *CglaeA*, and (C) *CgsptJ* in *C. globosum* using the *CgligD* deletion mutant for high-efficiency gene targeting. The probe used for Southern blotting analysis is shown as a blue bar. Lane 1, CGKW14 as a control; lane 2, *CgveA*-deficient CGKW14; lane 3, CGKW11 as a control; lane 4, *CglaeA*-deficient CGKW14; lane 5, CGKW11 as a control; lane 6, *CgsptJ*-deficient CGKW14. Each knockout strain was confirmed by independently duplicated experiments. *pyrG*, orotidine-5′-monophosphate decarboxylase gene; *hph*, hygromycin phosphotransferase gene.

structure introduced upon removal of the CgVeA activity in *C. globosum*. On the other hand, comparison to CGKW14 revealed that the *CgveA* disruption led to loss of production of chaetoglocin A (**5**), aureonitol (**6**), chaetoglobosin A (**7**), and cochliodone A (**8**) (Figure 2A, I vs II). The chemical structure of **2** was determined by full one- and two-dimensional NMR analysis (Figures S17−S20 and Table S7, Supporting Information) to reveal that it was 4′-epi-*N*-2-hydroxyethyl-azachaetoviridin A that was reported recently.[39] The chemical structures of **1**, **5**, **6**, and **8** were identified by HRESIMS and comparison of their NMR spectra to the reported characteristic spectra (Figures S15, S16, and S21−S26 and Tables S6 and S8−S10, Supporting Information).

The chemical structure of **1** is similar to terrequinone biosynthesized by the *tdi* gene cluster that carries a fungal NRPS gene *tdiA*.[40] Since *C. globosum* carries an NRPS gene, CHGG_03687, that is highly homologous to *tdiA* at 70% identity and 82% similarity, we suspected that CHGG_03687 might be involved in the biosynthesis of **1**. The transcriptional analysis revealed that the mRNA of CHGG_03687 was not detected in CGKW14 under the growth conditions described in the Materials and Methods section. However, with the *CgveA* deficiency, the mRNA was clearly transcribed (Figure 4A, lane 1 vs 2). To confirm that CHGG_03687 was involved in the production of **1**, we also deleted CHGG_03687. The HPLC profiles of the extract of this *CgveA*−CHGG_03687 double knockout strain showed the absence of **1** compared with the Δ*CgveA*/CGKW14 control (Figure 2B and Figure S51, Supporting Information), allowing us to assign the CHGG_03687-containing gene cluster as the cochliodinol

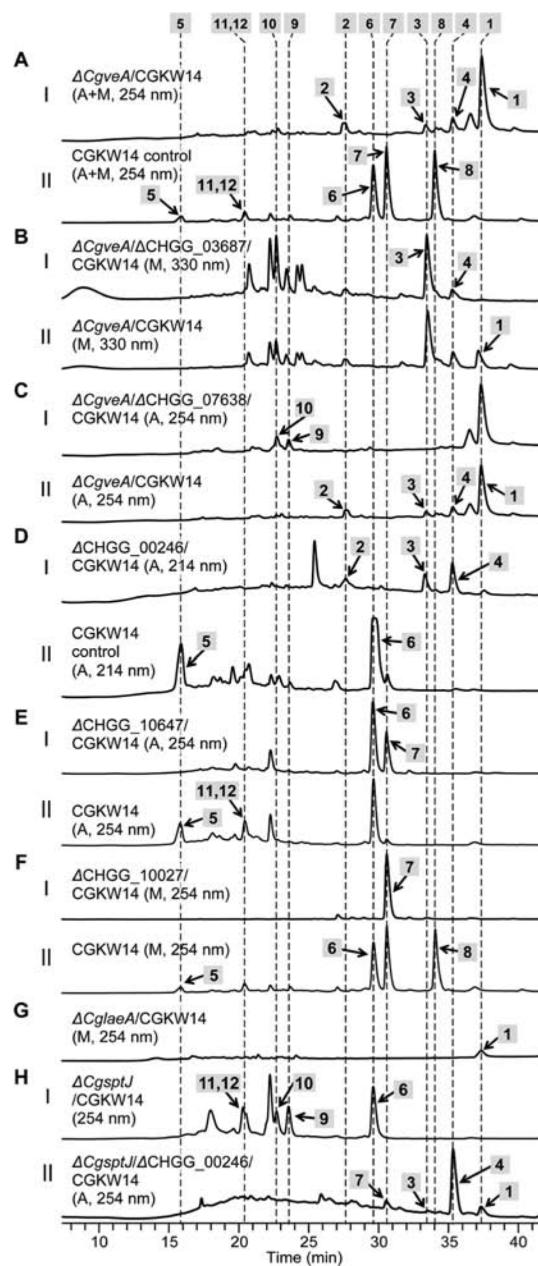

**Figure 2.** HPLC traces of metabolic extracts from the cultures of various *C. globosum* mutant strains for analyzing the effects of Δ*CgveA*, Δ*CglaeA*, and Δ*CgsptJ* and identification of the gene clusters responsible for the compounds found in the agar (A) or mycelial (M) extracts. Traces were measured at the wavelength designated within the parentheses. (A) I, Extract of the culture of Δ*CgveA*/CGKW14; II, CGKW14 as a control, both monitored at 254 nm. (B) Extract of the culture of Δ*CgveA*/ΔCHGG_03687/CGKW14 monitored at 280 nm. (C) Extract of the culture of Δ*CgveA*/ΔCHGG_07638/CGKW14 monitored at 280 nm. (D) I, Extract of the culture of ΔCHGG_00246/CGKW14; II, CGKW14 as a control, both monitored at 214 nm. (E) I, Extract of the culture of ΔCHGG_10647/CGKW14; II, CGKW14 agar extract as a control, both monitored at 254 nm. (F) I, Extract of the culture of ΔCHGG_10027/CGKW14; II, CGKW14 mycelial extract as a control, both monitored at 254 nm. (G) Extract of the culture of Δ*CglaeA*/CGKW14 monitored at 254 nm. (H) I, Extract of the culture of Δ*CgsptJ*/CGKW14; II, extract of the culture of Δ*CgsptJ*/ΔCHGG_00246/CGKW14, both monitored at 254 nm.

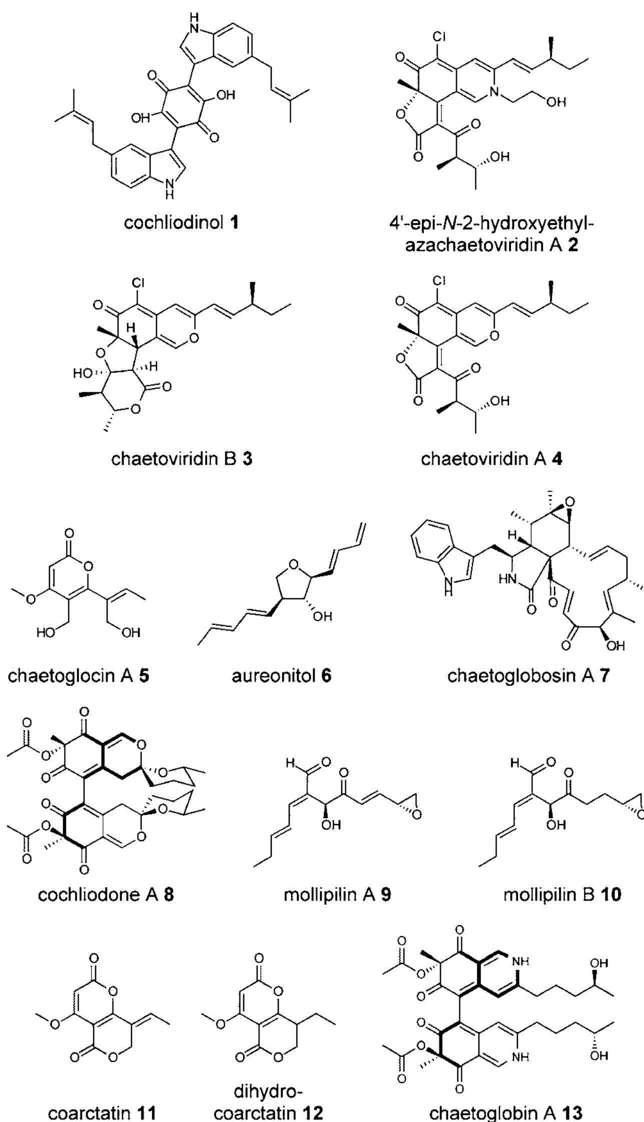


**Figure 3.** Chemical structures of secondary metabolites identified during the HPLC analyses of *C. globosum* strains carrying a deletion of either *CgveA*, *CglaeA*, or *CgsptJ* (Figure 2).

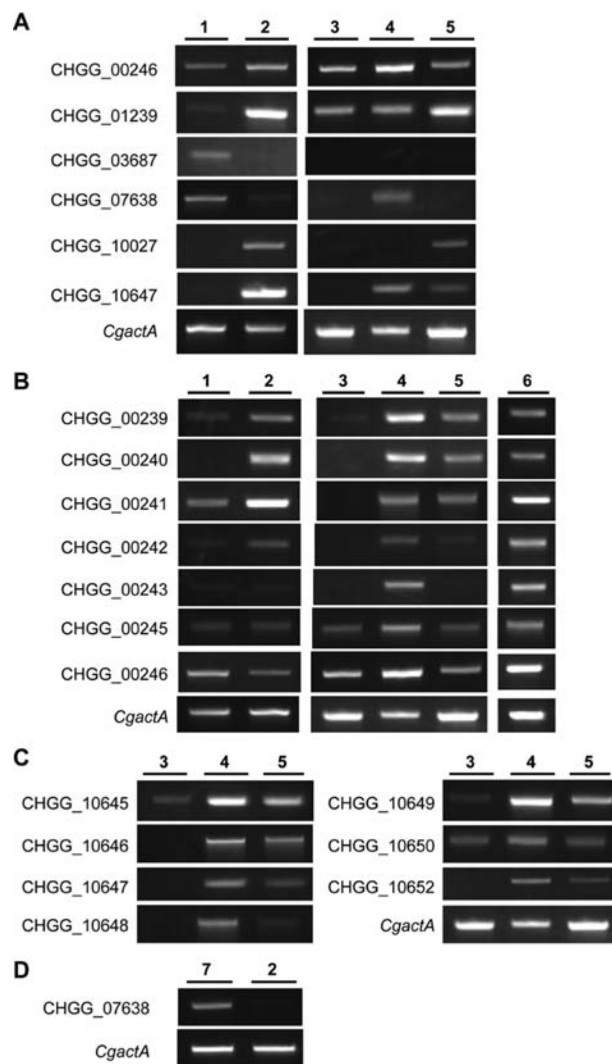


**Figure 4.** RT-PCR analysis of the effect of deleting *CgveA, CglaeA, CgsptJ*, CHGG_00246 and CHGG_07638 on the expression of other biosynthetic genes in *C. globosum*. Expression of the actin A gene (*CgactA*) was used as a positive control. Lane 1, *CgveA*-deficient CGKW14; lane 2, CGKW14 as a control; lane 3, *CglaeA*-deficient CGKW11; lane 4, *CgsptJ*-deficient CGKW11; lane 5, CGKW11 as a control; lane 6, CHGG_07638-deleted CGKW14; lane 7, CHGG_00246-deleted CGKW14. (A) Expression of CHGG_00246, CHGG_01239, CHGG_03687, CHGG_07638, CHGG_10027, and CHGG_10647 in *C. globosum* with a deletion of *CgveA* (lane 1), *CglaeA* (lane 3), or *CgsptJ* (lane 4). (B) Expression of seven genes proposed to be responsible for the biosynthesis of aureonitol **6** in *C. globosum* with a deletion of *CgveA* (lane 1), *CglaeA* (lane 3), *CgsptJ* (lane 4), or CHGG_07638 (lane 6). (C) Expression of seven genes proposed to be responsible for the biosynthesis of **5** in *C. globosum* with a deletion of *CglaeA* (lane 3) or *CgsptJ* (lane 4). (D) Expression of a predicted partially reducing polyketide synthase gene CHGG_07638 proposed to be responsible for the biosynthesis of chaetoviridins **2**, **3**, and **4** in *C. globosum* with a deletion of CHGG_00246 (lane 7).

biosynthetic gene cluster (Figure S11 and Table S1, Supporting Information).

The Δ*CgveA* mutant strain also showed that it was capable of biosynthesizing **2**−**4** under essentially the same culture conditions under which **1** was produced. Chaetoviridins have been previously isolated from *C. globosum*,[28] and the new compound **2** was also elucidated by NMR spectra to be a chaetoviridin-type of compound.[28,39,41] A previous study suggested that two PKSs are involved in the biosynthesis of the polyketide core.[28] Thus, we targeted CHGG_07638, a predicted partially reducing polyketide synthase (PR-PKS) existing in a close proximity with another PKS gene, for disruption (Supporting Method 1.1.3 and Figure S7, Supporting Information) to determine whether CHGG_07638 is involved in the production of **2**. The culture extract from the *CgveA*−CHGG_07638 double-knockout strain indeed lacked **2** (Figure 2C). Thus, we were able to confirm that CHGG_07638 was responsible for the biosynthesis of the polyketide core of **2**. We have reported a detailed investigation of the mechanism involving the biosynthesis of **2** and other chaetoviridin-type compounds **3** and **4** elsewhere recently.[32]

On the other hand, reduction in the productivity of some secondary metabolites was also observed in the Δ*CgveA* strain. UV traces from the HPLC analysis of the extract indicated that the yields of **5**, **6**, **7**, and **8** were clearly reduced from those found in the extract of CGKW11, the wild-type (Δ*CgligD*)

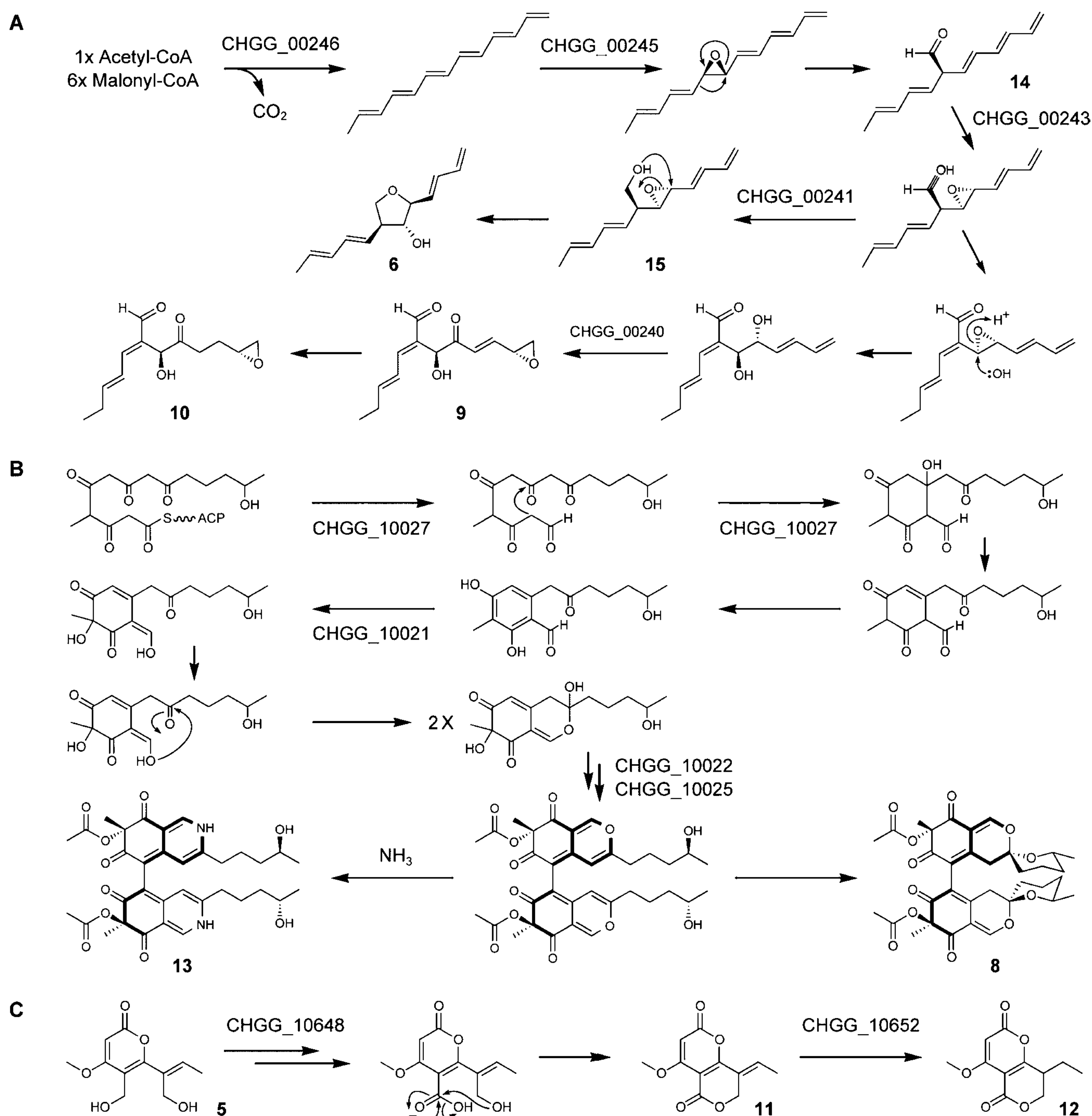


**Figure 5.** Proposed biosynthetic pathways of (A) aureonitol (**6**) and mollipilin A (**9**) and B (**10**), (B) cochliodones **8** and **13**, and (C) coarctatins **11** and **12**.

strain (Figure 2A and Figure S50, Supporting Information). In addition, a 30-day culture of *C. globosum* resulted in the formation of **13**. Analysis of the chemical structure of those isolated compounds by HRESIMS and NMR spectra revealed that **5**, **6**, **7**, and **8** were identical to previously known chaetoglocin A,[42] aureonitol,[43−46] chaetoglobosin A,[47] and cochliodone A,[48] respectively. Also, **13** was identified to be chaetoglobin A,[49] a compound closely related to **8**.[48] To identify the PKS and PKS−NRPS gene responsible for the biosynthesis of those compounds, we prepared four additional *C. globosum* mutant strains each carrying a deletion of one of the following PKS genes in CGKW14: CHGG_00246 (highly reducing PKS or HR-PKS), CHGG_01239 (PKS−NRPS hybrid), CHGG_10027 (nonreducing PKS or NR-PKS), and CHGG_10647 (NR-PKS). Based on the chemical structures of those compounds and *in silico* analysis of PKS and PKS−NRPS genes found in the *C. globosum* genome, CHGG_10647 (NR-PKS) was predicted to play a role in the biosynthesis of **5**. Similarly, CHGG_00246 (HR-PKS) and CHGG_10027 (NR-PKS) were considered to be involved in the biosynthesis of **6** and **8**, respectively. Deletion of the target gene in the knockout mutants was confirmed by Southern blotting (Figure S8, Supporting Information) and PCR (Figures S9 and S10, Supporting Information) analyses. HPLC analyses of the culture extracts from those mutant strains showed that the deficiency in CHGG_00246, CHGG_10647, and

CHGG_10027 was indeed correlated to the loss of production of **6**, **5**, and **8**, respectively (Figure 2D−F and Figures S52−S54, Supporting Information). Assignment of the proposed functions of the ORFs found in the gene clusters containing these PKS genes examined here is given in the Supporting Information (Figures S12−14 and Tables S2−S4). Regarding CHGG_01239, we reported elsewhere recently that it is responsible for the biosynthesis of **7**.[50]

**LaeA Deletion Leads to No New Activation of Biosynthetic Gene Cluster.** We also attempted to investigate the effect of deleting the *laeA* gene, which codes for a well-known global regulator controlling expression of secondary metabolite biosynthetic gene clusters in *A. nidulans*.[25] Thus, *C. globosum laeA* (*CglaeA*) was knocked out using the same homologous recombination method established earlier, and the extract of this mutant's culture was analyzed. Other than the minor presence of **1**, significant production of multiple natural products observed in the extract of CGKW11 (Figure S50, Supporting Information) was completely absent in the culture extract of the Δ*CglaeA*/Δ*CgligD* mutant (Figure 2G). Furthermore, we also observed a general down-regulation of gene expression in the secondary metabolite biosynthetic gene clusters compared with CGKW11 (Figure 4, lane 3 vs 5). Hence, similar to the previous findings in *A. nidulans*,[35,51] LaeA also functions in *C. globosum* as a global activator of secondary metabolism, presumably through its methyltransfer activity that leads to unfolding of heterochromatin into euchromatin to allow transcription of the biosynthetic gene clusters. Loss of epigenetic control incurred upon deletion of *CglaeA* made it globally unsuitable for expressing the genes responsible for the biosynthesis of secondary metabolites in *C. globosum*.

**SptJ Deletion Results in New Isolation of Two Compounds from *C. globosum*.** Next, we applied our homologous recombination-enabled Δ*CgligD C. globosum* strain to investigate the effect of deletion of CgSptJ, a predicted histone acetyltransferase homologous to *Saccharomyces cerevisiae* Spt10 that can install a chromatin mark associated with gene silencing and heterochromatin formation.[52] The UV traces from the HPLC analysis indicated that the mutant strain Δ*CgsptJ*/Δ*CgligD* (Figure 1C) produced two compounds, **9** and **10**, in addition to **11** and **12**, with a reduced production of **6** and a complete loss of production of **5** (Figure 2H, I and Figure S55, Supporting Information). To identify the gene clusters responsible for the biosynthesis of those compounds identified in the extract of the Δ*CgsptJ*/Δ*CgligD* strain, further investigations were conducted as described below.

**Biosynthesis of Aureonitol 6 and Related Compounds 9 and 10.** While analyzing the secondary metabolite profile of the Δ*CgsptJ* mutant strain, we successfully isolated two metabolites, **9** and **10**, that were never isolated from *C. globosum* before. The chemical structures of **9** and **10** were determined by HRESIMS and NMR spectra (Figures S27−S38 and Tables S11 and S12, Supporting Information). In a recent report, those compounds were also isolated from another fungus, *C. mollipilium*, and named mollipilin A (**9**) and B (**10**).[53] As described earlier, analysis of the previously prepared five *C. globosum* strains with deletion of the PKS/NRPS genes identified that CHGG_00246 is responsible for the biosynthesis of **6** (Figure 2D). However, deletion of CHGG_00246 in the Δ*CgsptJ* strain also led to the loss of formation of **9** and **10** (Figure 2H), suggesting that the HR-PKS CHGG_00246 furnished the polyketide core to **6**, **9**, and **10**. Not surprisingly, a general trend for an upregulation of the expression of the genes within the cluster containing CHGG_00246 was observed upon deletion of *CgsptJ* (Figure 4B, lane 4 vs 2 or 5). Thus, this biosynthetic gene cluster was examined further to determine a plausible biosynthetic mechanism for the formation of these compounds (Figure 5A; Figure S13 and Table S3, Supporting Information). The conjugated heptaketide backbone is likely assembled first by the iterative HR-PKS CHGG_00246. Epoxidation of the polyketide core can be performed by one of the cytochrome P450 or FAD-dependent monooxygenases (FMOs) present in the cluster, possibly CHGG_00245 as judged by close correlation in its pattern of transcription with CHGG_00246. A rearrangement involving the epoxide moiety can proceed to yield *R*-aldehyde **14**.[45] This intermediate is epoxidated once again, likely by a predicted FMO, CHGG_00243, and this epoxidation step serves as the point of divergence for the formation of **6** vs **9** and **10**. For the formation of **6**, the aldehyde in **14** must undergo a reduction to *R*-alcohol. This reduction reaction can be performed by CHGG_00241, a possible FAD-dependent oxidoreductase, to form **15**. The fact that deletion of CHGG_00241, CHGG_00243, and CHGG_00245 led to the loss of production of **6** (Figure S56C,D,E, respectively) supports the positioning of those genes within the biosynthetic pathway. A subsequent epoxide-opening cyclization in this *R*-alcohol leads to the formation of a tetrahydrofuran core in **6**. On the other hand, a series of isomerization, double epoxidation, epoxide opening, and oxidation of an alcohol group would lead to the transformation of **14** into **9**. Because deletion of CHGG_00240 does not affect the production of **6** (Figure S56, B vs F, Supporting Information), this FMO is implicated in the formation of the epoxide moiety of **9**. Lastly, **9** can be reduced by an oxidoreductase to form **10**, possibly by CHGG_00239, which is highly homologous to a 3-oxoacyl-[acyl-carrier-protein] reductase. The discovery of the tetrahydrofuran biosynthesis gene cluster now creates the basis for a detailed biochemical investigation of this rearrangement.

**Proposed Biosynthetic Pathway of Azaphilones 8 and 13.** Compound **8** was isolated from CGKW11, the wild-type (Δ*CgligD*) *C. globosum*. However, we found that the Δ*CgveA*/Δ*CgligD* mutant strain did not produce **8**. Subsequently, homologous recombination-mediated deletion of CHGG_10027, which codes for an NR-PKS, confirmed its involvement in the biosynthesis of **8** (Figure 2F vs A, II; Figure S14 and Table S4, Supporting Information). Also, a 30-day culture resulted in the formation of **13** (Figures S48 and S49 and Table S15, Supporting Information). The precursor of **8** can be converted into **13** via a nonenzymatic reaction[54] with ammonia present within the fungal cell to form the vinylogous *γ*-pyridone (Figure 5B, bottom row). Surprisingly, we found in our previous report that when CHGG_10027 was heterologously expressed in *S. cerevisiae*, this NR-PKS biosynthesized a tetraketide 6-methylorsellinic acid *in vivo*.[5,55] These experimental results suggest that the starter unit:ACP transacylase (SAT) in the CHGG_10027 is capable of accepting acetyl-CoA as a starting unit and adding four malonyl-CoA units for biosynthesizing 6-methylorsellinic acid in the absence of other biosynthetic enzymes coded within the gene cluster. Within the native context, however, CHGG_10027 also extends the starter unit with four malonyl-CoA units but accepts either heptanoyl-CoA or 5-hydroxyheptanoyl-CoA, not acetyl-CoA, as its starting unit. Lastly, the nascent chain is released by its terminal reductase domain as a cyclized aldehyde. Subsequent involvement of CHGG_10023 (enoyl

reductase), CHGG_10028 (acetyl transferase), and CHGG_10030 (enoyl reductase) leads to the formation of the monomeric unit of the dimeric precursor of **8** and **13**. Acetylation of the alcohol group on the ring system can be achieved by CHGG_10028, a predicted *O*-acetyltransferase,[56] and dimerization is likely performed by a fungal laccase-like CHGG_10025.[57]

**Biosynthetic Pathway of Chaetoglocin 5 and Coarctatins 11 and 12.** As described earlier, we found that deletion of *CgsptJ* led to the loss of production of **5** (Figure 2H, I). Instead, we observed a sharp increase of another UV peak in the extract from the Δ*CgsptJ*/Δ*CgligD* strain. The constituents of the new peak were identified to be coarctatin **11** and dihydrocoarctatin **12**[44,58] (Figures S39−S47 and Tables S13 and S14, Supporting Information). The PKS gene-knockout studies conducted earlier identified that an NR-PKS CHGG_10647 is responsible for the formation of **5**, **11**, and **12** (Figure 2E vs 2A, II). Furthermore, RT-PCR study revealed that deletion of *CgsptJ* in CGKW11 (Figure 4C, lane 4) led to an increased expression of all of the genes present in the gene cluster involving CHGG_10647 over the CGKW11 background (Figure 4C, lane 5). Upon examination of the proposed enzymatic activities encoded by the genes in this cluster (Figure S12 and Table S2, Supporting Information), we found a putative FAD-dependent oxidase CHGG_10648 that could catalyze an oxidation of **5** to set up the compound for a lactonization to form **11**. Subsequently, **11** can be converted into **12** by reduction of the double bond by a predicted short-chain dehydrogenase, CHGG_10652 (Figure 5C). Thus, upregulation of this gene cluster, especially the expression of CHGG_10648, seems to result in an active conversion of **5** into **11** and **12**, leading to the depletion of **5** in the culture (Figure 2H, I).

**Aureonitol 6 Can Regulate Biosynthesis of Another Secondary Metabolite.** During the course of the study, we noticed an inverse correlation between the transcription level of chaetoviridin **2**−**4** biosynthetic gene CHGG_07638 (PR-PKS) and the ability of the strain to produce **6**. It is known that when a polyketide biosynthetic gene cluster is knocked out, product yield from other polyketide biosynthetic gene clusters tends to increase.[59] This is presumably because the proportion of cellular pool of shared substrates, such as malonyl-CoA, allotted to each polyketide biosynthetic pathway can increase as the number of active pathways decreases. However, it also appeared plausible that **6** was regulating the transcription of genes in other clusters. Our earlier study showed that CHGG_00246 coded for the HR-PKS responsible for the biosynthesis of the polyketide core of **6**. Thus, to investigate whether **6** is capable of regulating secondary metabolism in *C. globosum*, specifically the chaetoviridin biosynthesis, we examined the transcriptional level of the PKS gene CHGG_07638 in the ΔCHGG_00246/CGKW14 strain. The RT-PCR results clearly indicated that expression of CHGG_07638 was upregulated when the strain lost its ability to biosynthesize **6** (Figure 4D).

To verify that it was in fact the secondary metabolite **6** that was affecting the transcription of chaetoviridin biosynthetic genes, we supplemented the growth medium with **6** and examined the ability of the ΔCHGG_00246/CGKW14 strain to produce chaetoviridins. Visual inspection of the plates revealed a clear loss of chaetoviridin-derived deep red color from the mycelium (Figure 6A). The degree of color loss was correlated to the increase in the concentration of **6** in the growth medium. Extracts from those mycelia also showed that

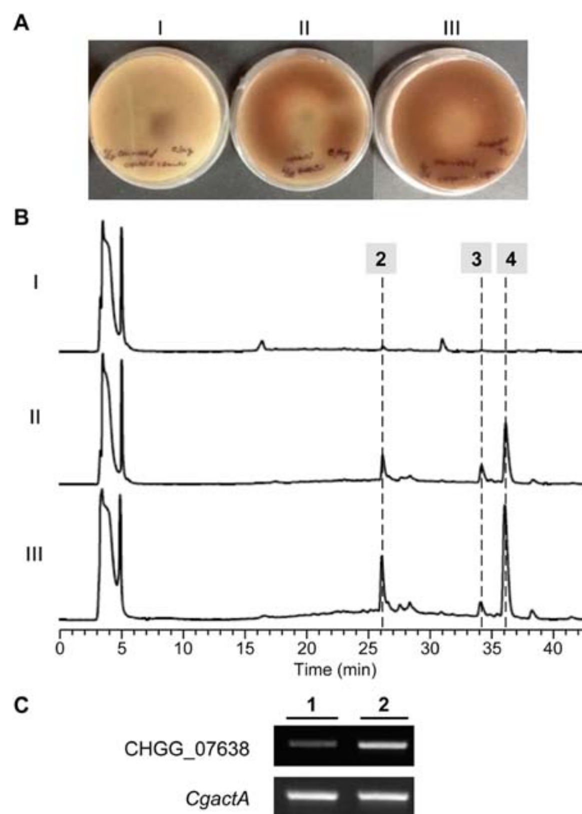


**Figure 6.** Assays on the effect of aureonitol (**6**) on the biosynthesis of chaetoviridins **2**, **3**, and **4**. (A) Mycelium color assay using ΔCHGG_00246/CGKW14 strain grown on an oatmeal agar plate supplemented with **6**. Each plate contained the corresponding compound at an incremental concentration of (I) 62.5 μg/mL, (II) 12.5 μg/mL, and (III) methanol as a negative control. (B) HPLC traces of the extract of mycelia grown on an oatmeal agar plate supplemented with **6**. Each plate contained **6** at an incremental concentration of (I) 12.5 μg/mL; (II) 1.25 μg/mL, and (III) methanol as a negative control. The strains were incubated for 4 days at 30 °C. All UV traces were monitored at 254 nm. (C) RT-PCR analysis of the effect of **6** on the expression of the chaetoviridin biosynthetic genes in the ΔCHGG_00246/CGKW14 strain. Expression of the actin A gene *CgactA* was used as a positive control. Lane 1, 12.5 μg/mL of **6**; lane 2, DMSO as a control.

the production of chaetoviridins **2**−**4** was suppressed in the presence of **6** in the growth medium (Figure 6B), with a complete suppression at 12.5 μg/mL of **6** in the growth medium (Figure 6B, I). Likewise, the transcription level of CHGG_07638 was also decreased markedly in the cells grown in the presence of **6** (Figure 6C). These results strongly support the notion that **6** acts as a transcriptional regulator that can control the activity of another biosynthetic gene cluster. When *C. globosum* became unable to produce **6** upon deletion of *CgveA*, repression on the transcription of the chaetoviridin biosynthetic genes exerted by **6** was removed. As a result, production of chaetoviridins **2**−**4** proceeded. Chaetoviridins were not produced in the Δ*CgsptJ*/Δ*CgligD* strain, because deletion of *CgsptJ* did not affect the strain's ability to produce **6**. However, deletion of CHGG_00246 from the Δ*CgsptJ*/Δ*CgligD* strain (Δ*CgsptJ*/CGKW14) again allowed production of **2**−**4** (Figure S55, Supporting Information).

Secondary metabolites are known to play a role in the regulation of metabolism and other cellular functions. For example, acyl homoserine lactones act as an autoinducer signal

for bacterial quorum sensing.[60] A-factor and related γ-butyrolactones,[61] as well as a complex nonribosomal peptide hormaomycin,[62] are known to act like hormones that can control secondary metabolism and morphological differentiation by binding to their receptors that interact with transcriptional regulators. A *Fusarium oxysporum* mycotoxin, fusaric acid, is known to repress quorum-sensing regulatory genes, which leads to suppression of the biosynthesis of an antifungal metabolite phenazine-1-carboxamide in *Pseudomonas chlororaphis* strain PCL1391.[63] More recently, 1,3-diaminopropane and spermidine were found to induce the biosynthesis of benzylpenicillin in *Penicillium chrysogenum* by increasing the expression of penicillin biosynthetic genes through stimulation of the expression of a global epigenetic regulator *laeA*.[64] However, these cases primarily involve intercellular communication or intracellular signaling achieved by targeting global transcription regulators. Although there is a report of a secondary metabolite acting as an inducer of transcription of its own biosynthesis,[65] to the best of our knowledge this is the first report of a secondary metabolite directing the production of different natural products by working as a pathway-specific regulator to control the gene expression in another secondary metabolite biosynthetic gene cluster.

To add another layer of complexity, a similar pattern of transcriptional correlation was also observed between the chaetoviridin and aureonitol biosynthetic genes. When CHGG_07638 associated with chaetoviridin biosynthesis was deleted, expression of the aureonitol biosynthetic genes showed a general increase of transcription (Figure 4B, lane 2 vs 6). In particular, expression of CHGG_00243 was significantly increased. Similarly, deletion of *CgsptJ* also led to a general increase of transcription of the aureonitol biosynthetic genes, including CHGG_00243 (Figure 4B, lane 4). Under both conditions, production of chaetoviridins (**2**−**4**) was not detected, but the formation of **6**, **9**, and **10** was observed (Figure 2C and H, I). These findings suggest that transcription products or regulators associated with the biosynthesis of chaetoviridins **2**−**4** or **2**−**4** themselves may also negatively influence the expression of the aureonitol biosynthetic genes and production of **6**, **9** and **10**. Further investigation into this complex relationship among biosynthetic pathways of different secondary metabolites is currently ongoing.

## ■ CONCLUSION

In conclusion, our results showed that construction of a *ligD*-deletion strain of *C. globosum* allowed us to perform highly efficient targeted genetic manipulation of this fungus. Through the study, we identified that deletion of genes coding for epigenetic regulators led to modulation of transcription of a number of biosynthetic gene clusters in *C. globosum*. Using this method, we were able to coax *C. globosum* to generate certain natural products it did not produce under standard culture conditions. Then, the same homologous recombination technique was used to modify specific gene clusters efficiently to identify which cluster is responsible for making which compound. Altogether, we were able to isolate a total of 13 compounds including two secondary metabolites, **9** and **10**, both newly isolated from *C. globosum*, that are associated with six gene clusters that have not been characterized to date. Our results suggest that deletion of the *ligD* homologue gene may be a powerful general method to convert other fungal species that are currently not amenable to molecular genetic studies into strains that can be rationally manipulated through targeted homologous recombination. Furthermore, while our current study has reinforced the notion that targeting epigenetic regulators, such as histone modifying factors, is an effective method of manipulating transcription of biosynthetic gene clusters, it also demonstrated that disruption of the light-responsive protein VeA is another highly effective approach in activating silenced or weakly active secondary metabolite biosynthetic gene clusters. Last but not least, our study also revealed that aureonitol **6** can act like a transcriptional regulator of the gene cluster responsible for the biosynthesis of chaetoviridins **2**−**4** in *C. globosum*, uncovering yet another aspect of the complex network of secondary metabolite biosynthetic pathway regulations in fungi. Taken together, we were able to show that our simple and flexible approach involving deletion of *ligD*-homologue and subsequent targeted disruption of epigenetic transcriptional regulators can greatly accelerate the postgenomic characterizations of underexplored secondary metabolite biosynthetic pathways in various fungi and other organisms.

## ■ ASSOCIATED CONTENT

### Supporting Information

Data from the NMR and MS of the compounds and additional experimental information. This material is available free of charge via Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES


(1) Bergmann, S.; Schümann, J.; Scherlach, K.; Lange, C.; Brakhage, A. A.; Hertweck, C. *Nat. Chem. Biol.* **2007**, *3*, 213.

(2) Bok, J. W.; Chiang, Y. M.; Szewczyk, E.; Reyes-Dominguez, Y.; Davidson, A. D.; Sanchez, J. F.; Lo, H. C.; Watanabe, K.; Strauss, J.; Oakley, B. R.; Wang, C. C.; Keller, N. P. *Nat. Chem. Biol.* **2009**, *5*, 462.

(3) Nützmann, H. W.; Reyes-Dominguez, Y.; Scherlach, K.; Schroeckh, V.; Horn, F.; Gacek, A.; Schümann, J.; Hertweck, C.; Strauss, J.; Brakhage, A. A. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 14282.

(4) Asai, T.; Yamamoto, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 2006.

(5) Ishiuchi, K.; Nakazawa, T.; Ookuma, T.; Sugimoto, S.; Sato, M.; Tsunematsu, Y.; Ishikawa, N.; Noguchi, H.; Hotta, K.; Moriya, H.; Watanabe, K. *ChemBioChem* **2012**, *13*, 846.
(6) Nakazawa, T.; Ishiuchi, K.; Praseuth, A.; Noguchi, H.; Hotta, K.; Watanabe, K. *ChemBioChem* **2012**, *13*, 855.
(7) Bode, H. B.; Müller, R. *Angew. Chem., Int. Ed.* **2005**, *44*, 6828.
(8) Stewart, E. J. *J. Bacteriol.* **2012**, *194*, 4151.
(9) Wenzel, S. C.; Müller, R. *Curr. Opin. Biotechnol.* **2005**, *16*, 594.
(10) Brakhage, A. A.; Schroeckh, V. *Fungal Genet. Biol.* **2011**, *48*, 15.
(11) Hansen, B. G.; Salomonsen, B.; Nielsen, M. T.; Nielsen, J. B.; Hansen, N. B.; Nielsen, K. F.; Regueira, T. B.; Nielsen, J.; Patil, K. R.; Mortensen, U. H. *Appl. Environ. Microbiol.* **2011**, *77*, 3044.
(12) Qiao, K.; Chooi, Y. H.; Tang, Y. *Metab. Eng.* **2011**, *13*, 723.
(13) An, Z.; Mei, B.; Yuan, W. M.; Leong, S. A. *EMBO J.* **1997**, *16*, 1742.
(14) Cichewicz, R. H. *Nat. Prod. Rep.* **2010**, *27*, 11.
(15) Bayram, Ö.; Braus, G. H. *FEMS Microbiol. Rev.* **2012**, *36*, 1.
(16) Eriksson, P. R.; Ganguli, D.; Clark, D. J. *Mol. Cell. Biol.* **2011**, *31*, 557.
(17) Shwab, E. K.; Bok, J. W.; Tribus, M.; Galehr, J.; Graessle, S.; Keller, N. P. *Eukaryotic Cell* **2007**, *6*, 1656.
(18) Binninger, D. M.; Skrzynia, C.; Pukkila, P. J.; Casselton, L. A. *EMBO J.* **1987**, *6*, 835.
(19) Ishibashi, K.; Suzuki, K.; Ando, Y.; Takakura, C.; Inoue, H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 14871.
(20) Mizutani, O.; Kudo, Y.; Saito, A.; Matsuura, T.; Inoue, H.; Abe, K.; Gomi, K. *Fungal Genet. Biol.* **2008**, *45*, 878.
(21) Johnson, M.; Zaretskaya, I.; Raytselis, Y.; Merezhuk, Y.; McGinnis, S.; Madden, T. L. *Nucleic Acids Res.* **2008**, *36*, W5.
(22) Cummings, W. J.; Celerin, M.; Crodian, J.; Brunick, L. K.; Zolan, M. E. *Curr. Genet.* **1999**, *36*, 371.
(23) Calvo, A. M.; Wilson, R. A.; Bok, J. W.; Keller, N. P. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 447.
(24) Yu, J. H.; Keller, N. *Annu. Rev. Phytopathol.* **2005**, *43*, 437.
(25) Bayram, O.; Krappmann, S.; Ni, M.; Bok, J. W.; Helmstaedt, K.; Valerius, O.; Braus-Stromeyer, S.; Kwon, N. J.; Keller, N. P.; Yu, J. H.; Braus, G. H. *Science* **2008**, *320*, 1504.
(26) Calvo, A. M.; Bok, J.; Brooks, W.; Keller, N. P. *Appl. Environ. Microbiol.* **2004**, *70*, 4733.
(27) Bok, J. W.; Keller, N. P. *Eukaryotic Cell* **2004**, *3*, 527.
(28) Takahashi, M.; Koyama, K.; Natori, S. *Chem. Pharm. Bull. (Tokyo)* **1990**, *38*, 625.
(29) Borges, W. S.; Mancilla, G.; Guimarães, D. O.; Durán-Patrón, R.; Collado, I. G.; Pupo, M. T. *J. Nat. Prod.* **2011**, *74*, 1182.
(30) Jiao, W.; Feng, Y.; Blunt, J. W.; Cole, A. L.; Munro, M. H. *J. Nat. Prod.* **2004**, *67*, 1722.
(31) Zhang, J.; Ge, H. M.; Jiao, R. H.; Li, J.; Peng, H.; Wang, Y. R.; Wu, J. H.; Song, Y. C.; Tan, R. X. *Planta Med.* **2010**, *76*, 1910.
(32) Winter, J. M.; Sato, M.; Sugimoto, S.; Chiou, G.; Garg, N. K.; Tang, Y.; Watanabe, K. *J. Am. Chem. Soc.* **2012**, *134*, 17900.
(33) Käfer, E. *Genetics* **1965**, *52*, 217.
(34) Wiemann, P.; Brown, D. W.; Kleigrewe, K.; Bok, J. W.; Keller, N. P.; Humpf, H. U.; Tudzynski, B. *Mol. Microbiol.* **2010**, *77*, 972.
(35) Bok, J. W.; Noordermeer, D.; Kale, S. P.; Keller, N. P. *Mol. Microbiol.* **2006**, *61*, 1636.
(36) Perrin, R. M.; Fedorova, N. D.; Bok, J. W.; Cramer, R. A.; Wortman, J. R.; Kim, H. S.; Nierman, W. C.; Keller, N. P. *PLoS Pathog.* **2007**, *3*, No. e50.
(37) Weidner, G.; d'Enfert, C.; Koch, A.; Mol, P. C.; Brakhage, A. A. *Curr. Genet.* **1998**, *33*, 378.
(38) Jerram, W. A.; McInnes, A. G.; Maass, W. S. G.; Smith, D. G.; Taylor, A.; Walter, J. A. *Can. J. Chem.* **1975**, *53*, 727.
(39) McMullin, D. R.; Sumarah, M. W.; Blackwell, B. A.; Miller, J. D. *Tetrahedron Lett.* **2013**, *54*, 568.
(40) Balibar, C. J.; Howard-Jones, A. R.; Walsh, C. T. *Nat. Chem. Biol.* **2007**, *3*, 584.
(41) Arai, N.; Shiomi, K.; Tomoda, H.; Tabata, N.; Yang, D. J.; Masuma, R.; Kawakubo, T.; Omura, S. *J. Antibiot. (Tokyo)* **1995**, *48*, 696.
(42) Ge, H. M.; Zhang, Q.; Xu, S. H.; Guo, Z. K.; Song, Y. C.; Huang, W. Y.; Tan, R. X. *Planta Med.* **2011**, *77*, 277.
(43) Marwah, R. G.; Fatope, M. O.; Deadman, M. L.; Al-Maqbali, Y. M.; Husband, J. *Tetrahedron* **2007**, *63*, 8174.
(44) Burrows, B. F.; Turner, W. B.; Walker, E. R. H. *J. Chem. Soc., Perkin Trans. 1* **1975**, 999.
(45) Saito, M.; Seto, H.; Yonehara, H. *Agric. Biol. Chem.* **1983**, *47*, 2935.
(46) Abraham, W.-R.; Arfmann, H.-A. *Phytochemistry* **1992**, *31*, 2405.
(47) Sekita, S.; Yoshihira, K.; Natori, S.; Kuwano, H. *Chem. Pharm. Bull. (Tokyo)* **1982**, *30*, 1629.
(48) Phonkerd, N.; Kanokmedhakul, S.; Kanokmedhakul, K.; Soytong, K.; Prabpai, S.; Kongsearee, P. *Tetrahedron* **2008**, *64*, 9636.
(49) Ge, H. M.; Zhang, W. Y.; Ding, G.; Saparpakorn, P.; Song, Y. C.; Hannongbua, S.; Tan, R. X. *Chem. Commun.* **2008**, 5978.
(50) Ishiuchi, K.; Nakazawa, T.; Yagishita, F.; Mino, T.; Noguchi, H.; Hotta, K.; Watanabe, K. *J. Am. Chem. Soc.* **2013**, *135*, 7371.
(51) Reyes-Dominguez, Y.; Bok, J. W.; Berger, H.; Shwab, E. K.; Basheer, A.; Gallmetzer, A.; Scazzocchio, C.; Keller, N.; Strauss, J. *Mol. Microbiol.* **2010**, *76*, 1376.
(52) Chang, J. S.; Winston, F. *Eukaryotic Cell* **2011**, *10*, 118.
(53) Asai, T.; Morita, S.; Shirata, N.; Taniguchi, T.; Monde, K.; Sakurai, H.; Ozeki, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 5456.
(54) Wei, W. G.; Yao, Z. J. *J. Org. Chem.* **2005**, *70*, 4585.
(55) Davison, J.; al Fahad, A.; Cai, M.; Song, Z.; Yehia, S. Y.; Lazarus, C. M.; Bailey, A. M.; Simpson, T. J.; Cox, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 7642.
(56) Garvey, G. S.; McCormick, S. P.; Alexander, N. J.; Rayment, I. *Protein Sci.* **2009**, *18*, 747.
(57) Niemetz, R.; Gross, G. G. *Phytochemistry* **2003**, *64*, 1197.
(58) Seto, H.; Shibamiya, M.; Yonehara, H. *J. Antibiot. (Tokyo)* **1978**, *31*, 926.
(59) Komatsu, M.; Uchiyama, T.; Omura, S.; Cane, D. E.; Ikeda, H. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2646.
(60) Ng, W. L.; Bassler, B. L. *Annu. Rev. Genet.* **2009**, *43*, 197.
(61) Horinouchi, S. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 283.
(62) Höfer, I.; Crüsemann, M.; Radzom, M.; Geers, B.; Flachshaar, D.; Cai, X.; Zeeck, A.; Piel, J. *Chem. Biol.* **2011**, *18*, 381.
(63) van Rij, E. T.; Girard, G.; Lugtenberg, B. J.; Bloemberg, G. V. *Microbiology* **2005**, *151*, 2805.
(64) Martín, J.; García-Estrada, C.; Kosalková, K.; Ullán, R. V.; Albillos, S. M.; Martín, J. F. *Fungal Genet. Biol.* **2012**, *49*, 1004.
(65) Inaoka, T.; Ochi, K. *J. Bacteriol.* **2007**, *189*, 65.