Chemistry & Biology
Article

Multifactorial Induction of an Orphan PKS-NRPS Gene Cluster in *Aspergillus terreus*

Markus Gressler,[1,4] Christoph Zaehle,[2,4] Kirstin Scherlach,[2] Christian Hertweck,[2,3,*] and Matthias Brock[1,*]
[1]Microbial Biochemistry and Physiology
[2]Department of Biomolecular Chemistry,
Leibniz Institute for Natural Product Research and Infection Biology e.V., Hans Knoell Institute, Beutenbergstr. 11a, 07745 Jena, Germany
[3]Friedrich Schiller University, 07743 Jena, Germany
[4]These authors contributed equally to this work
*Correspondence: Christian.Hertweck@hki-jena.de (C.H.), Matthias.Brock@hki-jena.de (M.B.)
DOI 10.1016/j.chembiol.2010.12.011

SUMMARY

Mining the genome of the pathogenic fungus *Aspergillus terreus* revealed the presence of an orphan polyketide-nonribosomal-peptide synthetase (PKS-NRPS) gene cluster. Induced expression of the transcriptional activator gene adjacent to the PKS-NRPS gene was not sufficient for the activation of the silent pathway. Monitoring gene expression, metabolic profiling, and using a *lacZ* reporter strain allowed for the systematic investigation of physiological conditions that eventually led to the discovery of isoflavipucine and dihydroisoflavipucine. Phytotoxin formation is only activated in the presence of certain amino acids, stimulated at alkaline pH, but strictly repressed in the presence of glucose. Global carbon catabolite repression by CreA cannot be abolished by positive-acting factors such as PacC and overrides the pathway activator. Gene inactivation and stable isotope labeling experiments unveiled the molecular basis for flavipucine/fruit rot toxin biosynthesis.

INTRODUCTION

Fungi are known as prolific reservoirs of natural products. Not only are these compounds important leads for the development of therapeutics, like penicillin or lovastatin (Hoffmeister and Keller, 2007), many secondary metabolites represent infamous toxins (Möbius and Hertweck, 2009). In recent years, with the advent of whole genome-sequencing projects, it has become increasingly obvious that the biosynthetic potential of fungi is much higher than what can be observed under standardized fermentation conditions (Brakhage and Schroeckh, 2011; Chiang et al., 2009a; Hertweck, 2009). In various studied Aspergilli the majority of genes coding for polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), and hybrids thereof (PKS-NRPS) remain dormant in the absence of particular stimuli (Brakhage et al., 2008). It seems plausible that particular environmental conditions are required to activate the expression of these otherwise cryptic or silent gene loci. Present challenges in mycological research, also from a biotechnological point of view, are to understand the molecular basis of gene regulation and to find ways to stimulate the activation of otherwise downregulated or silent biosynthesis gene clusters (Scherlach and Hertweck, 2009). Because it is often not feasible to transfer and express multiple pathway genes in a heterologous host (Schümann and Hertweck, 2006), several new avenues have been established to harness the biosynthetic potential of fungi. For example, variation of cultivation conditions, addition of epigenetic modifiers or epigenetics (Bok et al., 2009; Cichewicz, 2010), and even cocultivation with trigger organisms (Schroeckh et al., 2009) in many cases resulted in the remodeling of the secondary metabolome. However, a more targeted approach involves the ectopic expression of cluster-specific activator genes (Bergmann et al., 2007). Although this appears to be a generally applicable approach, care must be taken not to oversimplify the regulatory complexity behind fungal gene expression.

In the present study we shed light on the hidden metabolic potential of *Aspergillus terreus*, a ubiquitously distributed saprobiotic mold fungus, which has also been implicated in various diseases (Laham and Carpenter, 1982; Lass-Flörl et al., 2005). Here, we demonstrate that the induction of a cryptic PKS-NRPS gene locus in *A. terreus* in fact requires fine-tuned physiological conditions, and identify the encoded metabolite as a heavily rearranged hybrid molecule that belongs to an underexplored mycotoxin family.

RESULTS

Analysis of the Orphan PKS-NPRS Gene Cluster in *A. terreus*

Inspection of the recently sequenced genome of *A. terreus* (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) revealed a gene locus around ATEG00316-ATEG00331 by a SMURF analysis (www.jcvi.org/smurf/index.php) harboring the gene ATEG00325 that could code for a PKS-NRPS hybrid (Figure 1A). However, searches for the closest relative for each predicted gene product imply that the cluster may only comprise genes spanning the region from ATEG00325 to 00331 because other genes seem to code for proteins involved in stress response, protein production, and stability (see Table S1 available online). A more detailed analysis of the deduced gene product of the PKS-NRPS hybrid

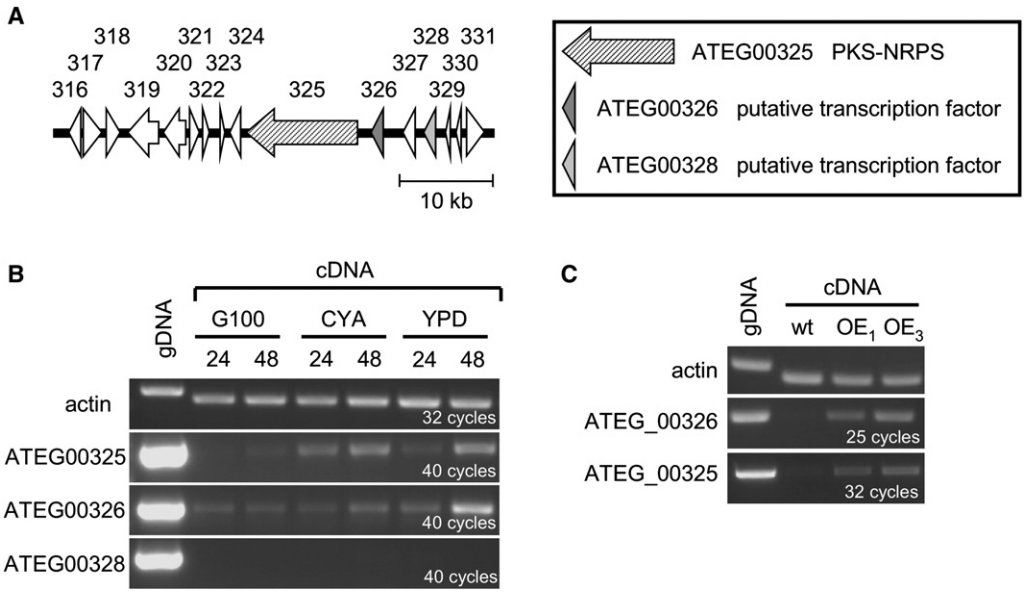

Figure 1. Organization of the PKS-NRPS Gene Cluster and Expression Analysis of Selected Genes

(A) Schematic presentation of the gene cluster with the PKS-NRPS ATEG00325 and the putative transcription factors ATEG00326 and ATEG00328 highlighted.

(B) Semiquantitative PCR for expression analysis of the PKS-NRPS and both transcription factors. SBUG844 was grown for 24 and 48 hr on glucose (G100), CYA, and YPD medium. cDNA was normalized against actin (32 cycles). The PKS-NRPS and ATEG00326 show similar expression patterns, but no induction of ATEG00328.

(C) Dependency of ATEG00325 expression on *gpdA* promoter controlled ATEG00326 expression. The wild-type SBUG844 (WT) and two strains carrying ATEG00326 under control of the *gpdA* promoter (OE_1 = single copy integration; OE_3 = triple integration) were grown for 48 hr on 100 mM glucose prior to analysis.

See also Figures S1, S2, S7, and S8, and Table S1.

from ATEG00325 shows typical motifs for the minimal set of catalytic domains for the PKS, namely ketosynthase (KS), acyltransferase (AT), acyl carrier protein (ACP), as well as signature NRPS domains for adenylation (A), condensation (C), a peptidyl carrier protein (PCP), and a C-terminal domain for reductive off-loading. The PKS fragment shows a remarkably long stretch between AT and ACP with weak homology to β-keto processing domains (Figure S1). Yet, a closer analysis indicated that these are devoid of motifs for essential catalytic sites and, thus, likely inactive (Figure S1). Furthermore, bioinformatic analysis by the program NRPS-PKS (Ansari et al., 2004) suggested that the A domain would have a preference for amino acids with an aliphatic substituent, such as leucine or isoleucine. Analysis of the flanking genes did not provide any additional clue about potential enzymatic-tailoring reactions. Taken together, the PKS-NRPS could produce a hybrid metabolite composed of a straight-chain, nonreduced (or only partially reduced) polyketide fused to an aliphatic amino acid. However, no such metabolites have been reported for *A. terreus*.

Under Standard Cultivation Conditions, *A. terreus* Does Not Produce PKS-NRPS Hybrid Molecules

Initially, we tested whether the orphan PKS-NRPS gene is expressed when culturing *A. terreus* under standard fermentation conditions. By semiquantitative RT-PCR, only a faint signal could be observed after prolonged incubation (Figure 1B). To verify whether this weak basal expression could result in metabolite formation, we compared the metabolic profiles of *A. terreus* wild-type variants and a mutant lacking an intact PKS-NRPS gene denoted SBUG844/Δ*akuB*/Δ00325 (Figure S2).

Metabolite profiles from strain SBUG844, strain SBUG844/Δ*akuB*, and the partial deletion mutant SBUG844/Δ*akuB*/Δ00325 were initially analyzed from solid media containing 50 mM glucose minimal medium, yeast peptone dextrose (YPD), or Czapek yeast autolysate (CYA) medium, and strains were grown for 7 days at 30°C on agar plates. Metabolites were extracted from agar plugs (Smedsgaard, 1997) and analyzed by LC-MS analysis. Although carbon source-dependent differences in the metabolite profiles were obtained, no marked differences among the three strains were observed (data not shown). Because secondary metabolites that are only produced in minute amounts might have been missed by the agar plug extraction, all three strains were additionally cultivated in liquid media, either as shake flask cultures or without agitation, and cultures were harvested after 65–137 hr. Although the number of metabolites detected by this method increased, no differences among the three strains were observed. Apparently, the basal expression of the PKS-NRPS gene is not sufficient for hybrid metabolite production.

gpdA Promoter-Controlled Overexpression of ATEG00326 Does Not Induce Metabolite Production

Adjacent to ATEG00325, two putative transcriptional regulator genes (ATEG00326 and ATEG00328) were detected. Semiquantitative RT-PCR showed no expression of ATEG00328, but a faint band for ATEG00326, which was similar to that observed for the

structural gene ATEG00325 (Figure 1B). Previous studies have shown that overproduction of transcriptional activators can lead to the activation of "silent" gene clusters (Bergmann et al., 2007; Chiang et al., 2009b), which tempted us to produce ATEG00326 under the control of the *A. terreus gpdA* promoter. This promoter is highly active in the presence of glucose and only induced to a minor extent on gluconeogenic carbon sources (data not shown).

The promoter was fused with ATEG00326, and the construct was used to transform the wild-type SBUG844. Southern analyses showed that strains with ectopic single, double, and triple integrations were obtained. Semiquantitative RT-PCR from mycelia grown on glucose minimal media (*gpdA*-inducing) revealed that the expression level of ATEG00326 increased with the number of integrations. In agreement with an activating effect of ATEG00326 on ATEG00325, the transcript levels for ATEG00325 increased in dependence of the ATEG00326 copy number. However, despite induction of ATEG00325, transcript levels remained at a rather low level (Figure 1C). No differences in the metabolic profiles of ATEG00326 overexpressing strains, wild-type, and the ATEG00325 deletion strain were observed. Thus, we concluded that, despite an apparent involvement of ATEG00326 in regulation of PKS-NRPS gene expression, activation is not sufficient to yield a significant proportion of the product. This indicates that specific physiological conditions might be required for product formation. Thus, to enable a rapid screening for such inducing physiological conditions, we generated a β-galactosidase producing reporter strain as a fusion of the ATEG00325 promoter with the *lacZ* gene from *E. coli*.

A *lacZ* Reporter Strain Reveals Physiological Conditions for Metabolite Production

β-Galactosidase reporter strains have been used to investigate gene expression in penicillin biosynthesis (Bergh et al., 1996). Here, we applied this tool to screen for ATEG00325 inducing conditions by fusing the ATEG00325 promoter with the *E. coli lacZ* gene. A transformant with a single ectopic integration denoted SBUG844/P00325:*lacZ* was selected for screening different cultivation conditions.

Besides the above mentioned media (glucose minimal medium, YPD, and CYA), we used potato dextrose broth (PDB), potato broth (PB), Sabouraud (Sab) medium, and minimal media supplemented with 0.5% olive oil, 1% casamino acids (CAs), or 1% peptone to screen for ATEG00325 induction. After 41 and 65 hr incubation, specific β-galactosidase activity was observed (Figure 2A), and no induction was detected on all sugar-rich media (glucose minimal medium, CYA, YPD, PDB, and Sab). In addition, β-galactosidase activity was not induced in the absence of glucose when olive oil served as sole carbon source. In contrast a sharp increase in β-galactosidase activity was observed when amino acid-containing media lacking glucose were used for cultivation, and activity was higher on peptone and CA than on PB medium (containing starch). Thus, we assumed that glucose might act as a repressor for ATEG00325 expression.

In agreement with the extremely low β-galactosidase activity in the presence of sugars, the metabolite profiles of SBUG844/Δ*akuB* and the partial deletion mutant, SBUG844/Δ*akuB*/Δ*00325*, did not show any difference. In contrast the HPLC

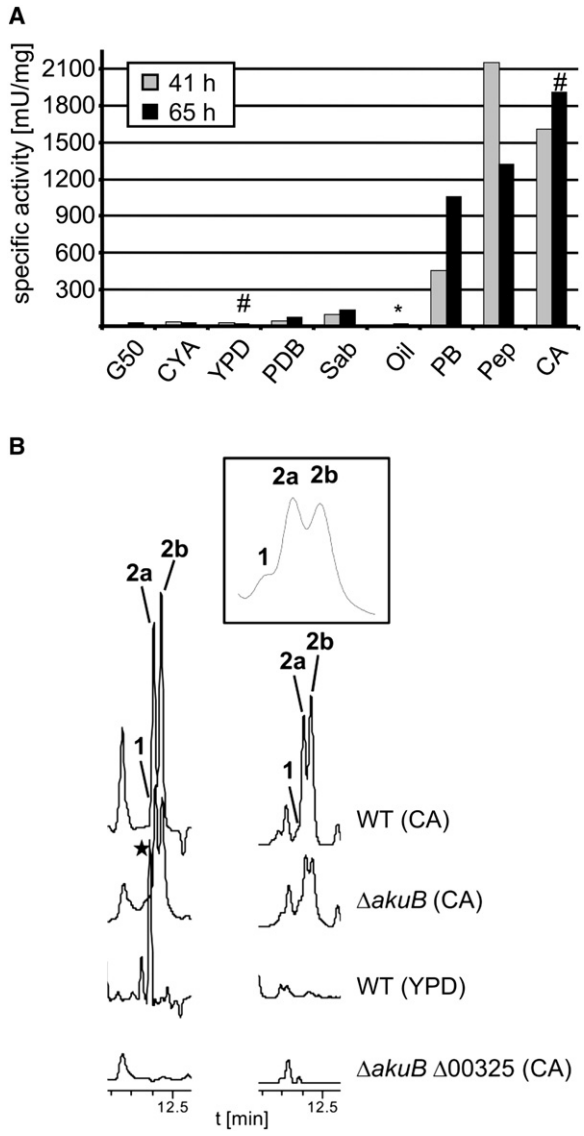

Figure 2. Expression Analysis of PKS-NRPS ATEG00325 and Metabolite Profiling

Gene expression was monitored by determination of β-galactosidase activities from the reporter strain SBUG844/P00325:*lacZ*.

(A) β-Galactosidase activities from 50 mM glucose (G50), CYA, YPD, PDB, Sab, 0.5% olive oil (Oil), PB, and media supplemented with 1% peptone (Pep) or CAs are shown. Except olive oil (only 65 hr; *), all strains were analyzed after 41 and 65 hr. Conditions selected for metabolite profiling are indicated by #.

(B) Comparative HPLC profiles from strains SBUG844, SBUG844/Δ*akuB*, and SBUG844/Δ*akuB*/Δ00325. Metabolites detected in strains with intact ATEG00325, but not in the partial deletion strain, are denoted as 1 and 2, whereby **2** splits into two isoforms **2a** and **2b**. The minor peaks detected in the deletion strain are unrelated to compounds 1 and 2a/b, as inferred from retention time and high-resolution mass spectrometry analysis. Left shows detection at 254 nm; right, nitrogen detection. Both compounds (**1** and **2**) contain nitrogen. Asterisk indicates an irrelevant peak. The inlay displays a magnification of the region of interest showing the separation of metabolites **1**, **2a**, and **2b**. The metabolite profiles from the reference strain A1156 are shown in the Figure S3.

See also Figures S5–S8.

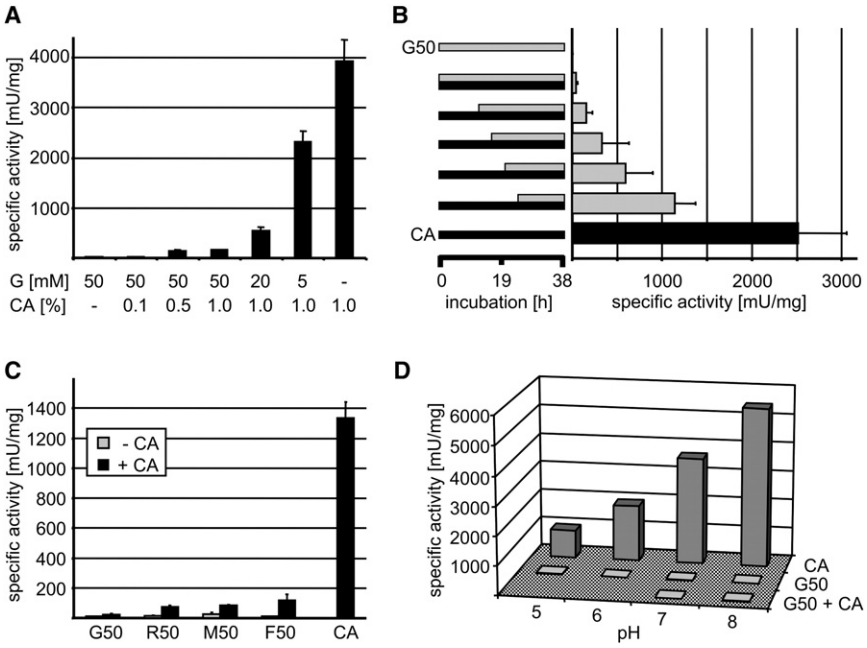

Figure 3. Sugar and pH-Dependent Expression of PKS-NRPS Gene ATEG00325

Gene expression was monitored by determination of β-galactosidase activities from the reporter strain SBUG844/P00325:*lacZ*.

(A) Expression in dependence of glucose (G) and CA concentrations (growth time = 28 hr).

(B) Inhibition of ATEG00325 expression on CA medium by glucose added at distinct time points. β-Galactosidase activity was determined after 38 hr. Time points of glucose addition are indicated by gray bars on the left side of the panel.

(C) Influence of different sugars (G, glucose; R, ribose; M, mannose; F, fructose; all 50 mM) on ATEG00325 expression. All sugars were tested alone or in combination with 1% CA. Activities were determined after 48 hr of incubation.

(D) pH-dependent expression of ATEG00325 in the presence of 1% CA, 50 mM glucose, or 50 mM glucose with 1% CA. Activities were determined after 48 hr. Error bars denote the standard deviation calculated from three biological replicates each measured in technical triplicates. See also Figures S7 and S8.

profiles of extracts from strain SBUG844/Δ*akuB* cultured in media containing only peptone or CAs showed at least one minor peak (11.8 min, *m/z* = 237) and two major peaks (12.0 and 12.2 min, both *m/z* = 239), which were completely absent from the metabolite profile of the partial ATEG00325 deletion strain (Figure 2B). Notably, according to the nitrogen detector, all of these metabolites contain nitrogen, which was expected for metabolites produced by a functional PKS-NRPS hybrid. Yet, in order to establish stable production conditions for these metabolites, more information on the regulation of gene expression and on optimal physiological conditions were required.

Various Sugars Inhibit ATEG00325 Expression

First, we investigated the specific inhibiting activity of glucose on ATEG00325 expression and tested the influence of different glucose and CA concentrations on *lacZ* expression from the reporter strain. The β-galactosidase activity was determined after 28 hr of growth. When 50 mM glucose was supplemented with 0.1%, 0.5%, or 1% CAs, induction of ATEG00325 expression remained repressed (Figure 3A). In contrast, decreasing glucose concentrations led to some induction of reporter expression, although even in the presence of 5 mM (which was completely consumed after 28 hr), the β-galactosidase activity was only 60% of that obtained in the complete absence of glucose. Thus, it can be concluded that even trace amounts of glucose inhibit ATEG00325 expression.

To test the time-dependent effect of glucose addition on ATEG00325 expression, 1% CA media were supplemented at different time points with glucose and observed over a total incubation period of 38 hr. As shown in Figure 3B, within the first 12 hr of incubation, addition of glucose strongly suppressed ATEG00325 expression. When added after 24 hr, significant amounts of β-galactosidase activity were obtained, but the specific activity remained 50% below the value without glucose. This indicates that glucose addition terminates ATEG00325 expression.

We then utilized different sugars (glucose, fructose, mannose, and ribose) to test their effect on ATEG00325 expression. None of the sugars was able to induce ATEG00325 promoter activity (Figure 3C). In combination with CAs the relative gene expression compared to CAs without sugar showed a decrease by a factor of 54 in the presence of glucose, 17 for ribose, 15 for mannose, and 11 for fructose. These data confirm that all sugars tested mediate a strong negative effect on ATEG00325 expression.

CreA Represses ATEG00325 Expression

CreA is a carbon catabolite repressor of filamentous fungi, repressing transcription of genes required for alternative carbon source utilization in the presence of glucose (Felenbok et al., 2001; Ilyés et al., 2004; Tamayo et al., 2008) by binding to the GC-rich consensus sequence 5′- (C/G)YGGRG-3′ in promoter regions (Panozzo et al., 1998).

CreA-mediated gene expression has not been studied for *A. terreus*, but glucose repression tempted us to search for a CreA homolog in the *A. terreus* genome and revealed a protein with 66% sequence identity to *A. nidulans* CreA (protein accession XP_001208483). Despite the relatively low overall identity due to a truncated C-terminal region (96 bp missing), the two DNA-binding zinc finger domains (Jacobs, 1992) are highly conserved in the *A. terreus* protein, with 95% and 96% identity and 100% similarity (Dowzer and Kelly, 1991). Thus, the high conservation of the zinc finger domains implies that the consensus sequence identified for *A. nidulans* CreA might also apply to CreA from *A. terreus*. Indeed, four putative CreA-binding sites were detected in the promoter of ATEG00325 (Figure 4). Although further analyses need to confirm the functionality of these binding sites in glucose-mediated repression of

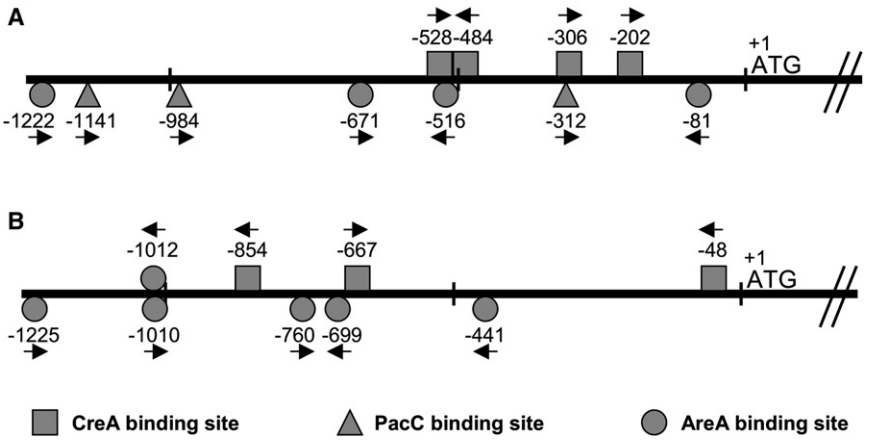

Figure 4. Schematic Presentation of Putative CreA, PacC, and AreA Binding Sites within the First 1250 bp of the ATEG00325 and ATEG00326 Promoter Regions

Arrows denote the orientation of the consensus sequence. Numbers denote the 5′ position of the consensus in respect to the translational start point. (A) The PKS-NRPS promoter (P00325) contains four AreA and CreA, and three PacC sites. (B) The promoter of the transcription factor (P00326) contains three CreA and six AreA-binding sites, but no consensus sequence for PacC binding.

See also Figures S7 and S8.

ATEG00325, these data are highly suggestive for an involvement of CreA.

Gene Expression Levels Depend on the Environmental pH

CAs revealed the strongest induction of ATEG00325, and during growth the pH increased to ≥9.0. To determine an effect of environmental pH on ATEG00325 expression, the pH was adjusted in the range between 5.0 and 8.0 by buffering media with sodium phosphate. SBUG844/P00325:*lacZ* was cultivated in media composed of glucose, glucose with CA (only pH 7.0 and 8.0), or only CA. After 48 hr of cultivation, the pH was retested, confirming that the initial pH had remained nearly constant. Activity determination showed that glucose persisted as a potent inhibitor of gene expression. However, on CA the specific β-galactosidase activity steadily increased with increasing pH of the medium (Figure 3D).

PacC is the main transcriptional regulator responding to alkaline pH in filamentous fungi (Denison, 2000). An *A. terreus* PacC homolog was detected by BLAST analysis at locus tag ATEG04740, but the *A. terreus* protein appeared C-terminally extended, probably due to an incorrect intron prediction overwriting a putative stop codon. However, PacC activation occurs by C-terminal truncations, and the N-terminal sequence containing three zinc finger domains is of major importance. The two DNA-binding fingers are identical in the *A. terreus* protein, and it is likely that the *A. nidulans* consensus sequence 5′-GCCARG-3′ (Tilburn et al., 1995) is also recognized. Three putative PacC-binding sites were detected in the promoter of ATEG00325 (Figure 4). Thus, increased expression of ATEG00325 at alkaline pH seems PacC dependent, but glucose repression dominates the effect of alkalization.

Distinct Amino Acids Are Sufficient but Not Essential to Activate ATEG00325 Expression

CAs were the best-suited inducers for ATEG00325 expression, and we were interested in elucidating the role of distinct amino acids on gene expression. A prescreening was performed with strains SBUG844/Δ*akuB* and SBUG844/Δ*akuB*/Δ00325 on agar plates, each containing one of the 20 proteinogenic L-amino acids as sole nutrient source and X-Gal to monitor β-galactosidase activity. Only Ala, Arg, Asn, Asp, Glu, Gln, and Pro showed different degrees of X-Gal conversion. In liquid cultures the strongest induction was observed by Asn, followed by Gln and Pro (Figure 5A), whereas Ala, Arg, Asp, and Glu remained at low activity levels. Addition of Arg to Asn as well as the addition of ammonium chloride or nitrate neither stimulated nor inhibited ATEG00325 gene expression (data not shown). Because the noninducing Asp is a precursor of Asn, we tested the combination of Asp with different nitrogen-containing compounds

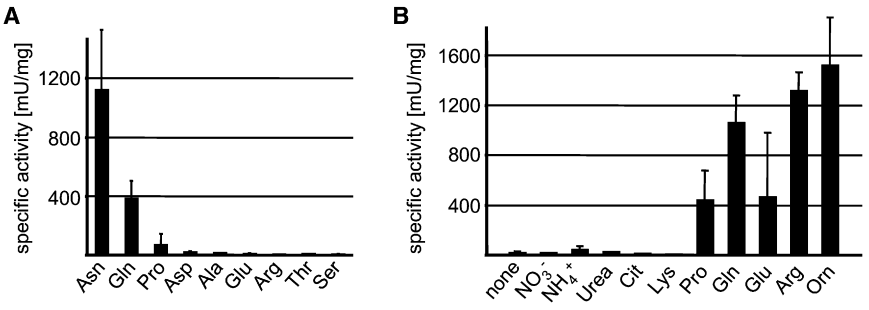

Figure 5. Amino Acid and Nitrogen Source-Dependent Expression of ATEG00325

Gene expression was monitored by determination of β-galactosidase activities from the reporter strain SBUG844/P00325:*lacZ*. Amino acids are denoted in the three-letter code.

(A) Single amino acids served as sole nutrient sources, and β-galactosidase activity was determined after 72 hr.

(B) The noninducing amino acid aspartate (50 mM) served as basal nutrient source, and media were supplemented with different nitrogen sources (10 mM). Activity was determined after 62 hr of cultivation. Cit, citrulline; Orn, ornithine. For a detailed explanation, refer to the text. Error bars denote the standard deviation calculated from three biological replicates each measured in technical triplicates.

See also Figures S7 and S8.

Table 1. Arginase and β-Galactosidase Activities from Reporter Strains Cultivated on Different Nutrient Sources

Nutrient Source	Arginase (mU/mg) P*agaA*:*lacZ*[a]	β-Galactosidase (mU/mg) P*agaA*:*lacZ*[a]	β-Galactosidase (mU/mg) P00325:*lacZ*[b]	β-Galactosidase (mU/mg) P00325:*lacZ* P*agaA*:00326[c]
1% CAs	ND	168 ± 79	1419 ± 225	1760 ± 166
50 mM glucose	50 ± 8	32 ± 11	7 ± 0	7 ± 2
50 mM arginine	2510 ± 129	733 ± 65	3 ± 0	1202 ± 415
50 mM arginine/50 mM glucose	1620 ± 288	429 ± 78	12 ± 1	51 ± 7

Errors denote the standard deviation from three independent biological replicates measured in technical triplicates. ND, not determined. See also Figures S4 and S8.

[a] Arginase and β-galactosidase activity were determined from strain SBUG844/P*agaA*:*lacZ* containing a fusion of the *agaA* promoter with the *E. coli lacZ* gene.

[b] β-Galactosidase activity was determined from strain SBUG844/P00325:*lacZ* containing a fusion of the ATEG00325 promoter with the *E. coli lacZ* gene.

[c] β-Galactosidase activity was determined from strain SBUG844/P00325:*lacZ* additionally containing a fusion of the *agaA* promoter with the transcription factor ATEG00326.

(Figure 5B). No activation was observed when Asp was used in combination with nitrate, ammonium, urea, citrulline, or Lys. Activity with Gln and Pro was enhanced, and ATEG00325 transcription was activated in combination with Glu, Arg, and ornithine. This indicates that intracellular amination of Asp might provide Asn-inducing gene expression.

In sum, single amino acids, such as Asn or Gln, are able to trigger gene expression on their own, but their presence can be substituted by a mixture of other amino acids, which implies a role of nitrogen sources in ATEG00325 activation. In this respect, gibberellin production of *Gibberella fujikuroi* is regulated by the GATA transcription factor AREA, which is involved in the derepression of genes involved in nitrogen utilization (Mihlan et al., 2003). Similarly, four putative GATA-binding sites are present in the promoter region of ATEG00325 (Figure 4) and additionally six sites in the promoter of the putative transcription factor ATEG00326.

Arginase Promoter-Controlled ATEG00326 Expression Enables PKS-NRPS Induction

In our initial attempts to induce ATEG00325 by overproducing the putative transcription factor ATEG00326, the requirement of glucose for high *gpdA* promoter activity might have hindered a strong induction. Thus, the putative arginase promoter (P*agaA*, ATEG04679) was analyzed because it was assumed to be inducible by arginine without strict glucose dependency. β-Galactosidase and arginase activity were determined from a reporter strain with a P*agaA:lacZ* fusion grown on media with glucose, arginine, and arginine with glucose. Both activity determinations showed consistent results with a strict requirement on arginine for activation and only a slight repression by glucose (Table 1). Thus, the transcription factor ATEG00326 was fused with P*agaA* and transferred into the reporter strain SBUG844/P00325:*lacZ* to investigate the effect of ATEG00326 expression on ATEG00325 expression. Results were compared to the parental strain SBUG844/P00325:*lacZ* (Table 1). On CA the activity level of the *agaA* promoter fusion strain only slightly increased over that of the parental strain, indicating that ATEG00325 is already induced near its maximum level. In contrast, on glucose neither the *agaA* promoter fusion strain nor the parental strain showed any induction of ATEG00325. When arginine served as sole nutrient source, a strong stimulation of expression of the ATEG00325 promoter was observed only in the strain carrying the P*agaA*:00326 fusion. However, supplementation with glucose completely repressed induction of ATEG00325. Induction in the absence of glucose was confirmed by identification of the two metabolites from the culture broth of this strain (data not shown). Semiquantitative RT-PCR was performed to study the induced expression of genes belonging to the cluster surrounding ATEG00325. This analysis revealed that ATEG00325, 00326, 00329, 00330, and 00331 were all induced by controlled overexpression of the transcription factor, and the induction pattern resembled that obtained from mycelium grown on CAs (Figure S4).

From these results we conclude that: (i) ATEG00326 acts as a transcriptional activator for ATEG00325 expression; (ii) ATEG00326 expression is sufficient to stimulate all genes required for product formation; but (iii) only in the absence of glucose, which mediates a dominant negative effect on ATEG00325 expression; and (iv) as assumed above, the cluster seems to span mainly the region starting at ATEG00325 and ending at ATEG00331.

A. terreus Is Capable of Producing Members of the Flavipucine Family of Mycotoxins

Expression and LC-MS analysis revealed that the expression of the PKS-NRPS gene correlates with metabolite formation, and production is dramatically increased in the presence of CAs at alkaline pH in the strict absence of glucose. To obtain larger metabolite quantities for a full structure elucidation, the fermentation of wild-type strain SBUG844 was upscaled (2 × 4 liters). Samples taken at 24, 36, 48, and 72 hr showed maximum production levels toward the end of fermentation.

After chromatographic purification of the nitrogen-containing metabolites **1** and **2**, their structures were fully elucidated (Figure 6A) by 1D and 2D nuclear magnetic resonance (NMR) spectroscopy and MS. The ^{1}H and ^{13}C NMR data obtained for **1** and **2** appeared to be highly similar, indicating that these compounds are related. From high-resolution electrospray ionization mass spectrometry (HRESI-MS), we deduced the molecular formula of $C_{12}H_{15}O_4N$ for **1** and $C_{12}H_{17}O_4N$ for **2**.

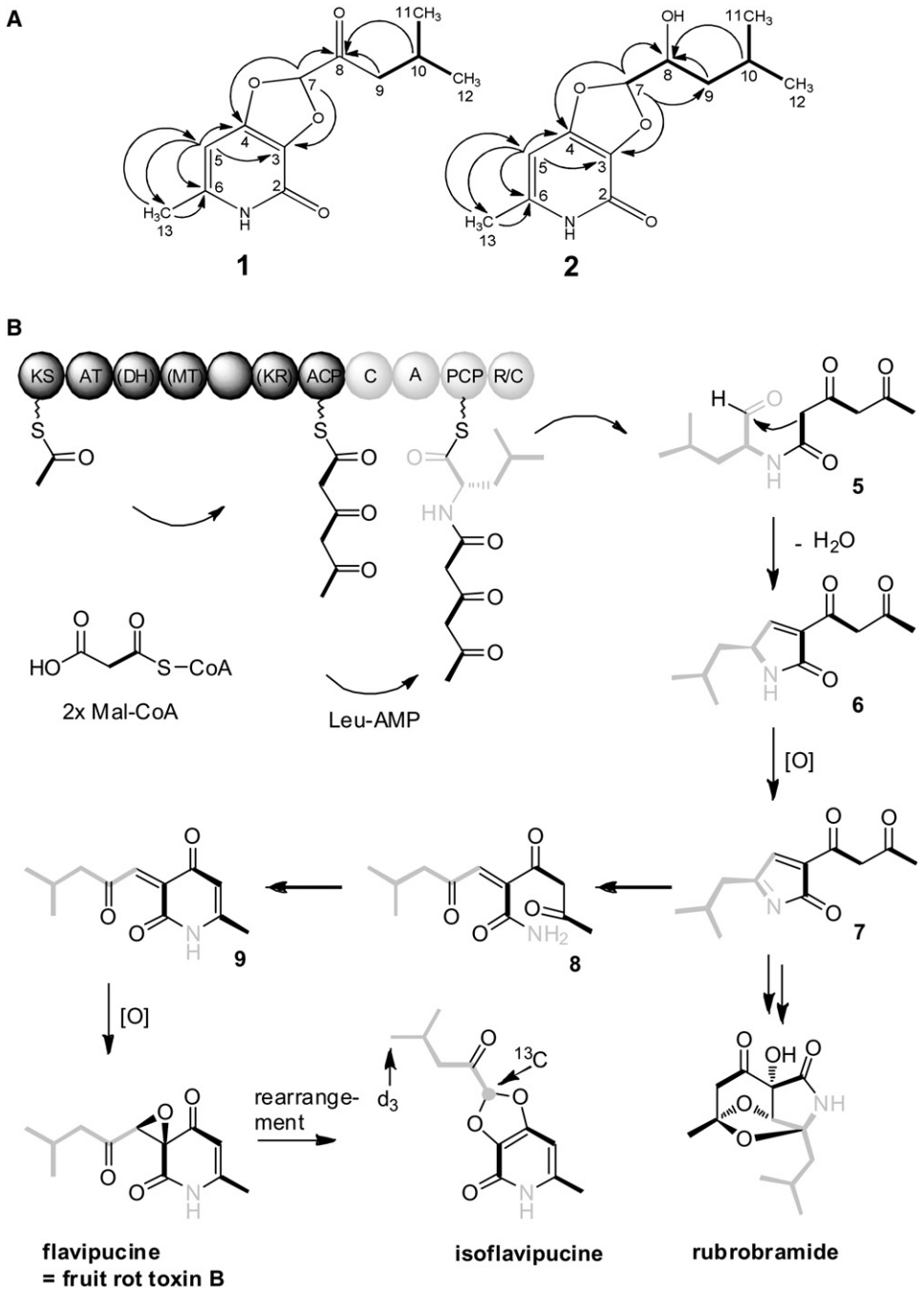

Figure 6. Metabolites Produced by ATEG00325 and Proposed Biosynthesis

(A) Structures of isoflavipucine **1** and dihydroisoflavipucine **2**.

(B) The domain architecture of ATEG00325 implies the formation of a hybrid molecule. Modules in brackets seem not to contribute to biosynthesis. After formation of linear PKS-NRPS product **5** by ATEG00325, the first cyclization could take place **6**. Starting from the following oxidation step **7** hydrolysis involving a trans-amidation step might lead to the formation of **9**, which is further oxidized to flavipucine. Formation of isoflavipucine from flavipucine requires an unusual rearrangement. Alternative rearrangement reactions could build up rubrobramide, representing a branching of flavipucine biosynthesis.

See also Figure S5 and Table S2.

Heteronuclear multiple bond correlation (HMBC) experiments revealed the common pyridone substructures for **1** and **2**. The unusual chemical shift of C-7 (Table S2) for a CH group with δ 113.4 ppm for compound **2** and δ 106.1 ppm in compound **1** indicates a dioxygen substitution. The structure of the branched aliphatic side chain of compounds **1** and **2** was deduced from correlation spectroscopy (H,H-COSY) data. ^{13}C NMR data for compound **1** showed a chemical shift of C-8 (δ 202.0 ppm) that

indicates the presence of a keto group, which is not present in compound **2** (Figure S5). From these analyses we concluded that metabolites **1** and **2** represent isoflavipucine and dihydroisoflavipucine, respectively.

Isoflavipucine (**1**), a constitutional isomer of the antibiotic flavipucine, is a known metabolite that has previously been isolated from *Aspergillus flavipes* (Casinovi et al., 1968; Findlay et al., 1977). Dihydroisoflavipucine (**2**) has been described as a semisynthetic product obtained through chemical reduction of isoflavipucine (Findlay et al., 1977), but it has not yet been isolated as a natural product. We succeeded in separating diastereomers of dihydroisoflavipucine by an achiral stationary HPLC phase (2a and 2b in Figure 2B), and using a chiral stationary phase allowed the separation of all four stereoisomers of **2** (Figure S6). The occurrence of all four possible stereoisomers implies that there is no stereochemical control over the formation of these two asymmetric centers during biosynthesis.

Isoflavipucine Is a PKS-NRPS Hybrid Metabolite Derived from Leucine

Although the formation of compounds **1** and **2** strictly correlates with the presence of the PKS-NRPS hybrid, their structures do not immediately provide an insight into the biosynthetic pathway. Former isotope-labeling experiments using [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate indicated that the pyridone moiety of flavipucine is derived from acetate (Grandolini et al., 1987). However, no labeling of the isobutyl side chain of the nitrogen heterocycle was observed, and its biogenetic origin has remained enigmatic. A plausible biosynthetic precursor for this side chain would be leucine. To support this hypothesis we performed labeling experiments with [1-^{13}C]L-leucine and [5-$^{2}H_3$]L-leucine. For this purpose we composed a synthetic medium containing L-asparagine and L-arginine, supporting both sufficient growth rates and high PKS-NRPS activity and supplemented the medium with unlabeled, deuterated, or ^{13}C-labeled L-leucine (2.5 mM), respectively. Samples were analyzed by LC-MS to compare the mass peaks for labeled and native **1**. The labeled sample resulting from the incorporation of [1-^{13}C]L-leucine showed an M+1 mass shift, whereas the sample resulting from the incorporation of [5-$^{2}H_3$]L-leucine showed an M+3 mass shift. To identify the position of ^{13}C labeling in **1**, the metabolite was purified by preparative LC-MS and analyzed by NMR. Comparison of carbon NMR data showed that the signal of C-7 in the ^{13}C NMR spectrum of isoflavipucine was enriched (calculated incorporation rate 92%). Detailed analysis of the ^{13}C NMR data of isoflavipucine (**1**) resulting from the incorporation of [1-^{13}C]L-leucine showed a doublet for C-8 at 202.1 and 201.8 ppm (J = 46.8 Hz), respectively (Figure S4). Consequently, both isotope-label experiments showed labeling of the aliphatic chain, and the carbonyl (C-1) of L-leucine is detected in metabolite **1**, indicating that the entire L-leucine backbone was incorporated, albeit disrupted.

DISCUSSION

In this study we have identified a cryptic PKS-NRPS gene cluster in *A. terreus* and unveiled its function using a multidisciplinary approach. Our strategy involved the generation of gene deletion mutants, the construction of reporter strains, screening for gene expression and metabolite production under a variety of culture conditions, and the overproduction of a transcription factor under the control of different inducible promoters.

Gene-activation strategies involving the overproduction of a pathway-specific transcription factor have proven helpful to identify new products from secondary metabolite gene clusters, which appear not inducible under standard laboratory conditions. An example for a successful application of this strategy is the identification of the PKS-NRPS derived metabolite aspyridone from *A. nidulans* (Bergmann et al., 2007). However, here, we show that such a simple approach might be insufficient. Our analyses revealed that glucose acts as a dominant repressing factor, even when the transcription factor was expressed under the control of the glucose-induced *gpdA* promoter. This negative effect of glucose, which is most likely mediated by the global carbon catabolite repressor CreA, cannot be abolished by other positive-acting factors such as PacC. The processed form of PacC (PacC^{27}) can directly act as an inducer of genes required for growth at alkaline pH and additionally acts as a repressor of genes required under acidic conditions (Penalva et al., 2008). In terms of secondary metabolite production, PacC was shown to stimulate penicillin production at alkaline pH (Brakhage et al., 2004). Similarly, the production of iso- and dihydroisoflavipucine in *A. terreus* is induced at alkaline pH but remains negatively dominated by glucose. Furthermore, the available nitrogen sources, especially amino acids, are important for gene activation, as indicated by our expression analyses and several AreA-binding sites in the promoters of ATEG00325 and ATEG00326. Thus, despite the ability of the transcription factor ATEG00326 to induce PKS-NRPS expression, regulators of primary metabolism, e.g., CreA, PacC, and AreA, possess a major impact.

In sum these results imply that the activation of downregulated or silent genes through the expression of activator genes and/or promoter exchange may not be sufficient for metabolite production. Thus, the use of a reporter system is strongly recommended to screen for nutritional and other physiological conditions, which might negatively affect gene expression or may be crucial for pathway induction.

Through our expression analyses and a targeted gene inactivation, we finally succeeded in the functional analysis of the, to our knowledge, only complete PKS-NRPS hybrid from *A. terreus* and showed that this multifunctional enzyme is involved in the biosynthesis of flavipucine-type molecules. A full characterization of the metabolites revealed the structures of isoflavipucine and dihydroisoflavipucine. Although isoflavipucine and flavipucine, the biosynthetic progenitors of dihydroisoflavipucine, have been isolated from *Aspergillus flavipes* (Casinovi et al., 1968; Findlay et al., 1977; Findlay and Radics, 1972; Grandolini et al., 1987), to our knowledge, they have not been reported as metabolites of *A. terreus*. Due to the close phylogenetic relation between the *Terrei* and *Flavipedes* sections (Varga et al., 2005), a conservation of secondary metabolite clusters among these species is well conceivable. Aside from that, also other ascomycetes such as *Macrophoma* species or *Cladobotryum rubrobrunnescens* produce flavipucine derivates or closely related compounds such as fruit rot toxin A (Sassa and Onuma, 1983) or rubrobramide (Wagner et al., 1998), respectively, but the responsible gene clusters have remained unknown. Due to

cytotoxic, antibiotic, and phytotoxic activities of these compounds, the identification of the corresponding biosynthesis gene cluster from *A. terreus* is also of high importance from medical and agricultural points of view.

In this respect the identification of the PKS-NRPS and the requisite metabolites, in conjunction with our isotope-labeling experiments, provide new insights into the biosynthesis of these unusual compounds. Inspection of the architecture of the multifunctional hybrid synthetase and the observed incorporation of labeled [1-^{13}C]$_L$-leucine and [5-$^{2}H_3$]$_L$-leucine allows us to propose a model for isoflavipucine (**1**) biosynthesis (Figure 6B).

First, the PKS part of the PKS-NRPS could assemble a triketide from an acetyl starter and two malonyl-CoA extender units. The poly-β-keto intermediate would then be fused to the leucine unit by the NRPS part. The resulting amide would be liberated from the PKS-NRPS through reductive release of the linear PKS-NRPS product from the enzyme complex. According to a comparative analysis of the release domain (Figure S1), we assume a member of the short-chain dehydratase/reductase (SDR) protein family containing a catalytic triad composed of serine, tyrosine, and lysine and a Rossmann fold for binding cofactors like FAD or NAD(P). Yet, the extended core motif described by Kleiger and Eisenberg (2002) could not be elucidated to determine if FAD or NAD(P) is bound. An important aspartate residue adjacent to the catalytic triad, which has also been described for the biosynthesis of cyclopiazonic acid (Liu and Walsh, 2009), could facilitate the postulated reductive release of the PKS-NRPS product. However, an alternative role of the reduction domain as a catalyst for a Dieckmann reaction for ring closure, as described in the biosynthesis of equisetin (Sims and Schmidt, 2008), cannot be excluded at this stage.

As in various related PKS-NRPS pathways, initially a tetramic acid derivative could be formed through heterocyclization. Various subsequent steps are required to transform this predicted intermediate into the final product. Most remarkably, because the C-N bond is cleaved and a keto group is found in place of the former amino group of leucine, the formation of flavipucine would involve, at least formally, a transamination step, which is unprecedented for this group of metabolites. Finally, an internal rearrangement with formation of the pyridine-2,4 (1*H*,3*H*)-dione leads to the deoxo precursor of flavipucine. In a synthetic model by heating the crystalline flavipucine at 139°C for 15 min (Findlay et al., 1977), it has been shown that flavipucine rearranges to yield the isosteric ketal isoflavipucine (**1**). The model for this intriguing oxidative cleavage is supported by our labeling experiments, which showed that the ketal carbon is derived from C-1 of leucine. Dihydroisoflavipucine is likely formed by a ketoreduction. In this context it should be noted that we detected all four possible stereoisomers of dihydroisoflavipucine, suggesting that both the epoxide-ketal rearrangement and the C-8 ketoreduction are not highly stereospecific. The exact mechanism of the heterocyclization steps will require more in-depth biochemical studies in the future. However, from the present results we can already conclude that flavipucine biosynthesis involves an unprecedented rearrangement cascade. Due to the close structural relationship of fruit rot toxin A and rubrobramide, it appears likely that all metabolites are produced by a similar PKS-NRPS. Because both metabolites, flavipucine and rubrobramide, can be isolated from *C. rubrobrunnescens* (Wagner et al., 1998), it is conceivable that rubrobramide emerges from an alternative rearrangement event involving the same biosynthetic precursor. Thus, the *A. terreus* PKS-NRPS exemplifies an important new member of the growing family of fungal PKS-NRPSs, which play key roles in the biosynthetic pathways leading to fusarin (Song et al., 2004), equisetin (Sims et al., 2005), cytochalasin (Schümann and Hertweck, 2007), tenellin (Eley et al., 2007), aspyridone (Bergmann et al., 2007), pseurotin (Maiya et al., 2007), and cyclopiazonic acid (Tokuoka et al., 2008). Finally, our work not only presents an approach for the systematic induction of a silent fungal pathway but also highlights the tight crosslink between primary and secondary metabolism.

SIGNIFICANCE

In the post-genomic era with a dramatically increasing number of sequenced fungal genomes, it has become obvious that the number of putative biosynthesis genes clearly outnumbers the metabolites observed under standardized fermentation conditions. Apparently, the majority of secondary metabolites are overlooked, and novel strategies are required to tap this vast biosynthetic potential. Various avenues to the activation of silent or downregulated pathways have been described, including global triggers, like exerting stress and employing epigenetics, and pathway-specific regulators. Herein, to our knowledge, we describe for the first time that expression of a pathway-specific activator gene alone may be insufficient for metabolite production because it may be negatively dominated by other factors. Indeed, activation strategies may be more complex than previously believed. Specifically, we disclosed that CreA-mediated carbon catabolite repression can override induced expression of a pathway-specific transcription factor gene. By using *lacZ* reporter strains and through systematically varying culture conditions, we found that the orphan biosynthesis gene cluster is only activated in the presence of certain amino acids such as asparagine, stimulated at alkaline pH, but strictly repressed in the presence of glucose. The finding that prerequisites for metabolite production can be multifarious at gene and transcript levels is of great importance for future genome-mining expeditions.

Furthermore, our work is an important addition to the body of knowledge on the biosynthetic capabilities of *A. terreus*. The pathogenic fungus possesses the potential to produce a great variety of secondary metabolites, yet its capability to produce toxins of the flavipucine/fruit rot toxin family has been unknown. Information about the genetic basis and regulation of phytotoxin production are significant in the area of fungal pathogenicity and agriculture.

Finally, functional genetics, bioinformatics, and isotope labeling provided the first insight into the biosynthesis of isoflavipucine and related hybrid molecules, involving a mechanistically intriguing rearrangement sequence.

EXPERIMENTAL PROCEDURES

Chemicals and Materials

Details for suppliers of chemicals, solvents, molecular biological kits, and equipment are listed in the Table S3.

Strains and Genome Information

The genome of the *A. terreus* strain A1156 (NIH2624) has been sequenced, and all locus tag and sequence information refers to this strain. Sequence information is available from the BROAD Institute website (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Strain A1156 was obtained from the Fungal Genetics Stock Center and was used in some experiments as reference strain. Most work was performed on *A. terreus* strain SBUG844, available from an in-house culture collection. Details on the relationship between SBUG844 and A1156 from sequence analyses can be found in Figure S7. From both strains, Δ*akuB* derivatives with defect in the nonhomologous end-joining repair mechanism (Kück and Hoff, 2010) were generated (A1156/Δ*akuB* and SBUG844/Δ*akuB*), which facilitated the targeted gene deletion by homologous recombination (data not shown). For plasmid propagation the *E. coli* strain DH5α (Invitrogen) was used.

Media and Cultivation Conditions

Aspergillus minimal media (AMM, http://www.fgsc.net/methods/anidmed.html) with nitrate as nitrogen source and adjusted to pH 6.5 generally served as basal medium. Solid media were prepared by adding 1.5%–2% agar prior to autoclaving. The following nutrient sources were added at concentrations and combinations as indicated in the respective experiments: 5–100 mM D-glucose; 50 mM D-fructose, D-mannose, or D-ribose; 0.1%–1% CAs; 1% peptone; 2.5–100 mM of proteinogenic L-amino acids; 0.5% olive oil. In the presence of amino acids, nitrate was generally omitted from the medium. In experiments requiring a fixed pH value, the medium was additionally buffered by supplementation with 150 mM sodium phosphate adjusted to the respective pH. As complex media, PDB, YPD (10 g/l yeast extract, 20 g/l D-glucose, 20 g/l peptone), PB, Sab, and CYA (Pitt, 1979) were used. All complex media except YPD were adjusted to pH 6.5. β-Galactosidase activity from solid media was determined by addition of 50 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) after autoclaving. Liquid media were inoculated with conidia suspensions at a final concentration of 1×10^6–2×10^6 conidia/ml and incubated on a rotary shaker at 30°C and 200 rpm, if not indicated otherwise.

For preparation of conidia suspensions, conidia were harvested in distilled water from AMM/glucose solid media incubated for 3 days at 37°C, and suspensions were filtered over 40 μm cell strainers. Conidia suspensions were stored at 4°C for a maximum of 2 weeks without significant loss of viability. Large-scale fermentations were performed as described in the Supplemental Experimental Procedures.

Molecular Biological Techniques

All oligonucleotides used in this study have been consecutively numbered (P1, P2, etc.), and detailed information is shown in Table S4. Details on kits and reagents used for molecular biological procedures are provided in the Supplemental Experimental Procedures. Chemically competent *E. coli* DH5α cells were transformed by standard techniques (Maniatis et al., 1982). *A. terreus* strains were transformed from protoplasts as described for *Aspergillus fumigatus* (Brock et al., 2008) except that 1.2 M sorbitol instead of 0.6 M KCl was used as osmotic stabilizer. As selection marker, either 180 μg/ml hygromycin B (Roche) or 0.1 μg/ml pyrithiamine (Sigma-Aldrich) was used.

Semiquantitative PCR

For comparative quantification of transcript levels, cDNA was used as a template, and amplification was performed in a SpeedCycler (Analytik Jena) using the GoTaq Polymerase (Promega). To adjust template concentrations, actin transcript levels (locus ATEG06973) were used as a reference (oligonucleotides P1 and P2). Transcripts for ATEG00325, ATEG00326, and ATEG00328 were analyzed by using oligonucleotides P3–P10. Bands were quantified by the Quantity One software from ethidium bromide-stained agarose gels as described previously (Brock et al., 2008).

Strain Constructions

Details on the cloning procedures for plasmid construction to obtain fungal transformants are given in the Supplemental Experimental Procedures. In brief, ATEG00325 deletion mutants were generated by replacing the first 1594 bp of the ATEG00325 coding region and 22 bp upstream the ATG start codon with the pyrithiamine resistance cassette (*ptrA*) from plasmid ptrA-pJET1 (Fleck and Brock, 2010) (Figures S8A–S8D). β-Galactosidase reporter strains were generated by using the *lacZ* gene from *E. coli* fused with the arginase promoter (P*agaA*, locus ATEG04679) or the ATEG00325 promoter (P00325), respectively. As selection marker, either the *ptrA* or the hygromycin B resistance cassette (*hph*) (Fleck and Brock [2010]) was used (Figures S8F and S8G). For generation of transcription factor overexpression strains, the *A. terreus gpdA* (P*gpdA,* locus ATEG10199) and *agaA* promoters were fused with the putative transcription factor ATEG00326. The *ptrA* or *hph* resistance cassette was used for transformation of strain SBUG844 and strain SBUG844/P00325:*lacZ*, respectively (Figures S8E and S8H). All transformants were checked by Southern blot analysis with digoxygenin-labeled probes as recommended in the manufacturer's protocol.

Cell-Free Extracts and Enzyme Assays

For preparation of cell-free extracts, mycelia were harvested from liquid cultures, washed with sterile tap water, pressed dry, frozen in liquid nitrogen, ground to a fine powder, and resuspended in 50 mM Tris/HCl buffer (pH 8.0). Debris was removed by centrifugation at 14,800 × g, and the cell-free extract was used for activity determination on a Lambda 25 UV/VIS double-beam spectrophotometer (Perkin Elmer). Protein concentrations were determined by the Bio-Rad Protein assay (Bio-Rad) as described in the manufacturer's protocol with bovine serum albumin as standard.

β-Galactosidase activity was determined by a continuous assay as described previously (Ebel et al., 2006). For determination of arginase activity, a coupled enzymatic assay with urease and L-glutamate dehydrogenase as helping enzymes was developed. Details are provided in the Supplemental Experimental Procedures. Activities were generally determined from three biological replicates measured in three technical replications. Error bars in figures represent the standard deviation obtained from all data points.

Analysis, Isolation, Purification, and Structure Elucidation of Isoflavipucine and Dihydroisoflavipucine

For metabolite extraction from liquid cultures, fluid-fluid extraction of culture broth with ethylacetate was performed. When mycelia were analyzed, cells were disrupted in ethylacetate by using an IKA-UltraTurrax T25 (Janke & Kunkel GmbH). The organic phase was collected and concentrated in vacuo.

Standard analysis of the extracts from mutants and wild-type strains was performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent) operating in alternating ionization mode and an Antek 8060 HPLC-CLN-detector (Antek Instruments GmbH). As stationary phase, a C8 column (Zorbax Eclipse XDB C8, 4.6 × 150 mm; 5 μm) with a binary solvent system consisting of methanol (solvent B) and water containing 0.1% formic acid (solvent A) was used. The following gradient with a flow rate of 1 ml/min was applied: 0.5 min, 10% B; 0.5–15 min, 10%–90% B; 15–17 min, 90% B; 17–17.5 min, 90%–100% B; 17.5–22 min, 100% B; and 22–23 min, 100%–10% B. For separation of stereoisomers a chiral stationary phase column (Phenomenex Lux Cellulose 2, 250 × 4.6 mm; 5 μm) with a solvent system consisting of heptane (55%) and methanol/ethanol 1:1 (45%) under isocratic conditions with a flow rate of 0.5 ml/min at 40°C was used. The crude ethylacetate extract solved in methanol was applied to silica gel chromatography (30 × 120 mm) for initial purification using a stepwise gradient: chloroform, chloroform/methanol (98:2, 95:5, 90:10, 50:50), and 100% methanol. For further purification of metabolites from silica gel chromatography, respective fractions were applied to preparative LC-MS using a Waters autopurification system (Waters GmbH) consisting of a 2525 binary gradient module, a 515 HPLC pump, a column fluidics organizer, a 2996 PDA, a ZQ 2000 mass detector, and a 2767 sample manager. The Waters X-Terra prep MS C18 column (19 × 50 mm; 5 μm) was equilibrated with 90% solvent A (water/0.1% formic acid) and 10% solvent B (methanol), with a flow rate of 20 ml/min. The following gradient was applied: 1 min, 10% B; 1–4 min, 10%–30% B; 4–20 min, 30% B; 20–23 min, 30%–100% B; 23–25 min, 100% B; and 25–27 min, 100%–10% B.

HRESI-MS was carried out on an Accela UPLC-system (Thermo Scientific) combined with an Exactive Mass Spectrometer (Thermo Scientific) operating in positive ionization mode. Separation was carried out on a Betasil C18 column (2.1 × 150 mm, 3 μm; Thermo Scientific) using water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, as binary solvent system. A flow

rate of 250 μl/min and the following gradient was used: 0–1 min, 5% B; 1–16 min, 5%–98% B; 16–19 min, 98% B; and 19–20 min, 98%–5% B.

NMR spectra were recorded on a Bruker Avance III 500 and a Bruker Avance III 600 spectrometer (Bruker BioSpin GmbH) equipped with a cryoprobe head using DMSO-d_6 as solvent and internal standard.

Feeding Studies

Feeding studies of SBUG844 were carried out in 100 ml AMM containing 25 mM arginine and asparagine, and supplemented at start of fermentation with 2.5 mM of [1-^{13}C]L-leucine, [5-$^{2}H_3$]L-leucine (both Cambridge Isotope Laboratories), or unlabeled L-leucine, respectively. Cultures were inoculated with 2 × 10^6 conidia/ml and incubated on a rotary shaker for a total of 72 hr at 30°C and 200 rpm. Addition of leucine was repeated after 48 hr of cultivation. Culture supernatants were extracted with ethylacetate. Extracts were applied to LC/MS and NMR analysis as described above. ^{13}C contents were calculated based on the natural ^{13}C content of unlabeled carbons. A description for the calculation is provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The nucleotide sequences of the region spanning ITS1 and ITS2 from *A. terreus* strains A1156 and SBUG844 as well as that of the 5′ untranslated region of ATEG00325 from strain SBUG844 have been deposited in the DDBJ/ EMBL/ GenBank nucleotide sequence database and assigned the accession numbers HQ380176, HQ380177, and HQ380178, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and four tables and can be found with this article online at doi:10.1016/j.chembiol.2010.12.011.

ACKNOWLEDGMENTS

We thank M. Cyrulies for assistance in large-scale fermentations, A. Perner for high-resolution MS, and F. Rhein and H. Heinecke for NMR measurements. All authors declare no conflicts of interest. This work was financially supported by the German science foundation DFG (grant BR 2216/4-1) and the Pakt für Forschung und Innovation.

Received: October 11, 2010
Revised: November 16, 2010
Accepted: December 13, 2010
Published online: January 13, 2011

REFERENCES

Ansari, M.Z., Yadav, G., Gokhale, R.S., and Mohanty, D. (2004). NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. Nucleic Acids Res. *32*, W405–W413.

Bergh, K.T., Litzka, O., and Brakhage, A.A. (1996). Identification of a major *cis*-acting DNA element controlling the bidirectionally transcribed penicillin biosynthesis genes *acvA* (*pcbAB*) and *ipnA* (*pcbC*) of *Aspergillus nidulans*. J. Bacteriol. *178*, 3908–3916.

Bergmann, S., Schümann, J., Scherlach, K., Lange, C., Brakhage, A.A., and Hertweck, C. (2007). Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. Nat. Chem. Biol. *3*, 213–217.

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., et al. (2009). Chromatin-level regulation of biosynthetic gene clusters. Nat. Chem. Biol. *5*, 462–464.

Brakhage, A.A., and Schroeckh, V. (2011). Fungal secondary metabolites—strategies to activate silent gene clusters. Fungal Genet. Biol. *48*, 15–22.

Brakhage, A.A., Spröte, P., Al-Abdallah, Q., Gehrke, A., Plattner, H., and Tüncher, A. (2004). Regulation of penicillin biosynthesis in filamentous fungi. Adv. Biochem. Eng. Biotechnol. *88*, 45–90.

Brakhage, A.A., Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., and Hertweck, C. (2008). Activation of fungal silent gene clusters: a new avenue to drug discovery. Prog. Drug Res. *66*, 1, 3–12.

Brock, M., Jouvion, G., Droin-Bergere, S., Dussurget, O., Nicola, M.A., and Ibrahim-Granet, O. (2008). Bioluminescent *Aspergillus fumigatus*, a new tool for drug efficiency testing and in vivo monitoring of invasive aspergillosis. Appl. Environ. Microbiol. *74*, 7023–7035.

Casinovi, C.G., Grandoli, G., Mercanti, R., Oddo, N., Olivieri, R., and Tonolo, A. (1968). A new antibiotic produced by a strain of *Aspergillus flavipes*. Tetrahedron Lett. *27*, 3175–3178.

Chiang, Y.M., Lee, K.H., Sanchez, J.F., Keller, N.P., and Wang, C.C. (2009a). Unlocking fungal cryptic natural products. Nat. Prod. Commun. *4*, 1505–1510.

Chiang, Y.M., Szewczyk, E., Davidson, A.D., Keller, N., Oakley, B.R., and Wang, C.C. (2009b). A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, asperfuranone, in *Aspergillus nidulans*. J. Am. Chem. Soc. *131*, 2965–2970.

Cichewicz, R.H. (2010). Epigenome manipulation as a pathway to new natural product scaffolds and their congeners. Nat. Prod. Rep. *27*, 11–22.

Denison, S.H. (2000). pH regulation of gene expression in fungi. Fungal Genet. Biol. *29*, 61–71.

Dowzer, C.E., and Kelly, J.M. (1991). Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol. Cell. Biol. *11*, 5701–5709.

Ebel, F., Schwienbacher, M., Beyer, J., Heesemann, J., Brakhage, A.A., and Brock, M. (2006). Analysis of the regulation, expression, and localisation of the isocitrate lyase from *Aspergillus fumigatus*, a potential target for antifungal drug development. Fungal Genet. Biol. *43*, 476–489.

Eley, K.L., Halo, L.M., Song, Z.S., Powles, H., Cox, R.J., Bailey, A.M., Lazarus, C.M., and Simpson, T.J. (2007). Biosynthesis of the 2-pyridone tenellin in the insect pathogenic fungus *Beauveria bassiana*. Chembiochem. *8*, 289–297.

Felenbok, B., Flipphi, M., and Nikolaev, I. (2001). Ethanol catabolism in *Aspergillus nidulans*: a model system for studying gene regulation. Prog. Nucleic Acid Res. Mol. Biol. *69*, 149–204.

Findlay, J.A., and Radics, L. (1972). Flavipucine [3′-isovaleryl-6-methylpyridine-3-spiro-2′-oxiran-2(1H),-4(3H)-dione], an antibiotic from *Aspergillus flavipes*. J. Chem. Soc. Perkin. 1 *16*, 2071–2074.

Findlay, J.A., Krepinsky, J., Shum, A., Casinovi, C.G., and Radics, L. (1977). Structure of isoflavipucine. Can. J. Chem. *55*, 600–603.

Fleck, C.B., and Brock, M. (2010). *Aspergillus fumigatus* catalytic glucokinase and hexokinase: expression analysis and importance for germination, growth, and conidiation. Eukaryot. Cell *9*, 1120–1135.

Grandolini, G., Casinovi, C.G., and Radics, L. (1987). On the biosynthesis of flavipucine. J. Antibiot. (Tokyo) *40*, 1339–1340.

Hertweck, C. (2009). Hidden biosynthetic treasures brought to light. Nat. Chem. Biol. *5*, 450–452.

Hoffmeister, D., and Keller, N.P. (2007). Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat. Prod. Rep. *24*, 393–416.

Ilyés, H., Fekete, E., Karaffa, L., Fekete, E., Sandor, E., Szentirmai, A., and Kubicek, C.P. (2004). CreA-mediated carbon catabolite repression of beta-galactosidase formation in *Aspergillus nidulans* is growth rate dependent. FEMS Microbiol. Lett. *235*, 147–151.

Jacobs, G.H. (1992). Determination of the base recognition positions of zinc fingers from sequence analysis. EMBO J. *11*, 4507–4517.

Kleiger, G., and Eisenberg, D. (2002). GXXXG and GXXXA motifs stabilize FAD and NAD(P)-binding Rossmann folds through C(alpha)-H… O hydrogen bonds and van der waals interactions. J. Mol. Biol. *323*, 69–76.

Kück, U., and Hoff, B. (2010). New tools for the genetic manipulation of filamentous fungi. Appl. Microbiol. Biotechnol. *86*, 51–62.

Laham, M.N., and Carpenter, J.L. (1982). *Aspergillus terreus*, a pathogen capable of causing infective endocarditis, Pulmonary mycetoma, and allergic bronchopulmonary aspergillosis. Am. Rev. Respir. Dis. *125*, 769–772.

Lass-Flörl, C., Griff, K., Mayr, A., Petzer, A., Gastl, G., Bonatti, H., Freund, M., Kropshofer, G., Dierich, M.P., and Nachbaur, D. (2005). Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. Br. J. Haematol. *131*, 201–207.

Liu, X., and Walsh, C.T. (2009). Cyclopiazonic acid biosynthesis in *Aspergillus* sp.: characterization of a reductase-like R* domain in cyclopiazonate synthetase that forms and releases cyclo-acetoacetyl-L-tryptophan. Biochemistry *48*, 8746–8757.

Maiya, S., Grundmann, A., Li, X., Li, S.M., and Turner, G. (2007). Identification of a hybrid PKS/NRPS required for pseurotin A biosynthesis in the human pathogen *Aspergillus fumigatus*. Chembiochem. *8*, 1736–1743.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Mihlan, M., Homann, V., Liu, T.W., and Tudzynski, B. (2003). AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi,* but its activity is not affected by NMR. Mol. Microbiol. *47*, 975–991.

Möbius, N., and Hertweck, C. (2009). Fungal phytotoxins as mediators of virulence. Curr. Opin. Plant Biol. *12*, 390–398.

Panozzo, C., Cornillot, E., and Felenbok, B. (1998). The CreA repressor is the sole DNA-binding protein responsible for carbon catabolite repression of the *alcA* gene in *Aspergillus nidulans* via its binding to a couple of specific sites. J. Biol. Chem. *273*, 6367–6372.

Penalva, M.A., Tilburn, J., Bignell, E., and Arst, H.N., Jr. (2008). Ambient pH gene regulation in fungi: making connections. Trends Microbiol. *16*, 291–300.

Pitt, J.I. (1979). The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces* (London: Academic Press).

Sassa, T., and Onuma, Y. (1983). Isolation and identification of fruit rot toxins from the fungus-caused *Macrophoma* fruit rot of apple. Agric. Biol. Chem. *47*, 1155–1157.

Scherlach, K., and Hertweck, C. (2009). Triggering cryptic natural product biosynthesis in microorganisms. Org. Biomol. Chem. *7*, 1753–1760.

Schroeckh, V., Scherlach, K., Nützmann, H.W., Shelest, E., Schmidt-Heck, W., Schuemann, J., Martin, K., Hertweck, C., and Brakhage, A.A. (2009). Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA *106*, 14558–14563.

Schümann, J., and Hertweck, C. (2006). Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. J. Biotechnol. *124*, 690–703.

Schümann, J., and Hertweck, C. (2007). Molecular basis of cytochalasan biosynthesis in fungi: gene cluster analysis and evidence for the involvement of a PKS-NRPS hybrid synthase by RNA silencing. J. Am. Chem. Soc. *129*, 9564–9565.

Sims, J.W., and Schmidt, E.W. (2008). Thioesterase-like role for fungal PKS-NRPS hybrid reductive domains. J. Am. Chem. Soc. *130*, 11149–11155.

Sims, J.W., Fillmore, J.P., Warner, D.D., and Schmidt, E.W. (2005). Equisetin biosynthesis in *Fusarium heterosporum*. Chem. Commun. (Camb.), 186–188.

Smedsgaard, J. (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J. Chromatogr. A *760*, 264–270.

Song, Z., Cox, R.J., Lazarus, C.M., and Simpson, T.J. (2004). Fusarin C biosynthesis in *Fusarium moniliforme* and *Fusarium venenatum*. Chembiochem. *5*, 1196–1203.

Tamayo, E.N., Villanueva, A., Hasper, A.A., de Graaff, L.H., Ramon, D., and Orejas, M. (2008). CreA mediates repression of the regulatory gene *xlnR* which controls the production of xylanolytic enzymes in *Aspergillus nidulans*. Fungal Genet. Biol. *45*, 984–993.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Penalva, M.A., and Arst, H.N., Jr. (1995). The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. *14*, 779–790.

Tokuoka, M., Seshime, Y., Fujii, I., Kitamoto, K., Takahashi, T., and Koyama, Y. (2008). Identification of a novel polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in *Aspergillus oryzae*. Fungal Genet. Biol. *45*, 1608–1615.

Varga, J., Tóth, B., Kocsube, S., Farkas, B., Szakacs, G., Teren, J., and Kozakiewicz, Z. (2005). Evolutionary relationships among *Aspergillus terreus* isolates and their relatives. Antonie van Leeuwenhoek *88*, 141–150.

Wagner, C., Anke, H., and Sterner, O. (1998). Rubrobramide, a cytotoxic and phytotoxic metabolite from *Cladobotryum rubrobrunnescens*. J. Nat. Prod. *61*, 501–502.