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ORIGINAL PAPER

# Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*

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**Abstract** Manipulation of the fungal epigenome is hypothesized to be an effective method for accessing natural products from silent biosynthetic pathways. A library of epigenetic modifiers was tested using the fungus *Aspergillus niger* to determine the impact of small-molecule inhibitors on reversing the transcriptional suppression of biosynthetic genes involved in polyketide (PKS), non-ribosomal peptide (NRPS), and hybrid PKS-NRPS (HPN) production. Examination of expressed sequence tag libraries from *A. niger* demonstrated that >70% of its PKS-, NRPS-, and HPN-encoding gene clusters were transcriptionally suppressed under standard laboratory culture conditions. Using a chemical epigenetic methodology, we showed that treatment of *A. niger* with suberoylanilide hydroxamic acid and 5-azacytidine led to the transcriptional upregulation of many secondary-metabolite-encoding biosynthetic gene clusters. Chemical epigenetic modifiers exhibited positional biases for upregulating chromosomally distal gene clusters. In addition, a phylogenetic-based preference was noted in the upregulation of reducing clade I PKS gene clusters, while reducing clade IV PKS gene clusters were largely unaffected. Manipulating epigenetic features in fungi is a powerful method for accessing the products of silent biosynthetic pathways. Moreover, this approach can be readily incorporated into modern microbial screening operations.

**Keywords** *Aspergillus niger* · Chemical epigenetics · Fungi · Gene expression · Natural products

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## Introduction

Among the many remarkable discoveries emerging from the genomics era is the revelation that most microorganisms, and in particular, filamentous fungi, possess far greater numbers of gene clusters encoding for the production of secondary metabolites than the numbers of natural products that have been isolated from these same organisms [1–3]. It is not uncommon for fungi to have dozens of unique gene clusters encoding for previously uncharacterized polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), and hybrid PKS-NRPS (HPN) natural products [4–11]. These transcriptionally suppressed gene

clusters, which are collectively referred to as silent biosynthetic pathways, generally remain unexpressed under a variety of laboratory culture conditions. Silent biosynthetic pathways are anticipated to be a rich source of chemically diverse compounds (e.g., novel organic moieties, multifaceted stereochemical features, unique heteroatom incorporation, etc.) with outstanding potential for generating novel therapeutic leads. Obtaining the products from transcriptionally suppressed gene clusters requires a new approach for efficiently probing silent biosynthetic pathways in a rational manner.

In an effort to obtain new drug leads from silent biosynthetic pathways, we have identified epigenetic modifying substances as promising tools for manipulating the secondary metabolism of fungi. Our group [12, 13] and others [14] have proposed that epigenome-level changes are extensively involved in controlling the transcriptional accessibility of gene clusters encoding for biosynthetic enzymes. Several proteins are required to facilitate chromatin remodeling in fungi, and their collective influence on gene expression is quite remarkable [15–22]. Chief among the chromatin interacting proteins are histones. These proteins undergo a wide-range of post-translational modifications [20, 22], with acetylation and methylation being two of the most frequently encountered and well studied of the histone alterations. While histone deacetylation is generally associated with gene silencing, the effects of methylation vary depending on which histone subtypes and specific amino acid residues are modified within nucleosomal complexes [15, 23, 24].

A variety of highly specific small-molecule probes are known to selectively inhibit the functions of histone-modifying proteins, and these compounds have found important roles as molecular tools for studying the epigenome [24]. Several families of histone acetyltransferases are responsible for catalyzing the covalent addition of acetyl groups to histones, while the reverse reaction is carried out by histone deacetylases (HDAC) and sirtuins [20, 22]. The hydroxamate-bearing natural product trichostatin A and its synthetic derivative, suberoylanilide hydroxamic acid (SAHA), are two noteworthy examples of potent HDAC inhibitors that have been successfully used as chemical epigenetic probes in a variety of eukaryotic systems, including filamentous fungi [24].

Another epigenetic modifying agent, 5-azacytidine (5-AZA), was originally developed as an antimetabolite that incorporated into RNA to cause a variety of dysfunctions related to production of tRNAs, rRNAs, and proteins [25]. Further studies have shown that 5-AZA has strong epigenetic modifying properties due to its inhibitory interactions with DNA methyltransferases, resulting in hypomethylation of DNA and chromatin restructuring [24, 26]. Interestingly, the epigenetic role of DNA methylation in fungi is still poorly understood and, in some cases, the very existence of DNA methylation in these organisms has been questioned [21, 27]. Two proposed explanations for how 5-AZA induces chromatin remodeling in fungi involve (1) inhibition of DNA methylation, which may occur at very low levels or transiently in fungi or (2) inhibition of histone methyltransferase (HMT) function [28, 29]. A HMT inhibitory role for 5-AZA is highly plausible given its structural similarity to other confirmed methyltransferase inhibitors, such as methylthioadenosine, sinefungin, and *S*-adenosylhomocysteine [24]. Nevertheless, the ability of 5-AZA to cause distinctive chromatin remodeling effects, even in the absence of detectable changes in DNA methylation, makes this and similar chemical epigenetic probes valuable molecular tools for inducing chromatin remodeling in fungi.

In the study reported here, we investigated the effects of the epigenome-modifying agents SAHA and 5-AZA as chemical tools for eliciting the expression of fungal silent biosynthetic pathways. *Aspergillus niger* was selected for this work since three separate genome sequencing initiatives completed in recent years [30, 31] have confirmed the presence of numerous PKS, NRPS, and HPN gene clusters in this fungus. The genome-wide impacts of the epigenetic modifiers SAHA and 5-AZA on the transcription of PKS, NRPS, and HPN biosynthetic gene clusters in *A. niger* were determined using real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). These data were compared to results from two expressed sequence tag (EST) libraries generated with *A. niger* that was grown under a wide variety of culture conditions. In conjunction with this work, we also addressed the remarkable diversity of PKS and HPN gene clusters in *A. niger* through a phylogenetic analysis of their respective ketosynthase domains. The results that emerged from this study strongly support the use of epigenetic modifying agents as an effective methodological approach for upregulating the transcription of secondary-metabolite-encoding biosynthetic gene clusters in fungi.

## Materials and methods

### Fungal strain and growth conditions

*Aspergillus niger* ATCC 1015 was obtained from the American Type Culture Collection. Suspensions of mycelia and spores were stored in 15% aqueous glycerol at −80°C. For the experiments, 100-µl samples of the cryopreserved *A. niger* stock cultures were lawned onto potato-dextrose plates (10 g dried potatoes, 5 g dextrose, 1000 ml DI water, 20 g agar) and the plates incubated at 25°C (12/12 h, light/dark). Liquid cultures of *A. niger* were prepared by inoculating potato-dextrose broth (10 g dried potatoes, 5 g dextrose, 1000 ml DI water) with uniform suspensions of

spores and mycelia harvested from the potato-dextrose plates. Cultures for compound screening were prepared by adding 100 µl of *A. niger* suspensions to test tubes containing 3 ml potato-dextrose broth and incubating the tubes at 25°C on a rotary shaker (170 rpm). Tube cultures received chemical epigenetic modifier treatments after 24 h, and the fungi were incubated an additional 6 days before being processed. Cultures for total RNA extraction were made by adding 2 ml of *A. niger* suspensions to 1-l Erlenmeyer flasks containing 250 ml potato-dextrose broth. Cultures were treated with epigenetic modifiers after incubating for 24 h (25°C on a rotary shaker at150 rpm), and they were allowed to continue growing for an additional 4 days before the RNA was extracted.

## Identification, domain analysis, and mapping of secondary metabolite gene clusters

The genome of wild-type *A. niger* ATCC 1015 was sequenced previously [31], and it has been published online (*Aspergillus niger* v1.0 Genome Database, http://genome.jgi-psf.org/Aspni1/Aspni1.home.html, accessed June 2008). Biosynthetic gene clusters were identified in the *A. niger* ATCC 1015 genome by nucleotide BLASTN and protein-nucleotide TBLASTN sequence searches for conserved domains representing PKS, NRPS, and HPN genes. The resulting list of putative hits was manually trimmed based on further sequence alignment and functional/mechanistic considerations. Provisional mapping of biosynthetic gene clusters was performed by nucleotide BLASTN sequence alignments of the PKS, NRPS, and HPN genes in *A. niger* ATCC 1015 with those published for *A. niger* CBS 513.88 [9]. Expanded 500-kb sequences bracketing each gene cluster were used to help corroborate the positional assignments for all PKS, NRPS, and HPN genes. When a homologous gene could not be identified in *A. niger* CBS 513.88, sequence alignments of the surrounding scaffolds were used for mapping. Mapping assignments for all PKS, NRPS, and HPN gene clusters were further substantiated by sequence alignment comparisons of all *A. niger* ATCC 1015 scaffolds against the *A. niger* CBS 513.88 genome [Electronic Supplementary Material (ESM) Table S1].

## Epigenetic modifier screening

Test tube cultures of *A. niger* (pre-incubated for 24 h) were treated with epigenetic modifiers dissolved in $H_2O$ or dimethyl sulfoxide. The solutions of test compounds were filter-sterilized (0.22 µ*M*) before being added to the cultures. After a 6-day incubation, the mycelia growth in each tube was qualitatively scored relative to the vehicle-only controls. Fungal growth was scored as "+++" (growth equivalent to control cultures), "++" (<50% reduction in growth), "+" (≥50% reduction in growth), or "−" (no growth observed).

After scoring all cultures, 3 ml of dichloromethane was added to each tube to extract secondary metabolites. The dichloromethane was removed by aspiration and the organic solvent evaporated in vacuo. Prior to analysis, all samples were defatted by partitioning the extracts between aqueous methanol (methanol:$H_2O$, 9:1) and hexane. After removal of the hexane, the aqueous methanol layers were evaporated in vacuo, yielding the crude mixtures of secondary metabolites that were used for the chemical analyses. Thin layer chromatography (TLC) was performed on the samples by spotting them onto silica gel plates that were developed in toluene–ethyl acetate–acetic acid (50:50:1 by volume). Secondary metabolites were visualized under UV irradiation at 254 and 365 nm and by sulfuric acid spray reagent. Additional visualization of secondary metabolite mixtures was performed on selected extracts by high-performance liquid chromatography ($C_{18}$ column using a 10–100% acetonitrile–water gradient with dual wavelength monitoring at 210 nm and 254 nm) and $^1$H neutron magnetic resonance analyses (extracts were dissolved in 650 µl $CD_3OD$, and spectra were generated by averaging 128 scans using a standard $^1$H pulse sequence). A list of all the epigenetic modifiers that were screened along with their effects on cell growth and secondary metabolite production are presented in ESM Table S2.

## Quantitative qRT-PCR analysis

An ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for all real-time qRT-PCR analyses of secondary metabolite gene cluster expression levels. Primers (ESM Table S3) were designed using the ABI Primer Express V3.0 (Applied Biosystems) and Primer3 [32] software packages. The *actin* gene (fgenesh1_pm.C_scaffold_6000043, Protein ID 48608) was chosen as the internal control based on its constitutive expression as an essential housekeeping gene. Data for all other genes were expressed relative to *actin* using the $2^{-\Delta\Delta CT}$ method [33]. The PCR analyses were optimized for each probe set using 1–8 ng cDNA per sample. All experiments were performed in sets of six replicates using RNA isolated from three replicates for each condition tested. RNA was prepared using the MP Biomedicals FastRNA Pro Red kit (MP Biomedicals, Irvine, CA) according to the manufacturer's instructions. RNA and cDNA concentrations were determined by reading the absorbance at 260 nm using 1-µl samples on a Nanodrop1000 (Thermo Scientific, Waltham, MA), while the overall quality of total RNA was determined using an Agilent 2100BioAnalyzer and RNA Nano Chips. All RNA samples were DNAse treated with Qiagen RNase-Free DNase (Qiagen, Valencia, CA) prior to

preparation of cDNA. The cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems), and real-time qRT-PCR was carried out using the Fast SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions.

### Analysis of KS domain phylogeny

Published gene sequences (ESM Table S4) were used to generate predicted protein sequences for KS domains in *A. niger* and other fungi. The regions between the conserved motifs MDPQQ and HGTGT were aligned using MUSCLE (100 iterations) [34] with additional post-run manual refinement. This resulted in an alignment of 351 amino acids, with 242 parsimony-informative, 39 parsimony-uninformative, and 70 constant characters. Analysis of the aligned sequences was performed using PAUP* 4.0. Settings for the maximum parsimony tree construction and MP bootstrap analysis were defined as follows: ACCTRAN (accelerated transformation) heuristic search option; all characters of equal weight; addition sequence randomized; number of replicates—10; branch-swapping algorithm set to TBR. Bootstrap analysis was performed with 100 replicates, and values of >50 are listed above their respective nodes. Neighbor-joining tree construction and bootstrap analysis using a neighbor-joining algorithm were performed, and bootstrap values for this analysis are presented below the afore-mentioned values in the nodes. In all analyses, the fatty acid synthase sequence NP_004095.4 from *Homo sapiens* was used as an out-group.

## Results

### Biosynthetic genes encoding for secondary metabolite production in *A. niger*

Prior annotation efforts (*Aspergillus niger* v1.0 Genome Database, http://genome.jgi-psf.org/Aspni1/Aspni1.home.html, accessed June 2008) of the estimated 11,200 putative genes in *A. niger* revealed the presence of several gene clusters presumed to be associated with the production of secondary metabolites. Our comprehensive examination of these and other suspected biosynthetic genes led to the identification of 33 PKS (Table 1), 15 NRPS (Table 2), and nine HPN (Table 3) gene clusters distributed throughout the genome of *A. niger* ATCC 1015. Several presumably non-functional biosynthetic genes and gene fragments were also encountered (e.g., missing essential domains, excessively short length, monomodular NRPSs, etc.), but these were excluded from further investigation. Analysis of the identified sequences allowed us to tentatively assign biosynthetic functions to the majority of the domains. However, several clusters (PKSs 1, 3, 8, 37, 39, 43, 44, 46, and 54; NRPS 22; HPNs 10, 28, and 47) possessed domains exhibiting atypical features (e.g., altered conserved domains), which precluded assigning their functional roles.

Comparative BLASTP analysis of the biosynthetic gene cluster domains in *A. niger* ATCC 1015 versus *A. niger* CBS 513.88 [9] revealed that four of the PKSs (3, 12, 32, and 53), one NRPS (31), and two HPNs (23 and 47) lacked significant homologies and thus can be considered unique to *A. niger* ATCC 1015. Conversely, *A. niger* CBS 513.88 harbors at least seven PKSs (An01g01130, An11g05940, An12g02730, An15g07920, An03g01820, An11g07310, and An07g01030) for which no homologs were identified in *A. niger* ATCC 1015. Two of the PKS gene clusters (3 and 25) identified in *A. niger* ATCC 1015 demonstrated excellent homologies to previously uncharacterized coding sequences (An15g02130 and An01g02030, respectively) in *A. niger* CBS 513.88.

### Identification of constitutively expressed secondary metabolite gene clusters in *A. niger*

Two EST libraries were generated for *A. niger* cultured under a wide variety of conditions. The first of these studies utilized wild-type *A. niger* N402 (4732 from the Fungal Genetics Stock Center) and resulted in the generation of 12,820 ESTs accounting for a total of 5,108 of the fungus's genes [35]. For this experiment, *A. niger* was grown on seven different carbon sources (glucose, bran, maltose, xylan, xylose, sorbitol, and lactose). Nucleotide BLASTN and protein-nucleotide TBLASTN searches of the EST database provided only a handful of sequences corresponding to PKS- and NRPS-containing biosynthetic pathways. Homologs for five PKS (2, 26, 32, 33, and 37) and six NRPS (6, 8, 15, 29, 30, and 31) transcripts were detected (ESM Table S5). No ESTs were identified for any HPN gene clusters.

The second library of ESTs was prepared using *A. niger* ATCC 1015 on an even more extensive assortment of media conditions, including arabinose, cellulose, corn fiber, glucose, hemicelluloses–protein, lactose, lignins, rapeseed meal, starch-maltose, wheat bran, and xylose along with other carbon-limited and carbon-nitrogen-limited media [31]. Examination of the ESTs using nucleotide BLASTN and protein-nucleotide TBLASTN searches identified transcripts from 16 PKS- and NRPS-related biosynthetic pathways. Of the 16 ESTs identified, 11 were identical to those observed in the *A. niger* N402 strain; the ESTs for the other five secondary-metabolite-encoding genes corresponded to PKSs 20 and 45, NRPSs 18 and 23, and HPN 16 (ESM Table S5). Interestingly, our results revealed that approximately 29% of PKS, NRPS, and HPN gene clusters in *A. niger* were expressed under a broad range of nutrient conditions using traditional culturing techniques.

**Table 1** Summary of the 33 PKS gene clusters and polyketide-like genes identified in the *Aspergillus niger* ATCC 1015 genome

| PKS gene identifier used in this study | *A. niger* ATCC 1015 gene name (JGI) | JGI scaffold number | Homologous *A. niger* CBS 513.88 gene (NCBI)[a] | Proposed domains[b] |
|---|---|---|---|---|
| PKS 1 (P1) | Aspni1:225587[e] | 1 | An01g06950 | KS AT DH(?) KR ACP |
| PKS 2 (P2) | Aspni1:225574 | 1 | An01g06930 | KS AT DH MT ER KR ACP |
| PKS 3 (P3) | Aspni1:171221 | 1 | No homolog | KS AT (?) KR ACP |
| PKS 4 (P4)[c] | Aspni1:55511 | 1 | An01g00060 | FAS subunit alpha |
| PKS 6 (P6) | Aspni1:118617 | 1 | An02g00450 | KS AT DH MT ER KR ACP |
| PKS 8 (P8) | Aspni1:37260 | 2 | An02g09430 | KS AT DH MT(?) ER KR(?) ACP |
| PKS 9 (P9)[c] | Aspni1:175936 | 3 | An06g00430 | PKS type III-like domain |
| PKS 12 (P12) | Aspni1:138585 | 4 | No homolog | KS AT DH ER KR ACP |
| PKS 13 (P13) | Aspni1:179079 | 4 | An11g03920 | KS AT DH MT ER KR ACP |
| PKS 14 (P14) | Aspni1:39026 | 4 | An11g04280 | KS AT DH KR ACP |
| PKS 15 (P15) | Aspni1:47991 | 4 | An11g05570 | KS AT DH MT ER KR ACP |
| PKS 20 (P20) | Aspni1:118629 | 4 | An11g09720 | KS AT MT KR ACP |
| PKS 25 (P25) | Aspni1:181803 | 6 | An15g02130 | KS AT DH MT ER(?) KR ACP |
| PKS 26 (P26) | Aspni1:210217 | 6 | An15g04140 | KS AT DH MT ER KR ACP |
| PKS 27 (P27) | Aspni1:118744 | 6 | An15g05090 | KS AT DH MT ER(?) KR ACP |
| PKS 30 (P30) | Aspni1:118666 | 9 | An12g07070 | KS AT DH ER KR ACP |
| PKS 32 (P32) | Aspni1:211885 | 10 | No homolog | KS AT MT KR ACP |
| PKS 33 (P33) | Aspni1:56896 | 11 | An09g05730 | SAT KS AT ACP ACP TE |
| PKS 34 (P34) | Aspni1:188697 | 11 | An09g05340 | KS AT DH ER KR ACP |
| PKS 36 (P36) | Aspni1:188817 | 11 | An09g01930 | KS AT DH MT ER KR ACP |
| PKS 37 (P37) | Aspni1:56946 | 11 | An09g01860 | SAT KS AT PT(?) ACP MT RED |
| PKS 38 (P38)[d] | Aspni1:43495 | 11 | An09g01290 | KS AT DH MT ER KR |
| PKS 39 (P39) | Aspni1:190014 | 12 | An12g02050 | SAT KS AT PT(?) ACP MT RED |
| PKS 40 (P40) | Aspni1:189378 | 12 | An12g02670 | KS AT DH MT ER KR |
| PKS 42 (P42) | Aspni1:44005 | 13 | An04g04340 | KS AT MT KR ACP |
| PKS 43 (P43) | Aspni1:118598 | 14 | An03g05140 | KS AT DH(?) MT ER KR ACP |
| PKS 44 (P44) | Aspni1:191422 | 14 | An03g05440 | SAT KS AT ACP ACP TE(?) |
| PKS 45 (P45) | Aspni1:191702 | 14 | An03g06380 | KS AT DH MT ER KR ACP |
| PKS 46 (P46) | Aspni1:128638 | 15 | An13g02430 | KS AT DH(?) ER KR ACP |
| PKS 48 (P48) | Aspni1:44965 | 16 | An10g00140 | KS AT KR ACP |
| PKS 53 (P53) | Aspni1:194381 | 19 | No homolog | SAT KS AT ACP MT |
| PKS 54 (P54) | Aspni1:51499 | 22 | An04g09530 | SAT(?) KS AT ACP |
| PKS 55 (P55) | Aspni1:118662 | 22 | An04g10030 | KS AT DH ER KR ACP |

PKS, Polyketide synthase; JGI, Joint Genome Institute

[a] Homologous genes in the *A. niger* CBS 513.88 genome were identified by protein BLASTP sequence comparisons. Gene clusters containing domains exhibiting >90% amino acid sequences similarities were considered homologous

[b] ACP, Acyl-carrier protein; AT, acyl transferase; DH, dehydratase; ER, enoylreductase; FAS, fatty acid synthase; KS, ketosynthase; KR, ketoreductase; MT, methyltransferase; PT, product template; SAT, starter unit ACP transacylase; TE, thioesterase; RED, reductase; (?), domain of uncertain assignment or undetermined function

[c] Although PKSs 4 and 9 are not incorporated in typical multi-domain biosynthetic gene clusters, their significant PKS-like homologies warranted notation

[d] The sequence of PKS 38 appears to be highly damaged and lacks several essential features for conveying functionality

[e] PKS 1 was assigned two JGI identifiers: Aspni1:225587 and Aspni1:196420

## Impact of epigenetic modifiers on secondary metabolite production in *A. niger*

The results of the EST library studies were supported by our observations that axenic cultures of *A. niger* generated only a modest portion of its genomically encoded natural products under in vitro culture conditions (data not shown). We initiated a screening of several epigenetic modifying agents (ESM Table S2) to determine which inhibitors were capable of increasing the number of secondary metabolites that were

**Table 2** Summary of the 15 non-ribosomal peptide gene clusters identified in the *A. niger* ATCC 1015 genome

| NRPS gene identifier used in this study | *A. niger* ATCC 1015 gene name (JGI) | JGI scaffold number | Homologous *A. niger* CBS 513.88 gene (NCBI)[a] | Proposed domains[b] |
|---|---|---|---|---|
| NRPS 4 (N4) | Aspni1:36929 | 2 | An02g05070 | A T C T C |
| NRPS 6 (N6) | Aspni1:207636 | 3 | An06g01300 | A T C A T C A T C C T C |
| NRPS 8 (N8) | Aspni1:52774 | 3 | An08g02310 | A T C C A A T C A T C C T C |
| NRPS 9 (N9) | Aspni1:118659 | 4 | An11g00050 | A T C A T C |
| NRPS 12 (N12) | Aspni1:182031 | 6 | An15g07530 | A T C A T C A T C |
| NRPS 14 (N14) | Aspni1:42205 | 9 | An12g07230 | A T C A T C A T TE |
| NRPS 15 (N15) | Aspni1:212679 | 11 | An09g01690 | A T C |
| NRPS 17 (N17) | Aspni1:43807 | 12 | An12g02840 | A T C A T C A T C C A T C |
| NRPS 18 (N18) | Aspni1:118599 | 12 | An05g01060 | A T C A T C A T C A T C C |
| NRPS 22 (N22) | Aspni1:44571 | 14 | An03g06010 | A T C A T(?) RED |
| NRPS 23 (N23) | Aspni1:191228 | 14 | An03g03520 | A T C T C |
| NRPS 26 (N26) | Aspni1:118635 | 17 | An04g06260 | A T C A T |
| NRPS 29 (N29) | Aspni1:118601 | 18 | An16g06720 | A T C C(?) A T C A T C A T |
| NRPS 30 (N30) | Aspni1:128584 | 19 | An03g00650 | A T C A T |
| NRPS 31 (N31) | Aspni1:55153 | 19 | No homolog | A T C A T C A T C A T C A T C |

NRPS, Non-ribosomal peptide synthase

[a] Homologous genes in the *A. niger* CBS 513.88 genome were identified by protein BLASTP sequence comparisons. Gene clusters containing domains exhibiting >90% amino acid sequences similarities were considered homologous

[b] A, NRPS adenylation domain; C, NRPS condensation domain; T, NRPS thiolation domain; for the definition of the other abbreviations, see footnote of Table 1

**Table 3** Summary of the nine hybrid PKS-NRPS (HPN) gene clusters identified in the *A. niger* ATCC 1015 genome

| HPN gene identifier used in this study | *A. niger* ATCC 1015 gene name (JGI) | JGI scaffold number | Homologous *A. niger* CBS 513.88 gene (NCBI)[a] | Proposed domains[b] |
|---|---|---|---|---|
| HPN 7 (H7) | Aspni1:118624 | 2 | An02g08290 | KS AT DH KR ACP C A T—RED and ER separate |
| HPN 10 (H10) | Aspni1:176722 | 3 | An08g03790 | KS AT DH KR(?) ACP C A T RED |
| HPN 11 (H11) | Aspni1:179585 | 4 | An11g00250 | KS AT DH ER KR ACP C(?) |
| HPN 16 (H16) | Aspni1:118644 | 4 | An11g06460 | KS AT KR C A T RED |
| HPN 23 (H23) | Aspni1:118581 | 5 | No homolog | KS AT ACP C A T RED |
| HPN 28 (H28) | Aspni1:41618 | 8 | An14g01910 | KS AT DH MT KR ACP C A(?) T (dysfunctional?) |
| HPN 29 (H29) | Aspni1:41846 | 8 | An14g04850 | A T KS AT |
| HPN 31 (H31) | Aspni1:187099 | 10 | An18g00520 | KS AT DH ER KR ACP C A T |
| HPN 47 (H47) | Aspni1:128601 | 15 | No homolog | KS AT DH KR(?) ACP C A T RED |

NCBI, National Center for Biotechnology Information

[a] Homologous genes in the *A. niger* CBS 513.88 genome were identified by protein BLASTP sequence comparisons. Gene clusters containing domains exhibiting >90% amino acid sequences similarities were considered homologous

[b] For a definition of the abbreviations, see footnotes to Tables 1 and 2

biosynthesized by the fungus. Cultures of *A. niger* ATCC 1015 were treated during early log-phase growth (24 h post-inoculation) with compounds (ten-fold steps from 1 n*M* to 1 m*M*). The treated cultures were allowed to incubate for an additional 6 days before they were extracted for profiling. Defatted dichloromethane extracts were prepared, giving us a simple, yet accurate means for qualitatively comparing the relative secondary metabolite diversity in treatment versus untreated control (vehicle-only) groups. Both SAHA and 5-AZA showed good activities, resulting in the production of several new secondary metabolites (ESM Table S2), and these two compounds were therefore selected for further investigation given their prior successful use in our laboratory for generating new natural products [12, 13].

## Real-time qRT-PCR analysis of secondary metabolite gene cluster expression

The effects of SAHA and 5-AZA administration on the expression of natural product biosynthetic gene clusters in *A. niger* ATCC 1015 were determined by real-time qRT-PCR analysis [33]. Pairs of forward and reverse primers were designed and tested for each of the PKS, NRPS, and HPN pathways (ESM Table S3), and those not providing acceptable comparative cycle threshold ($C_T$) values were redesigned. Three constitutively expressed genes (*actin*, *cox5*, and *sarA*) were evaluated as candidate housekeeping genes based on their prior successful use in *A. niger* [36]. All three housekeeping genes exhibited only modest changes in their respective expression levels upon treatment of *A. niger* with epigenetic modifiers (ESM Fig. S1). Consequently, *actin* was chosen as the reference gene for all subsequent experiments since its expression was quite robust, and its translational product is essential for cell growth/division.

The relative fold changes in expression for the PKS, NRPS, and HPN pathways following SAHA and 5-AZA treatments were systematically tested by real-time qRT-PCR using the $2^{-\Delta\Delta CT}$ method [33, 37] (Fig. 1). The effects of SAHA were much more pronounced than 5-AZA with most gene clusters showing significant changes in expression following the addition of the HDAC inhibitor. The expression levels of several PKS gene clusters were upregulated (>twofold) by SAHA treatment, including PKSs 6, 13, 14, 15, 25, 36, 37, 39, 42, 44, 46, 48, and 53. PKSs 36 and 37 exhibited the greatest changes in expression, with 37-fold and 71-fold increases, respectively, following SAHA treatment. PKSs 36 and 37 were also upregulated by 5-AZA; however, its effects were more subdued, causing fourfold and sixfold increases, respectively. In addition, NRPS gene clusters 4, 12, 15, 18, 23, and 26, as well as HPN gene clusters 11, 28, and 47 showed substantially increased expression in SAHA-treated cultures.

It is noteworthy that in a few selected cases, SAHA and 5-AZA had contradictory influences on biosynthetic gene cluster transcription (Fig. 1). This is best illustrated by PKSs 1, 3, 8, 27, 40, 44, and 54, NRPSs 17 and 29, and HPN 23 in which upregulation by SAHA was contrasted by decreases in expression caused by 5-AZA. Several small-molecule toxins targeting different cellular processes were also screened (cycloheximide, amphotericin B, and 5-fluorocytosine—data not shown), but none of these induced similar changes in natural product production, suggesting that the transcriptional upregulation of secondary metabolite gene clusters by SAHA and 5-AZA is due to selective inhibition of their respective epigenetic targets. However, we have not yet determined if SAHA and 5-AZA act directly by reversing the heterochromatin-mediated mechanism of biosynthetic gene cluster suppression or indirectly by altering genes that function as endogenous regulators of natural product production.

Despite numerous attempts using several redesigned primers, no transcriptional products were detected from PKSs 12 and 38 under vehicle-only, SAHA, and 5-AZA conditions. Closer inspection of PKS 38 revealed several coding errors that are assumed to compromise this gene cluster's function, including a damaged KS domain (missing/altered essential motifs), a missing ACP domain, and insertions of numerous putative intronic regions. In contrast, PKS 12 exhibited no unusual features that account for its lack of transcription, suggesting that this gene may be highly recalcitrant to expression. PKS-like genes 4 and 9 were excluded from the analysis since they were not part of the typical multi-domain clusters involved in secondary metabolite biosynthesis.

## Positional bias for epigenetic modulation of PKS, NRPS, and HPN gene cluster transcription

Employing a nucleotide BLASTN sequence alignment strategy, *A. niger* ATCC 1015 scaffolds were superimposed onto the *A. niger* CBS 513.88 chromosome map to assess the degree of overall genome similarity between the two fungal strains. A high level of synteny was observed between both fungi, allowing most scaffolds to be assigned with little difficulty (ESM Table S1). We next mapped the positions of the PKS, NRPS, and HPN gene clusters in *A. niger* ATCC 1015 by BLASTN alignment. The results were supported by analysis of PKS, NRPS, and HPN coding sequences using the Artemis Comparison Tool [38]. Flanking regions extending up to 500 kb surrounding each gene cluster were also examined to verify the proposed assignments. For genes with no observed homologs, flanking regions were used exclusively to determine a gene cluster's putative position. Employing this approach, we provisionally assigned all of the biosynthetic gene clusters to specific loci throughout the *A. niger* ATCC 1015 genome (Fig. 2).

We next examined whether SAHA and 5-AZA exhibited any positional biases for the PKS, NRPS, and HPN gene clusters that they most strongly affected. Treatment of *A. niger* with SAHA resulted in the strong transcriptional upregulation of 14 biosynthetic gene clusters, eight of which were positioned within 500 kb of their respective chromosomal termini (PKSs 6, 36, 37, 46, and 48; NRPS 15; HPNs 11 and 47). Moreover, three additional strongly upregulated gene clusters were found within <1 Mb of a chromosomal terminus (PKS 44; NRPSs 23 and 26), while two more were within <1.5 Mb of a chromosomal end (PKS 42; NRPS 4). PKS 39 was the only gene cluster strongly upregulated by SAHA that was not located near a

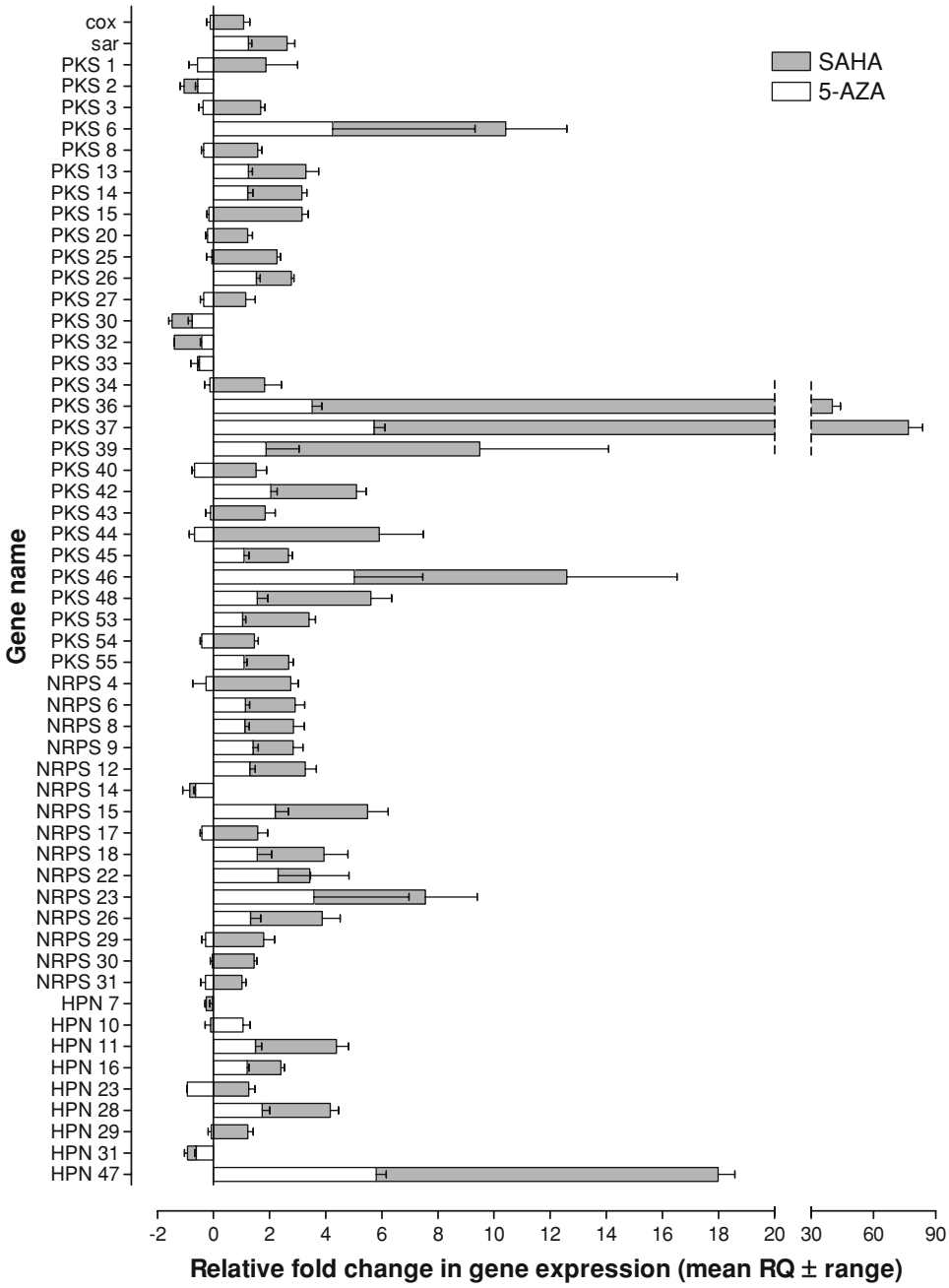


**Fig. 1** Real-time quantitative reverse-transcription-PCR (qRT-PCR) determination of the fold changes in the expression of the polyketide synthatase (*PKS*), non-ribosomal peptide synthase (*NRPS*), and hybrid PKS-NRPS (*HPN*) gene clusters in *Aspergillus niger* ATCC 1015. All data are presented relative to the expression of *actin.* PKSs 12 and 38 are not included since no transcripts were detected for these gene clusters. The RQ (relative quantification) values were determined using the using the $2^{-\Delta\Delta CT}$ method. Fold changes of <1 were expressed as their corresponding negative fold change to help distinguish genes exhibiting decreased expression. *SAHA* Suberoylanilide hydroxamic acid, *5-AZA* 5-azacytidine, *cox cox5*, *sar sarA*

chromosome's end (>2 Mb from terminus). A similar, but less pronounced trend was observed following 5-AZA treatment, which led to the strong upregulation of several gene clusters located within <1.5 MB of chromosomal termini (PKSs 6, 36, 37, and 46; NRPS 23; HPN 47).

## Phylogenetic analysis of ketosynthase domain diversity in *A. niger*

Ketosynthase (KS) domains associated with PKS (Table 1) and HPN (Table 3) gene clusters in *A. niger* ATCC 1015 were subjected to a phylogenetic analysis in an effort to (1) evaluate secondary metabolite diversity in the fungus and (2) determine if epigenetic modifiers selectively modulated the expression of specific gene cluster families. The KS domains from PKS and HPN gene clusters found in the *A. niger* CBS 513.88 mutant strain were included for comparative purposes. Classification of these genes was performed by comparing them to a diverse set of fungal KSs derived from PKS and HPN gene clusters having well-described biosynthetic functions [39]. Using this approach, we were able to assign the KS domains in *A. niger* ATCC 1015 to a series of discrete functional classes that had previously been proposed for other fungi [40]. A phylogenetic

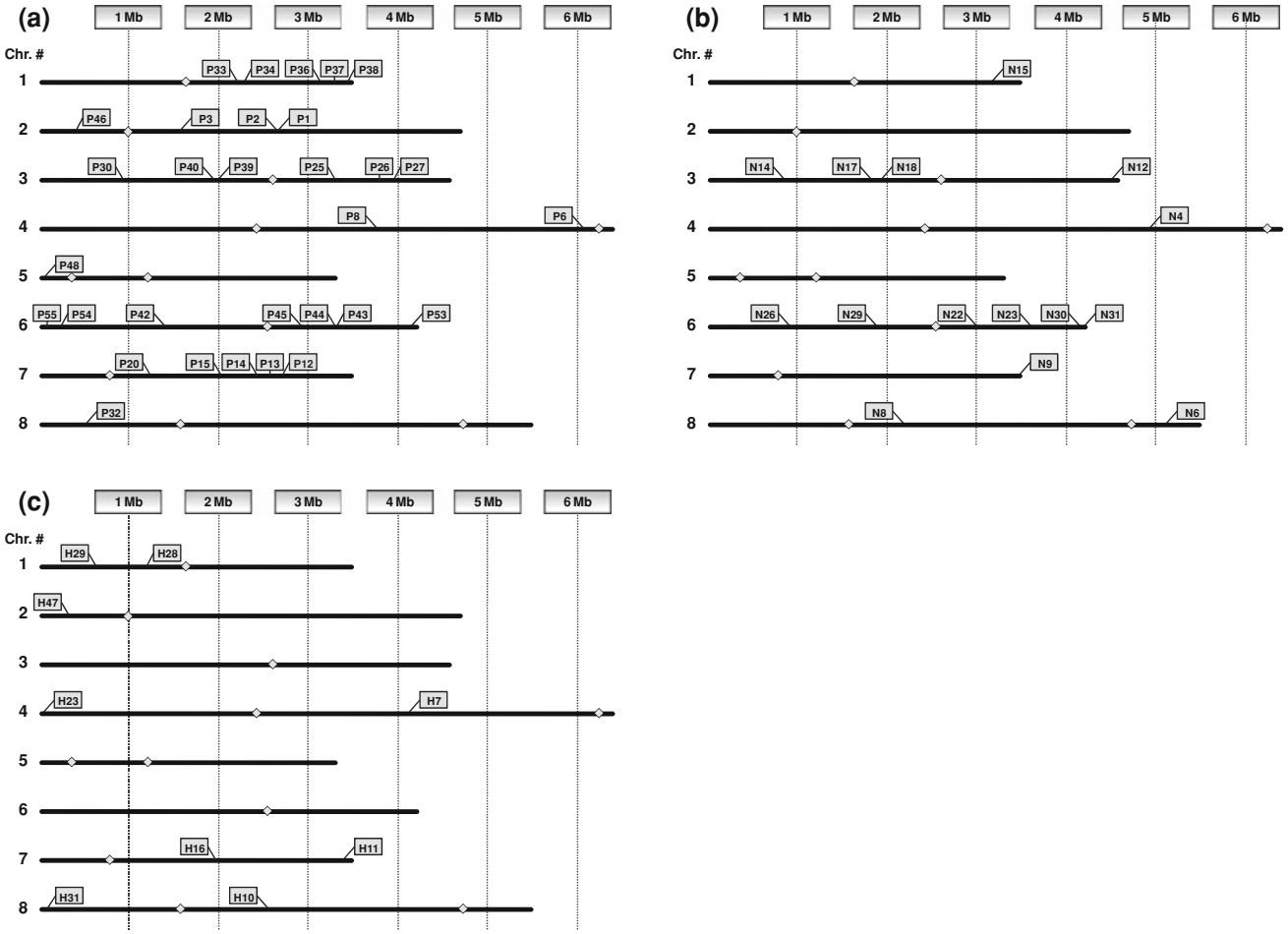


**Fig. 2** Mapping results for PKS (**a**), NRPS (**b**), and HPN (**c**) gene clusters in *A. niger* ATCC 1015. The published *A. niger* CBS 513.88 chromosome map [9] was used as a template for positioning scaffolds in *A. niger* ATCC 1015 by nucleotide BLASTN alignment analyses. Identifier *numbers* are the same as those presented in Tables 1–3 for PKS, NRPS, and HPN gene clusters, respectively

comparison of the predicted amino acid sequences for all of the KS domains showed that they were separable into two major sub-groups, with group 1 comprising reducing PKSs and group 2 comprising non-reducing and bacterial PKSs (Fig. 3). This clustering of KS domains is in general agreement with that reported previously for a multi-species analysis of fungal PKSs [40]; however, our results favor a closer association among bacterial-derived and non-reducing KSs than that proposed for a *Xylaria* sp. [41]. Unfortunately, all of these analyses, including the one presented here, suffer from poor bootstrap values at this critical node, and further experimental data will be required to resolve this issue.

The majority of KS domains (32 of the 40 included in this cladistic study) were determined to represent the group 1-reducing PKSs. These genes were divided further into four distinct sub-groups, reducing clades I–IV, based on a comparison to a diverse set of orthologous fungal KS domains (Fig. 4). The ten PKS gene clusters associated with reducing clade I KS domains exhibited significant diversity in their constitutive functional domains. In contrast, the three reducing clade II PKSs were devoid of enoylreductase (ER) domains, as noted in other fungi [40]. The KS domains for six of the HPNs were also associated with reducing clade II, and these gene clusters likewise lacked ER domains. Reducing clade III comprised two HPNs (11 and 31) that share a common multi-domain design (KS, AT, DH, ER, KR, and ACP domains); however, the NRPS portions of these clusters are dissimilar (Table 3). We speculate that the phylogeny of the KS domains derived from HPNs is more influenced by their respective PKS architecture than by that of their NRPS components (i.e., clade II HPNs lacking ER domains were clustered, while HPN 11 and HPN 31 containing ER domains clustered separately). A total of 11 PKSs were grouped in reducing clade IV, and these gene clusters also exhibited significant domain variability. A modest discrepancy is apparent regarding our re-assignment of *Cochliobolus heterostrophus* PKS2 and *Botryotinia fuckeliana* PKS8 to reducing clade I versus its previously proposed affiliation

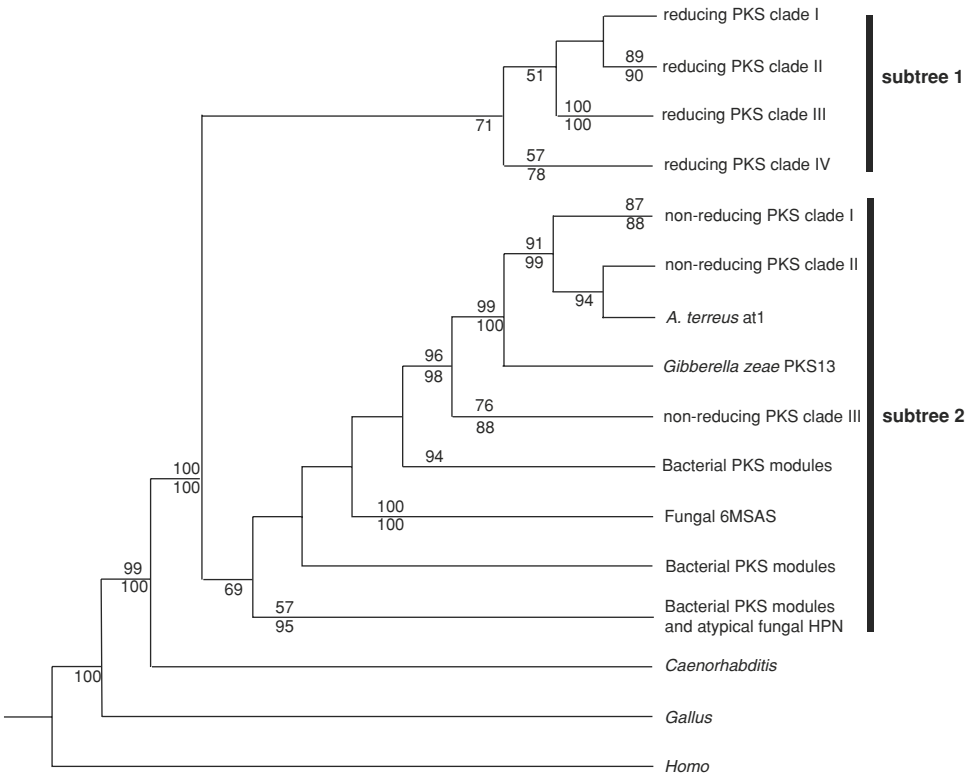


**Fig. 3** Overview of the phylogenetic analysis performed on ketosynthase (KS) domains associated with PKS and HPN gene clusters in *A. niger* ATCC 1015. Bootstrap values of >50 from maximum parsimony (*top value*) and neighbor-joining (*bottom value*) algorithms following 100 repetitions are indicated at their respective *nodes*. Two major clades were resolved: group 1 comprising reducing PKSs (subtree 1, Fig. 4) and group 2 comprising non-reducing and bacterial PKS (subtree 2, Fig. 5). Clade nomenclature is adapted from that proposed by Kroken et al. [1]

with reducing clade III [40]. Given the relatively small number of KSs clustering in reducing clade III, further sampling will be needed to accurately resolve the key features that define this group.

Six KS-domain-containing gene clusters were found grouped into non-reducing clades I–III (Fig. 5). These PKS gene clusters were distinguished by the presence of clearly defined starter unit acyl-carrier protein transacylase (SAT) domains. The substrate selectivity and diversity of SAT domains have been recently discussed in detail [42]. The distribution of PKSs from *A. niger* ATCC 1015 among the three clades showed that two PKSs were in clade I, one PKS was associated with clade II, and three additional PKSs were present in clade III. In other fungi, the non-reducing PKSs are reported to be associated with the production of a wide variety of aromatized products, including anthraquinone, naphthopyrone, and complex perylenequinone metabolites [39, 40].

Of the two remaining KS domains, the one associated with PKS 48 was found within a cluster of distinct fungal secondary metabolite encoding genes and identified as a 6-methylsalicylic-acid-type PKS. The last KS domain came from an atypical HPN gene cluster (HPN 29) that strongly associated with a clade of bacterial-like secondary metabolite biosynthetic gene clusters. The domain architecture identified for this cluster is highly unusual since the NRPS-associated genes (adenylation and thiolation domains) precede the PKS-type genes (ketosynthase and acyl transferase domains).

It is clear from this phylogenetic study that KS-containing gene clusters in *A. niger* ATCC 1015 are extremely diverse. Moreover, our investigation has revealed that the assemblage of KS domains in *A. niger* ATCC 1015 accounts for one or more representative examples from each of the major classes of PKS and HPN gene clusters documented throughout the entire fungal kingdom [40]. Although our dataset was limited to a single organism, we did observe a noteworthy non-uniform response in the transcription of KS-domain-containing gene clusters following the administration of SAHA to *A. niger*. Whereas most of the reducing clade I PKS gene clusters (8/10) were upregulated by SAHA, nearly all of the reducing clade IV PKSs (10/11) were non-responsive. This curious response pattern will be further scrutinized in due course.

## Discussion

Whole-genome sequencing data obtained from a variety of fungi demonstrate that these microorganisms harbor large numbers of PKS, NRPS, HPN, and other natural product biosynthetic gene clusters. Most of these pathways remain suppressed under in vitro culture conditions, meaning that only a fraction of a fungi's biosynthetic potential is generally accessible for drug screening applications. Based on

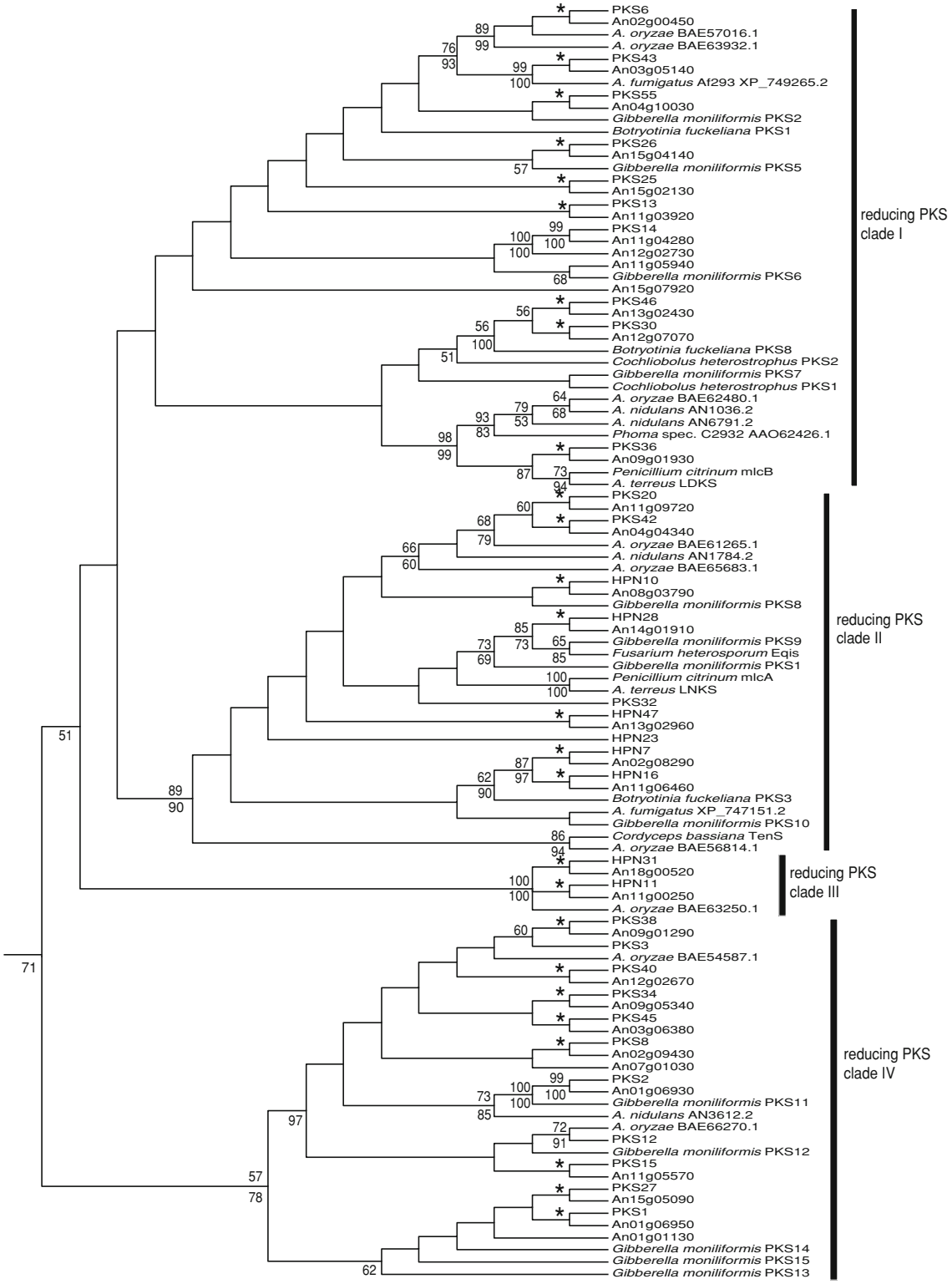


**Fig. 4** Subtree 1: phylogeny of the KS domains from the group 1-reducing PKSs in *A. niger* ATCC 1015. The cladogram was obtained by maximum parsimony analysis. Bootstrap values of >50 from maximum parsimony (*top value*) and neighbor-joining (*bottom*) algorithms following 100 repetitions are indicated at their respective *nodes*. Four major clades, reducing clades I–IV were identified. The KS domains from both PKSs and HPN gene clusters are included. *Asterisks* indicate 100% matches to homologous KS domains in the *A. niger* CBS 513.88 genome

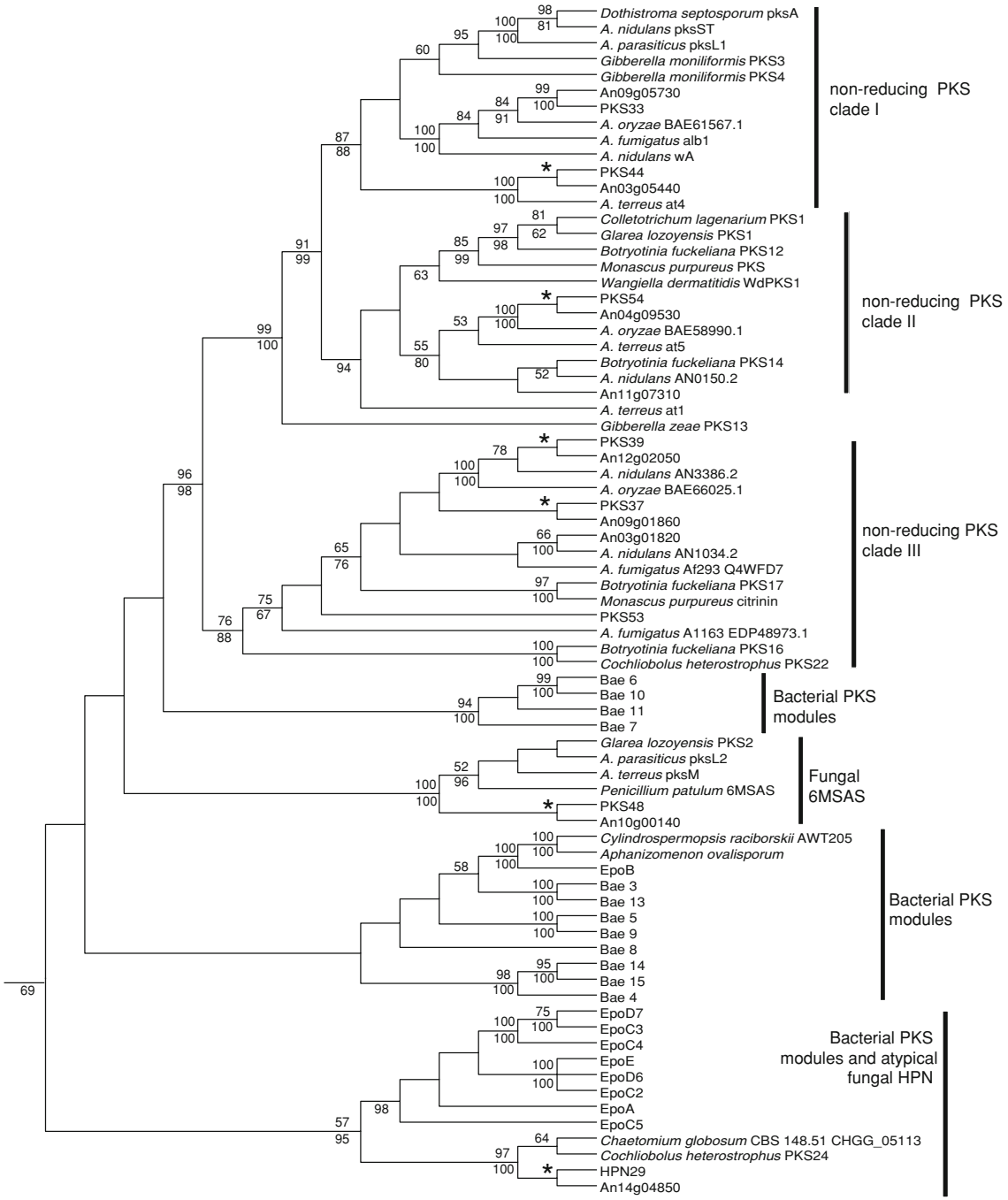


**Fig. 5** Subtree 2: phylogeny of the KS domains from the group 2-non-reducing and bacterial PKSs in *A. niger* ATCC 1015. The cladogram was obtained by maximum parsimony analysis. Bootstrap values of >50 from maximum parsimony (*top value*) and neighbor-joining (*bottom*) algorithms following 100 repetitions are indicated at their respective *nodes*. Three major non-reducing PKS clades (non-reducing clades I–III) were identified along with two clades representing bacterial PKSs, one clade of fungal 6-methylsalicylic-acid-type PKSs, and one clade of bacterial-like secondary metabolite biosynthetic gene clusters. The KS domains from both PKSs and HPN gene clusters are included. *Asterisks* indicate 100% matches to homologous KS domains in the *A. niger* CBS 513.88 genome

our observations, we conservatively estimate that fungi express less than half of their natural-product-encoding biosynthetic pathways under standard shake-flask and static culture conditions.

Incredibly, even *Neurospora crassa*, which has been intensively studied since the 1920s and considered to be devoid of any secondary metabolites, has more recently been shown to possess no less than seven PKS, four NRPS, and several terpene biosynthetic gene clusters that are believed to fulfill a variety of important functions [6]. With the exceptions of fungi from the sub-phyla Saccharomycotina and Taphrinomycotina [43], most fungi that have been

sequenced to date carry a variety of biosynthetic gene clusters encoding for the production of uncharacterized natural products. These compounds are expected to possess a wide range of unique biological activities and, therefore, have significant value as drug discovery leads [2, 3, 44]. The strict transcriptional suppression of secondary metabolites is conjectured to protect fungi from the potentially deleterious (autotoxic) effects of natural products, and their biosynthesis is only elicited when an appropriate environmental trigger is encountered. Based on emerging molecular evidence [14], we had predicted that the expression of many secondary metabolite pathways is transcriptionally controlled through epigenetic mechanisms [12, 13]. Consequently, we consider the chemical epigenetic manipulation of silent gene clusters to be a highly promising approach for procuring novel drug leads from fungi. This methodology has many significant advantages over currently available molecular-based techniques and cultivation-dependent approaches [2, 44]. Namely, our chemical epigenetic approach offers significantly greater simplicity, broad-spectrum applicability, and an ability to be readily incorporated into modern microbial screening programs.

The precise molecular mechanisms involved in upregulating the expression of silent secondary-metabolite-encoding pathways are likely more complex than what we presumed based on the singular inhibition of specific cellular targets by chemical epigenetic modifying agents. For example, in *Saccharomyces cerevisiae*, the histone deacetylase (HDAC) inhibitor trichostatin A and several HDAC deletion mutants demonstrated a wide variety of transcriptional effects [16]. These included both the upregulation and downregulation of genes involved in cell cycle progression, as well as amino acid and carbohydrate metabolism. Curiously, the effects of trichostatin A on a subset of *S. cerevisiae* genes were detectable within 15 min of exposure to the inhibitor, suggesting that trichostatin A has other, as yet undefined, effects on modifying gene transcription (e.g., direct transcriptional activation).

Similar to our observations, other researchers have also noted that the impact of HDAC inhibitors and HDAC mutants were most pronounced on genes located within the distal regions of fungal chromosomes. For example, Bernstein et al. [16] reported that deletion of the yeast HDAC, *RPD3*, preferentially altered the transcription of genes positioned near the termini of chromosomes. Moreover, deletion of the transcriptional regulator *laeA*, whose methyltransferase product was suggested to alter chromatin structure [45], has been shown to decrease the expression of many telomere proximal NRPS gene clusters in *Aspergillus fumigatus* [46]. The dynamics of histone-mediated heterochromatin formation and gene quelling in fungi is proposed to function in a generally linear fashion, exerting an initial silencing influence on the most distally located genes, which progresses proximally over multiple cell division cycles [47]. In view of the complex processes involved in the continual shifting of DNA between euchromatin and heterochromatin states, our data suggest the need for considering the roles of other potential signaling pathways linking outwardly unrelated nuclear and cytoplasmic events to the transcriptional regulation of secondary-metabolite-encoding biosynthetic gene clusters.

For nearly a century, natural products have served as an unrivaled resource in the quest for new drug entities. However, recent concerns and misconceptions in the pharmaceutical industry about the long-term value of natural products as a discovery platform have all but eliminated secondary metabolites from most research and development programs [48–51]. It is likely that the folly of this collective decision will further impair an already flagging drug-discovery pipeline [52]. Fortunately, emerging techniques in the field of natural products discovery, such as the genomic and epigenetic methods described in this study, offer an opportunity to reinvigorate the fundamental role of secondary metabolites as a drug discovery tool. We anticipate pursuing further studies to characterize how fungi coordinate epigenetic processes as a means for regulating natural product biosynthesis in situ. The results are expected to have far-reaching applications for efficiently directing the production of new secondary metabolites from fungi.

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