Histone Deacetylase Activity Regulates Chemical Diversity in Aspergillus

E. Keats Shwab,1 Jin Woo Bok,1 Martin Tribus,2 Johannes Galehr,2 Stefan Graessle,2 and Nancy P. Keller1*

Plant Pathology Department, University of Wisconsin—Madison, 1630 Linden Drive, Madison, Wisconsin 53706;1 and Division of Molecular Biology, Biocenter, Innsbruck Medical University, Fritz-Pregl Strasse 3, A-6020 Innsbruck, Austria2

Received 22 May 2007/Accepted 27 June 2007

Bioactive small molecules are critical in Aspergillus species during their development and interaction with other organisms. Genes dedicated to their production are encoded in clusters that can be located throughout the genome. We show that deletion of hdaA, encoding an Aspergillus nidulans histone deacetylase (HDAC), causes transcriptional activation of two telomere-proximal gene clusters—and subsequent increased levels of the corresponding molecules (toxin and antibiotic)—but not of a telomere-distal cluster. Introduction of two additional HDAC mutant alleles in a ΔhdaA background had minimal effects on expression of the two HdA-regulated clusters. Treatment of other fungal genera with HDAC inhibitors resulted in overproduction of several metabolites, suggesting a conserved mechanism of HDAC repression of some secondary-metabolite gene clusters. Chromatin regulation of small-molecule gene clusters may enable filamentous fungi to successfully exploit environmental resources by modifying chemical diversity.

A distinguishing characteristic of filamentous fungi is their ability to produce a wide variety of small molecules that aid in their survival and pathogenicity. These include compounds, such as pigments, that play a role in virulence and protect the fungus from environmental damage, as well as toxins that kill host tissues or hinder competition from other organisms. These secondary metabolites (SM) (27) can also impact host tissues or hinder competition from other organisms. The genus Aspergillus, whose members include toxin-producing pathogens (Aspergillus flavus and A. fumigatus) and pharmaceutical-producing species (A. nidulans and A. terreus), is renowned for prodigious metabolite production and serves as the model for natural-product exploration. Detailed comparison of the genomes of several aspergilli indicates a genomic landscape in which the greatest diversity between species is represented in these SM clusters (28).

There has been considerable debate as to the role that gene clustering plays in the secondary metabolism of fungi. Such gene arrangement must be advantageous to the fungus; if natural selection did not favor clustering, one would assume that processes such as gene translocation and unequal crossing over would have caused dispersal over evolutionary history. Support for horizontal transfer from prokaryotes, in which genes are often arranged into operons, exists for the penicillin biosynthetic cluster (9) but not for other fungal clusters. A prokaryotic gene transfer hypothesis is weakened by the fact that fungal SM genes often contain introns and employ codon usage typical of other fungal genes. Another hypothesis holds that clustering provides a selective advantage to the cluster itself in that the arrangement makes propagation of the genes by means of horizontal transfer more successful (40). However, the importance of horizontal transfer of gene clusters among fungi has not been adequately explored to determine the merit of this hypothesis. This study focuses instead on the hypothesis that a common regulatory mechanism(s) underlies the SM cluster motif (25). Specifically, we have investigated whether clustering of SM biosynthetic genes allows coregulation through localized modification of chromatin structure.

SM clusters in Aspergillus species tend to be located near the telomeres of chromosomes (28), and a recent genome examination of the rice blast fungus Magnaporthe oryzae located at least two SM clusters within 40 kb of its telomeres (32). This locational bias may reflect in part an increased efficiency of epigenetic regulation at chromosomal subtelomeres—telomere-adjacent regions characterized by repeated DNA sequences (31). Though little is known about subtelomeric gene regulation events in aspergilli or other filamentous fungi, SM cluster regulation has been shown to be location dependent. Translocation of an A. parasiticus SM cluster gene to a chromosomal location outside of its native cluster can exempt it from coregulation with the rest of the cluster (14). The characterization of the protein LaeA also supports the case for chromatin-based regulation of SM clusters. LaeA acts as a global transcriptional regulator of SM clusters in several aspergilli and appears to be a protein methyltransferase with limited homology to histone methyltransferases (4). Importantly, LaeA also demonstrates a positional bias, as transfer of genes into or out of an SM cluster leads to gain or loss, respectively, of transcriptional regulation by LaeA (5). Chromatin regulation of gene expression is thought to be directed by modifications of histones, such as methylation and acetylation, that form the language of a combinatorial code. Histone modification patterns likely control the interaction of histones with transcriptional activators and repressors (23).

* Corresponding author. Mailing address: Plant Pathology Department, Russell Laboratories, University of Wisconsin—Madison, 1630 Linden Drive, Madison, WI 53706, Phone: (608) 262-9795, Fax: (608) 263-2626, E-mail: npk@plantpath.wisc.edu.

† Published ahead of print on 6 July 2007.
HdaA is responsible for the majority of HDAC activity in effects of histone deacetylation on small-molecule production. Therefore, this group of enzymes was chosen to study the HDACs have been studied in particular detail (20, 38, 39). and HstA is an HDAC with homology to the NAD subcategory of HDACs that is apparently unique to fungi (39), chromosomal regions (24). In the model fungus lencing, while hyperacetylation is more commonly associ-
tends to be associated with heterochromatin and gene si-
(HDACs). As a general rule, hypoacetylation of histones

Arguably the most widely studied and best understood histone modification is acetylation. Histone acetylation states are dynamic and are controlled by the opposing actions of histone acetyltransferases and histone deacetylases (HDACs). As a general rule, hypoacetylation of histones tends to be associated with heterochromatin and gene silencing, while hyperacetylation is more commonly associated with euchromatin and gene activation (34, 39). Hypoacetylation of chromatin is also predominant in subtelomeric chromosomal regions (24). In the model fungus A. nidulans, the HDACs have been studied in particular detail (20, 38, 39). Therefore, this group of enzymes was chosen to study the effects of histone deacetylation on small-molecule production. We examined the effects of HDAC loss on the three best-characterized SM clusters in A. nidulans, the sterigmatocystin (ST) (a member of the carcinogenic and insecticidal aflatoxins) cluster (8), the penicillin (PN) (an antibiotic) cluster (7), and the terraqueonine A (TR) (an antitumor agent) cluster (6). All three clusters are positively regulated by LaeA (3, 4). We created isogenic lines differing only in loss of one or more of the HDAC genes, hdaA, hosB, and hstA. The class 2 enzyme HdaA is responsible for the majority of HDAC activity in A. nidulans (38). HosB is an enzyme belonging to the HOS3-like subcategory of HDACs that is apparently unique to fungi (39), and HstA is an HDAC with homology to the NAD⁺-dependent sirtuin class. Sirtuin HDACs are known to be involved in the formation of heterochromatin in a broad range of species, including a number of fungi (10).

### Materials and Methods

**Fungal strains.** Knockout procedures for hdaA, hosB, and laeA have been described previously (4, 38). hstA deletion mutants were generated by replacing the gene with the selection marker argB in the A. nidulans strain A89 (Table 1). PCR with primers Asir2kof and Asir2kor (Table 2) was used to amplify hstA and flanking sequence from the cosmID09. The amplification product was ligated into a pGEM-T vector, and the coding sequence of hstA was eliminated with BamHI/NarI and replaced by a BamHI/ClaI-excised fragment. The resulting hstA deletion construct consisted of argB and ~1,400 bp of the flanking region upstream and downstream of hstA. Transformation of A. nidulans was performed after elimination of the pGEM vector with Aap1 and Spel. The excised fragment was gel purified, and 7.5 µg of the DNA fragment was used for the transformation procedure. Putative deletion strains were verified by PCR and Southern blot analysis.

**TABLE 1. Genotypes of A. nidulans strains used in this study**

| Strain       | Genotype                                                                 |
|--------------|---------------------------------------------------------------------------|
| RDIT 2.3     | hdaA::pyrG rbiA veA1                                                    |
| RJW 61.4     | hdaA::pyrG rbiA chA1 yA2 veA1 ppyG89                                   |
| RJW 61.9     | hdaA::pyrG rbiA veA1 argB argB                                              |
| RJW 61.5     | hstA::argB veA1 argB veA1 argB                                              |
| RJW 61.2     | hstA::argB veA1 argB argB argB                                              |
| REK 9.22     | hstA::argB argB argB argB argB                                              |
| REK 9.21     | hstA::argB argB argB argB argB                                              |
| TJW 65.7     | hstA::argB argB argB argB argB                                              |

### TABLE 2. PCR primer sets used in hstA knockout and to generate probes for Northern blot assays

| Gene  | Primer | Sequence                           |
|-------|--------|------------------------------------|
| hstA  | Asir2of | 5’ACGAGAAATATATCTCCCG3’ |
| Actin | Actf   | 5’CTCTCCCTCCTCTCTCCACCC3’ |
| AN2547.3 | penSF3 | 5’TCTCTGGTAGTAACTGAGCCG3’ |
| AN7830.3 | upSTR  | 5’CGGCGGAGCTTGGTGGCCG3’ |
| penDE  | penDEF | 5’AGCAGTTCTCCTTCCCTCCGG3’ |
| tdiA   | tdiAF  | 5’TCTCTGCTGATCACCGAGAAGCC3’ |
| tdiB   | NAI1   | 5’TCTCTACTCGCCATCCGCC3’ |
Table 1 lists all of the *A. nidulans* strains used for this study. All strains were maintained as glycerol stocks. Some strains are not discussed in the text but were used for sexual crosses to obtain the strains of interest. Sexual crosses of *A. nidulans* strains were conducted according to standard methods (30). Strain genotypes were identified by PCR amplification of the correct allele, followed by confirmation of the allele by Southern blot analysis according to standard procedures (36). Strains of *Alternaria alternata* and *Penicillium expansum* were wild-type strains provided by the laboratory of Craig Grau at the Department of Plant Pathology, University of Wisconsin—Madison.

**SM analysis.** Published procedures were used to extract and analyze ST; its precursor, norsolorinic acid (NOR) (regulated identical to ST and derived from the same cluster) (12); PN; and TR (3, 4). Prior to SM extraction, all *A. nidulans* strains were point inoculated onto solid glucose minimal medium (GMM) in 10-cm-diameter petri dishes and incubated for 72 h at 37°C (37). ST extractions were also performed at 48 h, yielding similar results (data not shown). Prior to SM extraction, *A. alternata* and *P. expansum* extractions were point inoculated onto solid GMM in 10-cm-diameter petri dishes and incubated for 72 h at 37°C on solid potato dextrose agar (Difco Laboratories, Sparks, MD). Solid-medium plates for all fungal strains were point inoculated with approximately 10⁴ spores/well. For experiments involving H₂O₂, the chemical was added to media following autoclaving, after the media had cooled to 37°C; 30% (wt/wt) H₂O₂ in water was added to 50 ml of liquid GMM to produce final concentrations of 0, 1, 2, or 3 mM. Dilutions of H₂O₂ were made with water so that an equal volume was added to the media to produce each of these concentrations. For experiments involving H₂O₂, the chemical was added to media following autoclaving, after the media had cooled to 50°C; 30% (wt/wt) H₂O₂ in water was added to molten GMM agar to produce final concentrations of 1, 2, or 3 mM. Dilutions of H₂O₂ were made with water so that an equal volume was added to the media to produce each of these concentrations. RNA blots were hybridized with a 0.7-kb SacII-KpnI fragment from the plasmid pRB7 containing the stcU coding region, a 1.3-kb EcoRV-Xhol fragment from plasmid pWJ19 containing the efb8 coding region, or a 1.1-kb EcoRI-HindIII fragment from the plasmid pUCHH(458) containing the ipnA coding region. PCR with gene-specific primers was used to generate probes for actin, AN2647.3, AN7830.3, penDE, tldA, and tldB. Primer sequences are shown in Table 2. All experiments were performed in duplicate or triplicate.

**Statistical analysis.** For statistical analyses, a probability of type I error of less than 0.01 was considered statistically significant. Where only two treatments were compared, the significance of variation was determined using Student’s t test. Where more than two treatments were compared, analysis of variance was used to determine the significance of overall variability among treatments, followed by a Neumann-Keuls test to compare individual pairs of treatments. The Microsoft Excel data analysis package was used to perform analysis of variance and t tests. Neumann-Keuls tests were performed by hand.

**RESULTS**

**Location of clusters.** Several HDACs are known to regulate genes located in subtelomeric regions of other genomes (15, 19, 21, 22, 34, 41); thus, we were interested in identifying the locations of the three SM clusters in our study. Two of the three clusters are within 100 kb of the telomere: the ST cluster is 90 kb from the chromosome IV telomere, and the PN cluster is 30 kb from the chromosome VI telomere. In contrast, the
TR cluster is 700 kb distal from the nearest telomere on chromosome V (Fig. 1). Both the ST and PN clusters functionally fulfill the definition of subtelomeric, as the intervening sequence between the clusters and telomere is characterized by repeated DNA found at the majority of subtelomeric regions. Such repeated sequences were not identified in the areas surrounding the TR cluster (Fig. 1). Subtelomere lengths vary considerably among eukaryotes; while Kluyveromyces lactis subtelomeres span only about 30 kb (17), those of humans may reach over 300 kb in length (33), and in trypanosomes, subtelomeres span only about 30 kb (17), those of humans vary considerably among eukaryotes; while Kluyveromyces lactis subtelomeres span only about 30 kb (17), those of humans may reach over 300 kb in length (33), and in trypanosomes, those of humans vary considerably among eukaryotes; while Kluyveromyces lactis subtelomeres span only about 30 kb (17), those of humans may reach over 300 kb in length (33), and in trypanosomes, those of humans vary considerably among eukaryotes; while Kluyveromyces lactis subtelomeres span only about 30 kb (17), those of humans may reach over 300 kb in length (33), and in trypanosomes, although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster.

Production of SMs and expression of biosynthetic genes in the ∆hdaA mutant. Prior experimentation had shown HdaA to exhibit most of the detectable HDAC activity in A. nidulans (38), and thus, our first studies examined the effect of loss of this allele on SM production. Production levels of ST; its precursor, NOR; PN; and TR were compared between the wild type and the ∆hdaA strain. The ∆hdaA mutant, which showed a wild-type growth rate, had increased production of subtelomeric metabolites (ST and PN) but unaltered TR levels (Fig. 2). As expected, NOR production paralleled ST production in the mutant (data not shown).

We used representative genes of the ST and PN clusters to examine whether the increased ST and PN production in the ∆hdaA strain correlated with mRNA production. For the ST cluster, these genes included aflR, which encodes a DNA-binding transcription factor required for expression of biosynthetic genes in the cluster (18, 43), and stcU (formerly verA), encoding a ketoreductase essential for ST production (26). aflR expression should correlate with expression of the entire cluster, while expression of stcU serves as an additional check, as this gene is known to be under the control of AflR and the two genes are located toward opposite ends of the cluster (Fig. 1). For the PN cluster, we examined two of the three genes in the cluster, ipnA and penDE (antA), encoding an isopenicillin N synthetase and an isopenicillin N acyltransferase, respectively. Both genes are essential for PN production (7).

Transcription of the ST cluster is not readily observed until between 40 and 48 h of growth in the wild type (Fig. 3A), but stcU and aflR were strongly up-regulated in the ∆hdaA strain after only 36 h of growth, in contrast to no effect on an adjacent noncluster gene (AN7830.3) (Fig. 1 and 3A) and actin control (Fig. 3A). Neither the wild type nor the ∆hdaA strain showed transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A). Similar results were obtained for the PN cluster. Although some level of PN cluster expression was observed in the wild type at even the earliest time points examined, transcription of ST genes at 24 h, and by 48 h, both strains showed approximately equal amounts of ST gene expression (Fig. 3A).
genes in cultures of wild-type *A. nidulans* grown in liquid media containing concentrations of the oxygen radical-producing compound H$_2$O$_2$ ranging from 0 mM to 3 mM (Fig. 5) at times showing the greatest hdaA effects (e.g., 36 h for the ST cluster and 24 h for the PN cluster) (Fig. 3). Figure 5 demonstrates that neither aflR nor ipnA was affected by H$_2$O$_2$ treatment, nor were flanking genes or actin. Concomitantly, H$_2$O$_2$ had no discernible effect on ST production (Fig. 5C). Interestingly, H$_2$O$_2$ did appear to affect expression of tdiB, for which low concentrations stimulated and high concentrations inhibited expression (Fig. 5B). As the presence of H$_2$O$_2$ does not produce effects resembling those of the hdaA strain, we conclude that the effects on SM cluster expression observed in this mutant are not the result of oxidative stress.

**Effects of HDAC loss on the haeA phenotype.** Since loss of laeA (haeA) leads to a repression of global SM production in all aspergilli examined (4), we investigated whether LaeA might function by interfering with and/or activating heterochromatin formation, postulating that the haeA phenotype would be rescued by HDAC loss. Addition of hdaA to a haeA background resulted in wild-type levels of NOR and PN production in the double mutant (Fig. 6); however, the increase in production was not equivalent to that of the hdaA mutation alone. Thus, the loss of LaeA appears not to affect the function of HdaA, and vice versa. This suggests that, although HdaA and LaeA have opposing effects on ST and PN production, they operate through different mechanisms. This observation is supported by the fact that LaeA, unlike HdaA, positively regulates the telomere-distal TR cluster in addition to the two subtelomeric clusters (3, 4). Global regulation of both telomere-proximal and -distal SM clusters by LaeA is also seen in *A. fumigatus* (29).

As the single hstA and hosB mutations did not affect production of NOR or PN (Fig. 4), we did not expect to see any effect on metabolite production when these alleles were placed in the haeA background. Unexpectedly, the hosB haeA mutant did show increased production of PN over haeA alone, suggesting that this HDAC may play a role in regulating PN production under some circumstances. Combination of the three HDAC loss-of-function mutants with hdaA increased NOR, but not PN, production over the hdaA haeA double mutant, again arguing for an additive effect of all three HDACs on repression of the ST cluster.

**Effects of HDAC inhibition on SM production in other fungal genera.** To determine whether HDAC regulation of SM clusters might extend to other species, we investigated fungi of two genera notable for their small-molecule arsenals. Representative isolates of the species *A. alternata* and *P. expansum* were treated with the class 1 and class 2 HDAC inhibitor TSA (35, 42). TSA treatment resulted in a statistically significant ($P < 0.01$) increase of numerous unidentified SMs in both species (Fig. 7). Thus, HDACs may function in the regulation of secondary metabolism among a broad range of fungal genera.

**DISCUSSION**

The data presented here constitute strong evidence for a role for HdaA in suppression of cluster-derived small-molecule
production in *A. nidulans* and suggest that a role for HDAC in SM regulation may be conserved in other filamentous fungi. Elimination of HdaA, a major HDAC of *A. nidulans*, results in early and increased gene expression of two telomere-proximal small-molecule clusters and production of their corresponding metabolites. Transcriptional suppression by HdaA is precise, since neither actin nor the nearest expressed flanking genes to the ST and PN gene clusters were up-regulated in the \( \Delta hdaA \) mutant. In this respect, HdaA presents cluster demarcation specificity similar, although in an opposing fashion, to that of LaeA (3, 6). However, in contrast to LaeA, where location of LaeA-regulated clusters ranges from telomere proximal to internal on chromosomal arms in both *A. nidulans* and *A. fumigatus* (3, 29), our current evidence supports a role for HdaA only in regulating telomere-proximal clusters.

This is congruent with findings in *Saccharomyces cerevisiae*, which, though devoid of SM clusters, does exhibit HDAC-dependent regulation of select subtelomeric genes. Hda1, the *S. cerevisiae* homologue of HdaA, demonstrates an overall targeting bias for telomere-proximal genes (34). This HDAC is known to be involved in silencing of the subtelomeric adhesin gene *FLO11*. Significantly, relocation of *FLO11* to a telomere-distal region has been shown to release the gene from epigenetic silencing, suggesting that proximity to the telomere is important for such silencing to occur (21). Hda1 also is known to regulate clusters of metabolically unrelated subtelomeric genes, known as HAST (Hda1-affected subtelomeric) domains. These are comprised of a variety of genes that are activated in...
response to adverse environmental conditions, including genes involved in gluconeogenesis, fermentation, alternate carbon source utilization, and responses to various types of stress (34).

Epigenetic regulation of subtelomeric regions is also critical in the pathogenicity of several microbes. A prime example is the regulation of the subtelomeric var gene clusters of the malaria agent Plasmodium falciparum. HDAC-mediated regulation of these genes allows the protozoan to vary antigen display on the surfaces of infected host cells, thus evading the immune response (19). The subtelomeric EPA gene clusters of Candida glabrata, involved in biofilm formation and essential for pathogenicity, are regulated epigenetically by the NAD+/H11001-dependent HDAC Sir2p (15). These genes are turned on in response to the low-nicotinic acid environment of the host urinary tract, resulting in adhesion and subsequent infection (16). Thus, this system allows adhesin production only in the proper environment.

Although it is the sirtuin HDAC that is involved in C. glabrata subtelomeric gene regulation, under our conditions, we did not find a significant effect of hstA (or hosB) loss on NOR or PN production. However, the combination of these deletions with ΔhdaA did cause an increase in NOR production (Fig. 4), and the combination of ΔhosB with ΔlacA also resulted in greater production of PN than in the ΔlacA mutant alone (Fig. 6). It is interesting that ΔhstA and ΔhosB demonstrate a synergistic effect with ΔhdaA, but neither of the former HDAC mutants has a significant individual effect on SM production. Studies with yeast have shown that cooperative repression of chromosomal regions by multiple HDACs is common. While the S. cerevisiae HDACs Hda1, Rpd3 (a homologue of the A. nidulans HDAC RpdA [20]), and Sir2 (a homologue of HstA) are each involved in the suppression of a unique set of genes, they also contribute to the silencing of numerous shared genes (1). Likewise, the Schizosaccharomyces pombe HDACs Clr3, Clr6, Sir2, and Hos2 (homologous to A. nidulans HdaA, RpdA, HstA, and HosA [20], respectively) repress both unique and shared genes (22, 41). The genes repressed by Clr3 and Sir2 demonstrate particularly strong overlap and also tend to overlap with genes regulated by the heterochromatin-associated protein Swi6 (41). Similar to the pattern we observed for NOR production in the triple HDAC mutant (Fig. 4), disrup-

FIG. 6. Production of NOR and PN in HDAC mutant strains with ΔlacA genetic backgrounds. (A) Production of NOR by ΔlacA mutant strains with additional individual ΔhdaA, ΔhstA, or ΔhosB mutations or with all three HDAC knockouts. NOR was extracted from 72-h cultures on solid media in triplicate. (B) Production of PN by ΔlacA mutant strains with additional individual ΔhdaA, ΔhstA, or ΔhosB mutations or with all three HDAC knockouts. PN was extracted from 72-h liquid shake cultures in triplicate and quantified using a bacterial growth inhibition assay. For the histograms, wild-type (WT) production levels were assigned a value of 1, and all other production levels are presented relative to the WT. Different letters above the bars represent statistical differences at $P < 0.01$. The error bars represent ±1 standard deviation.

FIG. 7. Effects of TSA on secondary metabolism of A. alternata and P. expansum. (A) Histogram of relative SM production levels in TSA-treated and untreated cultures. SM production levels in the absence of TSA were assigned a value of 1, and TSA-treated production levels are presented relative to untreated levels. The numbers on the x axis of the graph correspond to metabolites indicated on the TLC plates shown in panels B and C. The differences presented for individual compounds represent statistical differences at $P < 0.01$. The error bars represent ±1 standard deviation. SMs were extracted from 72-h cultures on solid media with or without 1 μM TSA in triplicate.
tion of the genes encoding both S. pombe Clr3 and Clr6 results in upregulation of many genes to a degree higher than that resulting from individual mutation of either gene, and often to an extent greater than a merely additive effect. A large portion of these synergistically regulated genes were found to be subtelomeric (22). While the effects of hstA and hosB on ST/NOR and PN production were not as dramatic as those observed for hdaA, this is not to say that other natural products are not more strongly affected by mutation of these genes. More comprehensive studies are required to determine the extent to which these HDACs are involved in SM regulation. It should be noted that A. nidulans also possesses at least two additional HDACs, RpdA and HosA, that may well be important in this complex regulatory process.

Our data regarding the effects of the HDAC inhibitor TSA on the secondary metabolism of Fusarium and Penicillium provide evidence that HDAC-mediated regulation of small-molecule production may be a widespread phenomenon in filamentous fungi, which we speculate may have evolved as a tool to allow SM production under optimal environmental conditions. Pragmatically, as a variety of chemical HDAC inhibitors are readily available, treatment of fungi with such compounds could potentially provide a means of increasing production of beneficial metabolites and could also aid in the identification of novel natural products that may not have been previously detected due to low production levels under normal growth conditions.

The findings of our study support a role for epigenetic regulation of SM clusters. We hypothesize that epigenetic regulation of secondary metabolism is an efficient way for filamentous fungi to ensure that energetically costly molecules are synthesized only when production is likely to be advantageous. For example, HdaA-mediated repression of SM clusters occurs early in development (Fig. 3). It has long been observed that SM production is generally nil in the initial exponential growth phase and increases when nutrients are limited and growth is restricted (11). A global mechanism(s) to suppress expression of SM clusters during initial vegetative growth and yet allow SM production once sufficient biomass is established should yield competitive advantages for filamentous fungi and provide the fungus with an efficient means of responding to changing foraging and competitive pressures. Evidence suggests that at least two such mechanisms may be operating in the aspergilli: telomere-proximal SM cluster suppression by HdaA and a less spatially limited positive regulation by LaeA. Certainly, loss of laeA in A. fumigatus yields a less pathogenic organism with an efficient means of responding to changing metabolism—from biochemistry to genomics. Nat. Rev. Microbiol. 6:189–198.

ACKNOWLEDGMENTS

Funding has been provided for this research and publication by the USDA Cooperative State Research, Education and Extension Service (CSREES) project WIS049621, NSF MCB-0316393, and NIH 1 R01 AI065728-01 to N.K., as well as the Austrian Science Foundation (P19750) and Tyrolean Science Foundation (0404/225) to S.G. This article is dedicated to the memory of Ann Henry Keller.

REFERENCES

1. Bernstein, B. E., J. K. Tong, and S. L. Schreiber. 2000. Genomewide studies of histone deacetylase function in yeast. Proc. Natl. Acad. Sci. USA 97:13708–13713.
2. Bok, J. W., S. A. Balajee, K. A. Marr, D. Andes, K. F. Nielsen, J. C. Frisvad, and N. P. Keller. 2005. LaeA, a regulator of morphogenetic fungal virulence factors. Eukaryot. Cell 4:1574–1582.
3. Bok, J. W., D. Hofmeister, L. A. Maggio-Hall, R. Murillo, J. D. Glasner, and N. P. Keller. 2006. Genomic mining for Aspergillus natural products. Chem. Biol. 13:31–37.
4. Bok, J. W., and N. P. Keller. 2004. LaeA, a regulator of secondary metabolism in Aspergillus spp. Fungal Genet. Biol. Cell 5:527–535.
5. Bok, J. W., D. Noordermeer, S. P. Kale, and N. P. Keller. 2006. Secondary metabolic gene cluster silencing in Aspergillus nidulans. Mol. Microbiol. 61:1656–1665.
6. Booschard, S., M. Weber, A. Kempf-Sontag, N. P. Keller, and D. Hofmeister. 8 January 2007. Accurate prediction of the Aspergillus nidulans terrequinone gene cluster boundaries using the transcriptional regulator LaeA. Fungal Genet. Biol. doi:10.1016/j.fgb.2006.12.010. [Epub ahead of print].
7. Brakhage, A. A. 1997. Molecular regulation of penicillin biosynthesis (Aspergillus (Emericella) nidulans). FEMS Microbiol. Lett. 148:S1–10.
8. Brown, D. W., J.-H. Yu, H. Kelkar, M. Fernandes, T. C. Neshit, N. P. Keller, T. H. Adams, and T. J. Leonard. 1996. Twenty-five co-regulated transcripts define the sterigmatocystin gene cluster in Aspergillus nidulans. Proc. Natl. Acad. Sci. USA 93:1418–1422.
9. Buades, A., and M. Aoya. 1996. Phylogenetic analysis of the isopenicillin-N-synthetase horizontal gene transfer. J. Mol. Evol. 42:537–542.
10. Buck, S. W., C. M. Gallo, and J. S. Smith. 2006. Diversity in the Sir2 family of deacetylases. Nat. Rev. Mol. Cell Biol. 7:263–274.
Penalva, M. Perneta, C. Price, B. L. Pritchard, M. A. Quail, E. Rabbinowitsch, N. Rawlin, M. A. Rajandream, U. Reichard, H. Renaud, G. D. Robson, S. Rodriguez de Cordoba, J. M. Rodriguez-Pena, C. M. Ronning, S. Rutter, S. L. Salzberg, M. Sanchez, J. C. Sanchez-Ferrero, D. Saunders, K. Seeger, R. Squares, S. Squares, M. Takeuchi, F. Tekaia, G. Turner, C. R. Vazquez de Aldana, J. Weidman, O. White, J. Woodward, J. H. Yu, C. Fraser, J. E. Galagan, K. Asai, M. Machida, N. Hall, B. Barrell, and D. W. Denning. 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438:1151–1156.

29. Perrin, R., N. Federova, J.-W. Bok, J. Wortman, R. A. Cramer, Jr., H. Kim, W. Nierman, and N. Keller. 2007. Mapping the chromosomal landscape of co-regulated pathogenicity factors in Aspergillus fumigatus. PLoS Pathog. 3:e50.

30. Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. P. Macdonald, and A. W. J. Buxton. 1953. The genetics of Aspergillus nidulans. Adv. Genet. 5:141–238.

31. Pryde, F. E., H. C. Gorham, and E. J. Louis. 1997. Chromosome ends: all the same under their caps. Curr. Opin. Genet. Dev. 7:822–828.

32. Rehmeyer, C., W. Li, M. Kusaba, Y. S. Kim, D. Brown, C. Staben, R. Dean, and M. Farman. 2006. Organization of chromosome ends in the rice blast fungus, Magnaporthe oryzae. Nucleic Acids Res. 34:4685–4701.

33. Riethman, H., A. Ambrosini, and S. Paul. 2005. Human subtelomere structure and variation. Chromosome Res. 13:505–515.

34. Robyr, D., Y. Suka, I. Xenarios, S. K. Kurdistani, A. Wang, N. Suka, and M. Grunstein. 2002. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell 109:437–446.

35. Rundlett, S. E., A. A. Carmen, R. Kobayashi, S. Bavykin, B. M. Turner, and M. Grunstein. 1996. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA 93:14503–14508.

36. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

37. Shimizu, K., and N. P. Keller. 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. Genetics 157:591–600.

38. Tribus, M., J. Galehr, P. Trojer, G. Brosch, P. Loidl, F. Marx, H. Haas, and S. Graessle. 2005. HdaA, a major class 2 histone deacetylase of Aspergillus nidulans, affects growth under conditions of oxidative stress. Eukaryot. Cell. 4:1736–1745.

39. Trojer, P., E. M. Brandtner, G. Brosch, P. Loidl, J. Galehr, R. Linzmaier, H. Haas, K. Mair, M. Tribus, and S. Graessle. 2003. Histone deacetylases in fungi: novel members, new facts. Nucleic Acids Res. 31:3971–3981.

40. Walton, J. D. 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fungal Genet. Biol. 30:167–171.

41. Wiren, M., R. A. Silverstein, I. Sinha, J. Walfridsson, H. M. Lee, P. Laurensen, L. Pillus, D. Robyr, M. Grunstein, and K. Ekwall. 2005. Genome-wide analysis of nucleosome density histone acetylation and HDAC function in fission yeast. EMBO J. 24:2906–2918.

42. Yoshida, M., M. Kijima, M. Akita, and T. Beppu. 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. 265:17174–17179.

43. Yu, J. H., R. A. Butchko, M. Fernandes, N. P. Keller, T. J. Leonard, and T. H. Adams. 1996. Conservation of structure and function of the aflatoxin regulatory gene aflR from Aspergillus nidulans and A. flavus. Curr. Genet. 29:549–555.