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Cryptic *Aspergillus nidulans* Antimicrobials▽

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Secondary metabolite (SM) production by fungi is hypothesized to provide some fitness attribute for the producing organisms. However, most SM clusters are "silent" when fungi are grown in traditional laboratory settings, and it is difficult to ascertain any function or activity of these SM cluster products. Recently, the creation of a chromatin remodeling mutant in *Aspergillus nidulans* induced activation of several cryptic SM gene clusters. Systematic testing of nine purified metabolites from this mutant identified an emodin derivate with efficacy against both human fungal pathogens (inhibiting both spore germination and hyphal growth) and several bacteria. The ability of catalase to diminish this antimicrobial activity implicates reactive oxygen species generation, specifically, the generation of hydrogen peroxide, as the mechanism of emodin hydroxyl activity.

For decades, natural products produced by bacteria and fungi have been mined for use as antimicrobials. The easily accessible compounds (e.g., β-lactams, macrolides) were the first to be mined and patented for drug development. Recently, advances in genome sequencing have revealed an arsenal of untapped secondary metabolite (SM) gene clusters in both kingdoms (3, 25, 26). In many of these organisms the number of SM gene clusters (20 to 50) far exceeds the number of known metabolites, hence, the concept of "silent" SM gene clusters (7, 12, 14). The inaccessibility to these compounds in laboratory settings is hypothesized to be due to an inability to replicate the conditions that induce SM activation in nature.

In their ecological habitat, microbes are constantly being challenged, often by other microbes. These microbial interactions activate SM clusters that produce bioactive compounds that offer protection for the organism, niche security, and a myriad of other benefits (27). One case in point is the *Aspergillus fumigatus* gliotoxin gene cluster. Although most widely referred to for its role as a toxin in invasive aspergillosis (15), gliotoxin is a potent antifungal and likely helps secure *A. fumigatus* dominance in soil environments (1, 8, 16, 19, 21). *A. fumigatus* self protects from endogenous gliotoxin by expressing a gliotoxin reductase, GliT, also known to exhibit oxidase activity, encoded in the cluster (29, 31). Because the gliotoxin gene cluster is expressed under most laboratory conditions, this metabolite was identified decades ago (36). However, most antimicrobial gene clusters are silent in laboratory environments, presumably because there is little to no competition and therefore no need for protection. Until recently, the hurdle of silent gene cluster activation in laboratories has limited the mining resources for drug discovery and hindered any understanding of the ecological role of these SMs. With the advent of genome sequencing and recent advances in epigenetic modifications, formerly silent gene clusters can be activated, resulting in the production of novel compounds.

Epigenetic modification has been best illustrated in the model organism *Aspergillus nidulans*. The secondary metabolome of this fungus contains at least 25 SM gene clusters, many of which are characteristically silent in the laboratory environment, and therefore considerable numbers of its natural products are unknown (7, 12, 14). However, through chromosomal remodeling of *A. nidulans*, an array of novel bioactive compounds can be retrieved. For example, deletion of *cclA*, which encodes a protein (CclA) that is involved in histone 3 lysine 4 (H3K4) methylation, results in the activation of the several gene clusters yielding emodin derivatives, including monodictyophenone and the antiosteoporosis polyketides F9775A and F9775B, which are derived from orsellinic acid (5, 28).

At the same time that loss of CclA was found to induce these gene clusters, coculture of *A. nidulans* with *Streptomyces hygroscopicus* resulted in the induction of several cryptic SMs, including the F9775 metabolites (32). We hypothesized that the *cclA* deletant unveiled microbial SM induction pathways utilized by *A. nidulans* during interaction with other microbes. The purpose of the present study was to test this hypothesis by examining potential antimicrobial activities of compounds released by activated pathways against possible competitor microbes (Table 1). We found that a derivative of emodin, 2-hydroxyemodin (2-OH), effectively inhibits the growth of *A. fumigatus*, *Aspergillus flavus*, *Candida albicans*, *Bacillus cereus*, and *Micrococcus luteus.* We suggest that the process of SM

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TABLE 1. Strains used in this study

Organism	Name	Source of strain
Aspergillus fumigatus	WT (Af293)	Nancy Keller laboratory
Aspergillus fumigatus	AF41	David Denning laboratory
Aspergillus fumigatus	AF72	David Denning laboratory
Aspergillus fumigatus	F14532	David Denning laboratory
Aspergillus fumigatus	F14403	David Denning laboratory
Aspergillus fumigatus	F16216	David Denning laboratory
Aspergillus fumigatus	F11628	David Denning laboratory
Aspergillus fumigatus	F12776	David Denning laboratory
Aspergillus fumigatus	F13747	David Denning laboratory
Aspergillus flavus	WT (NRRL3357)	Nancy Keller laboratory
Aspergillus nidulans	WT (RJW64; *veA1*)	Nancy Keller laboratory
Aspergillus nidulans	*cclA*Δ (RAAS74.29; *pyroA4* Δ*cclA*::*pyroA veA1*)	Nancy Keller laboratory
Aspergillus nidulans	*cclA*Δ *mdpG*Δ (LO2149; *pyrG89 pyroA4* Δ*nkuA*::*argB riboB2* Δ*stcJ*::Af*riboB* Δ*cclA*::Af*pyroA* Δ*mdpG*::Af*pyrG veA1*)	Berl Oakley laboratory
Aspergillus nidulans	*alcA(p)*:*mdpE* [LO2333; *pyrG89 pyroA4* Δ*nkuA*::*argB riboB2* Δ*stcJ*::Af*riboB* Δ*mdpE*::Af*pyrG-alcA(p)*::*mdpE veA1*]	Berl Oakley laboratory
Pseudomonas aeruginosa	PAO1	Nancy Keller laboratory
Bacillus cereus	U85	Nancy Keller laboratory
Micrococcus luteus	*M. luteus*	Nancy Keller laboratory

cluster activation due to competition in nature can be mimicked through epigenetic modifications, resulting in an arsenal of novel compounds that can be characterized for their ecological roles and mined for new therapeutics.

MATERIALS AND METHODS

Microbial growth conditions. *Aspergillus*, *Candida*, *Pseudomonas*, *Bacillus*, and *Micrococcus* strains (Table 1) were maintained as frozen glycerol stocks at −80°C and grown at 37°C on glucose minimal medium (GMM) or yeast extract-peptone-dextrose medium (YPD).

Preparation of test organisms. *Aspergillus* spp. were point inoculated onto GMM and incubated at 37°C for 3 to 5 days. Spore suspensions were prepared as follows: 7 ml of 0.01% Tween–water was added to each plate, and then spores were isolated by gently scraping the surface of each plate with a sterile plastic spreader. The spore suspensions were quantified using a hemocytometer. *Candida albicans* and bacterial species were grown on YPD agar plates at 37°C for 24 h. Immediately prior to use, cells were removed from the plate with a sterile applicator, suspended in sterile phosphate-buffered saline, and counted using a hemocytometer.

Morphology. For macroscopic phenotype analysis, 10^4 spores in 10 μl Tween-water were point inoculated onto GMM with supplements or lactose minimal medium (LMM) with cyclopentanone and supplements for induction of the *alcA* promoter. Cultures were grown at 37°C for 4 days. For microscopic analysis, spores were added to 25 ml of GMM with supplements to a final concentration of 10^6 spores/ml. Cultures were grown for 20 h at 37°C shaking at 250 rpm. Cultures were then shifted either to fresh GMM plus supplements or LMM plus cyclopentanone and supplements and grown at 37°C shaking at 250 rpm for an additional 4 h. Microscopic hyphal morphology was then viewed at 400× magnification.

Inhibition analysis. To determine the effect of coculturing *Aspergillus* spp. strains on growth, 5 μl of a 10^4 spores/ml suspension of *A. nidulans* wild type, *cclA*Δ, or *cclA*Δ *mdpG*Δ strains was point inoculated on GMM plates with supplements and *A. fumigatus* wild-type (WT) strain AF293 or *A. nidulans* WT. The *alcA(p)*::*mdpE* inoculations were made on LMM with cyclopentanone and supplements for induction of the *alcA* promoter. Plates were incubated for 3 days at 37°C, and radial growth was assessed visually for signs of growth inhibition at 3 to 5 days postinoculation. Images shown in the figure insets were obtained with a dissecting microscope at 50× magnification.

Preparation of crude extracts and purified metabolites. *A. nidulans* wild-type and *cclA*Δ strains were inoculated on GMM agar and incubated at 37°C for 4 to 5 days, and then mycelia and supporting agar were excised from the plates with a sterile blade as described by Bok et al. (5). In general, 10 to 20 plates, or 20 ml of agar, were extracted. Briefly, the mycelia and agar were homogenized for 10 s in a blender containing 250 ml of methanol. An additional 500 ml of methanol was added to the slurry and was stirred slowly overnight at room temperature. The slurry was filtered through Miracloth and three layers of Whatman filter paper and using a vacuum apparatus to remove agar and mycelia. The filtrate was then evaporated using a Rotavapor R-210. The evaporated material was suspended in 200 ml of a 1:1 mixture of water and ethyl acetate and separated using a 250-ml separatory funnel, and the aqueous and organic fractions were collected and evaporated using the Rotavapor. These crude fractions were stored in sealed glass vials at −80°C. Purified metabolites were collected as described by Bok et al. (5).

Extract testing. Crude extracts and purified metabolites were assayed for antifungal activities against various strains of *A. fumigatus* and *A. nidulans*, as well as *A. flavus*, *Pseudomonas aeruginosa*, *B. cereus*, *M. luteus*, and *C. albicans*, by using a 96-well plate format as previously described (24). Briefly, spores, yeast, or bacteria (1×10^3/well) were added to triplicate wells containing 200 μl of RPMI with morpholinepropanesulfonic acid (MOPS) and various concentrations of crude extracts or purified metabolites from the *cclA*Δ strain. Plates were incubated for 24 to 48 h, and MICs were determined by visual examination of wells. In preliminary experiments, we determined that there was no solvent-related inhibitory activity against the test species from the evaporated organic fraction of the crude extracts prior to medium addition. Similarly, the addition of dimethyl sulfoxide (DMSO) at comparable concentrations as observed with the addition of purified metabolites had no inhibitory activity against the fungi tested. For quantification of germinated spores, three biological replicates and two technical replicates of 100 spores were counted for each treatment. Spores were considered germinated if an apparent germ tube was seen emerging from the spores.

Catalase inhibition assay. To determine if the observed 2-OH antifungal activity was due to generation of reactive oxygen species, catalase (Sigma-Aldrich, St. Louis, MO) was added at various concentrations to test wells containing 2-OH. Neither catalase nor high concentrations of the protein bovine serum albumin exhibited antimicrobial activity against the fungi tested.

RESULTS

Competition growth assays. Our laboratory previously showed that the loss of CclA, which is involved in H3K4 methylation, turns on cryptic secondary metabolite gene clusters in *A. nidulans*. This resulted in the production of several silent SMs, including monodictyophenone, emodin and emodin derivatives, and the antiosteoporosis polyketides F9775A and F9775B (5). The expansive armamentarium of SMs produced by fungi is thought to provide an adaptive advantage, allowing them to compete with other microbiota and colonize diverse ecological niches. We hypothesized that the cryptic SM gene clusters (e.g., the monodictyophenone cluster emodin derivatives) turned on in the *A. nidulans cclA*Δ strain might

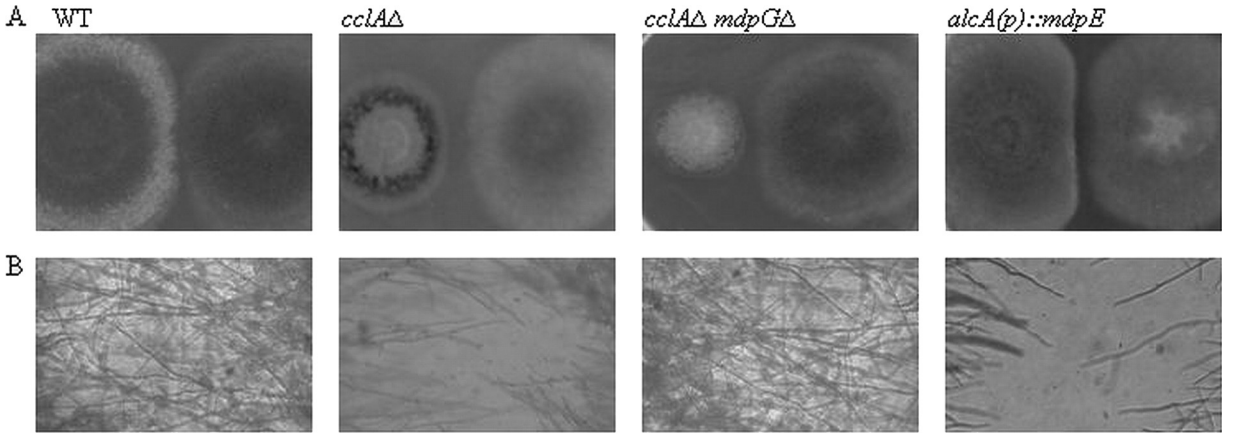

FIG. 1. *A. nidulans cclA*Δ inhibits the growth of *A. fumigatus* strains on GMM agar. Both 2-OH-overproducing strains [*cclA*Δ and *alcA(p)*::*mdpE*] prevented hyphal intercalation of the radial growth of both *A. nidulans* (data not shown) and *A. fumigatus* wild-type strains. In contrast, the mycelia of wild-type *A. nidulans* and *A. fumigatus* strains overlapped with no signs of inhibitory activity. The *cclA*Δ *mdpG*Δ strain, which failed to produce 2-OH, showed interactions similar to wild type. All strains were grown on GMM with the exception of the confrontation between *A. nidulans alcA(p)*::*mdpE* and *A. fumigatus*, which was conducted on LMM with cyclopentanone.

produce SMs with antimicrobial activities against other fungi and bacteria.

To investigate this hypothesis we cocultured the wild type and three *A. nidulans* mutants [*cclA*Δ upregulated in monodictyophenone and other SM clusters, *alcA(p)*::*mdpE* upregulated in just the monodictyophenone cluster, and *cclA*Δ *mdpG*Δ unable to express metabolites in the monodictyophenone cluster but upregulated in other SMs] with wild-type *A. fumigatus* on GMM agar medium (WT, *cclA*Δ, and *cclA*Δ *mdpG*Δ) or LMM agar with cyclopentanone [*alcA(p)*::*mdpE*]). Interestingly, we observed that the radial growth of *A. fumigatus* mycelia did not extend into regions of the plate containing strains where the monodictyophenone cluster was expressed [*cclA*Δ and *alcA(p)*::*mdpE*] (Fig. 1). In contrast, the mycelia of wild-type *A. nidulans* and *A. fumigatus* strains overlapped with no signs of inhibitory activity. Inhibition by the *cclA*Δ strain was abrogated in a strain lacking the backbone PKS for this cluster (*cclA*Δ *mdpG*Δ). Similar results were observed with the mutant series against wild-type *A. nidulans* (data not shown). These results indicated that upregulation of the monodictyophenone gene cluster produced SMs with inhibitory activities against wild-type strains of *A. fumigatus* and *A. nidulans*.

Next, crude extracts were prepared from *A. nidulans cclA*Δ and wild-type strains grown on GMM agar at 37°C. The aqueous and organic fractions of the crude extracts were then assessed for antifungal activities against *A. fumigatus* wild-type AF293 in a 96-well microtiter plate-based assay. *A. fumigatus* spores (1×10^3 CFU/well) were added to wells containing various concentrations of the crude extracts, and germination was assessed microscopically over time to evaluate inhibitory activity. Although aqueous extracts had no effect on fungal growth (data not shown), we did observe that organic extracts from *A. nidulans cclA*Δ, but not *A. nidulans* wild type, possessed potent antifungal activity and largely inhibited the germination of *A. fumigatus* spores (Fig. 2A and C). This result was consistent with our hypothesis that an SM(s) with antifungal activity was produced as a result of the loss of CclA.

Antimicrobial activities of purified compounds from *A. nidulans cclA*Δ. We previously identified several organically soluble SMs produced by the *A. nidulans cclA*Δ mutant strain, including desacetylaustin, dehydroaustinol, citreorosein, emodic acid, 2-OH, 2-aminoemodin, emodin, and the polyketides F9775A and F9775B (5). We speculated that one or more of these SMs might mediate the inhibitory activities observed with the crude extracts. First, each of the compounds was purified and assessed for antifungal activity against *A. fumigatus* spores in a 96-well microtiter plate assay at concentrations ranging from 9 μM to 567 μM. We observed that of the nine compounds tested, only 2-OH exhibited significant antifungal activity against *A. fumigatus*, and 2-OH activity reconstituted the inhibition of germination effects seen with treatment with *cclA*Δ extracts (Fig. 2B and C).

To further investigate whether the antifungal activity exhibited by 2-OH was specific for *A. fumigatus*, or if 2-OH exhibited broader antimicrobial activity, we assessed 2-OH activity against three fungi (*A. fumigatus*, *A. flavus*, and *C. albicans*) and three bacteria (*P. aeruginosa*, *B. cereus*, and *M. luteus*). We observed that 2-OH exhibited robust antimicrobial activities against both aspergilli, *C. albicans*, and two of the three bacterial species tested (Table 2). *A. flavus* (MIC_{100}, 142 to 284 μM) was more resistant than *A. fumigatus* (MIC_{100}, 35 to 71 μM), which was more resistant than *C. albicans* (MIC_{100}, 9 to 18 μM). 2-OH was also quite effective against *B. cereus* (MIC_{100}, ≤9 μM) and *M. luteus* ($MIC_{100,}$ ≤9 μM), while *P. aeruginosa* showed significantly higher resistance to 2-OH (MIC, 284 to 567 μM) (Table 2).

2-OH mechanism of action. To begin the investigation of potential mechanisms of action, we assessed the inhibitory activities of 2-OH against a collection of *A. fumigatus* strains with known antifungal drug resistance to voriconazole, itraconazole, or posaconazole. The rationale for this approach was that if a strain with a known phenotype (and corresponding genotype) exhibited resistance to 2-OH, then we could gain insights into the mechanism by which 2-OH kills *A. fumigatus*. All of the *A. fumigatus* drug-resistant strains assessed exhibited

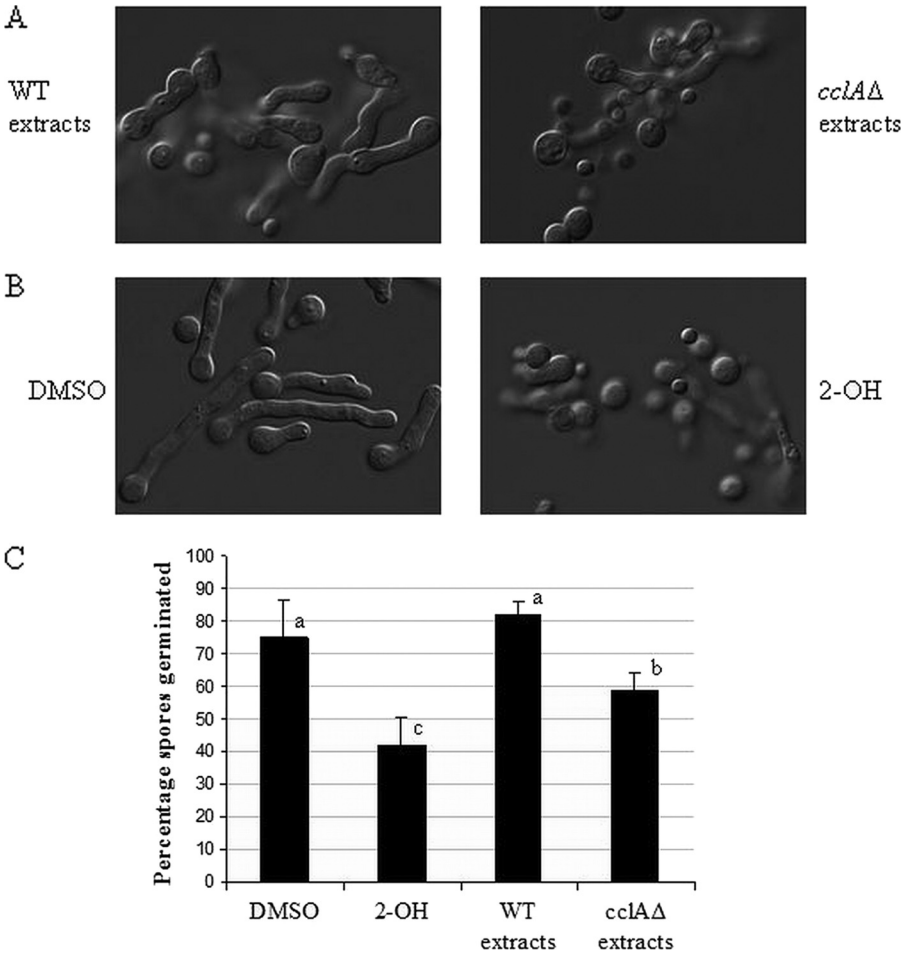

FIG. 2. AF293 germination and germling morphology are affected by *cclA*Δ but not wild-type extracts. (A) Germination of spores is inhibited by addition of organic extracts from *cclA*Δ strains, but not from WT strains. These effects included decreased percentages of germination and decreased conidial swelling. (B) Addition of purified 2-OH reconstitutes these effects. DMSO is the control solvent for 2-OH. (C) Quantification of the percentage of germinated spores for each treatment. Letters (a to c) indicate differences with statistical significance ($P < 0.05$) according to the Tukey-Kramer multiple comparison test.

susceptibility to 2-OH that was comparable to wild-type *A. fumigatus* (data not shown). This suggested that the mechanism of activity was novel and independent of defined pathways known to be involved in resistance to azole drugs.

TABLE 2. 2-OH exhibits inhibitory activities against *M. luteus*, *B. cereus*, *P. aeruginosa*, *A. fumigatus*, *A. flavus*, and *C. albicans* at various concentrations

2-OH concn (μM)	Growth of species following the indicated treatment[a]					
	M. luteus	*B. cereus*	*P. aeruginosa*	*A. fumigatus*	*A. flavus*	*C. albicans*
567	−	−	−	−	−	−
284	−	−	+	−	−	−
142	−	−	+	−	+	−
71	−	−	+	−	+	−
35	−	−	+	+	+	−
18	−	−	+	+	+	−
9	−	−	+	+	+	+
Untreated control	+	+	+	+	+	+

[a] The symbols + and − denote growth and no growth, respectively. Growth was assessed by microscopic visualization of wells 48 h posttreatment.

Emodin (1,3,8-trihydrozy-6-methylanthraquinone) is a free radical generator that, when used as a therapeutic agent, can cause immunosuppression, vasorelaxation, and hypolipidemia (37). The production of free radicals, specifically, hydrogen peroxide, is thought to contribute to the immunosuppressive activities of emodin (17). 2-OH has also been shown to specifically produce hydrogen peroxide and not superoxide (18). Therefore, we hypothesized that hydrogen peroxide might account for the fungicidal activity of 2-OH against *A. fumigatus.* To test this hypothesis, we assessed whether catalase, which catalyzes the reduction of hydrogen peroxide to water and oxygen, could diminish the inhibitory activity of 2-OH against *A. fumigatus*. In agreement with our hypothesis, we observed that catalase did effectively abrogate the inhibitory activity of 2-OH against *A. fumigatus* (Table 3). The complete loss of antifungal activity in the presence of catalase suggested that the inhibitory activity of 2-OH was dependent on the production of hydrogen peroxide. Interestingly, challenge of *A. fumigatus* with 2-OH did not result in increased endogenous catalase activity (data not shown), in accordance with the sensitivity to this metabolite.

TABLE 3. Catalase abrogates the inhibitory activity of 2-OH[a]

Additive (amt or concn)				Growth[b]
Catalase (U)	2-OH (μM)	BSA (μg)	H_2O_2 (mM)	
0	0	370	0	+
10,000	0	0	0	+
0	60	0	0	−
5,000	60	0	0	+
0	60	370	0	−
0	0	0	0.5	+
0	0	0	1	+
0	0	0	1.5	−
0	0	0	2	−
5,000	0	0	2	+

[a] AF293 spores (1×10^3 total CFU) were added to wells with the indicated additives. Addition of 60 μM 2-OH resulted in growth inhibition of spores similar to that caused by addition of 1.5 mM H_2O_2. Bovine catalase at a concentration of 5,000 units/well was able to eliminate the growth inhibition by 2-OH and H_2O_2.

[b] The symbols + and − denote growth and no growth, respectively. Growth was assessed by microscopic visualization of wells 48 h posttreatment.

TABLE 4. *A. nidulans* wild-type, *cclA*Δ, *cclA*Δ *mdpG*Δ, and *alcA(p)*::*mdpE* strains exhibit similar patterns of susceptibility to 2-OH[a]

2-OH treatment (μM)	Growth of strain in RPMI with indicated 2-OH concn				Growth of *alcA*::*mdpE* in RPMI + 30 mM cyclopentanone
	WT	*cclA*Δ	*cclA*Δ *mdpG*Δ	*alcA*::*mdpE*	
567	−	−	−	−	−
284	−	−	−	−	−
142	+	−	+	−	−
71	+	+	+	+	+
35	+	+	+	+	+
18	+	+	+	+	+
9	+	+	+	+	+
Untreated control	+	+	+	+	+

[a] The patterns of susceptibility suggest that the monodictyophenone gene cluster does not include a resistance-encoding gene to protect the fungus from the metabolites produced by this pathway. The symbols + and − denote growth and no growth, respectively. Growth was assessed by microscopic visualization of wells 48 h post treatment.

Lack of self-resistance activity. Frequently, fungal SM gene clusters include a gene encoding a protein that imparts a resistance mechanism for the fungus against the respective SM produced by the cluster. For instance, *gliT* in the gliotoxin gene cluster encodes a reductase that detoxifies gliotoxin in order to protect *A. fumigatus* against endogenous gliotoxin (29, 31). We were interested to see if a *cclA*Δ strain (overproducing 2-OH), a *cclA*Δ strain, *mdpG*Δ strain (unable to produce 2-OH or other metabolites in this pathway due to deletion of *mpdG* encoding the monodictyophenone cluster polyketide synthase [10]), or an *alcA(p)*::*mdpE* strain (overproducing 2-OH due to overexpression of *mdpE* encoding the monodictyophenone pathway-specific transcription factor [10]) showed variances in susceptibilities to 2-OH. Table 4 shows that there was little difference in 2-OH sensitivity between these three *A. nidulans* strains and the wild type. The slight increase in sensitivity of *cclA*Δ may be suggestive of the endogenous overproduction of 2-OH in this strain, although this does not appear to be the case for the *alcA(p)*::*mdpE* strain. Regardless, this suggests that the monodictyophenone gene cluster does not include a gene that encodes resistance to 2-OH-mediated growth inhibition.

We also speculated that the reduced rate of growth and/or altered morphology of all three *A. nidulans* mutants might give insight into SM function in *A. nidulans*. Macroscopic and microscopic examination of all three strains compared to the wild type revealed a dominant impact of *cclA*Δ on diameter growth that was not alleviated in the double *cclA*Δ *mpdG*Δ strain (Fig. 3). However, loss of monodictyophenone cluster expression in *cclA*Δ did result in an increase in altered colony morphology. Because induction of the *alcA(p)*::*mdpE* allele led to decreased/delayed conidial production, it is possible that one or more of the monodictyophenone cluster-generated metabolites has a negative impact on conidiophore development.

DISCUSSION

Historically, the vast majority of antimicrobial compounds have either been natural products or derived from natural products. These include a diverse spectrum of drugs, including antibiotics, antifungals, antiparasitics, lipid control agents, immunosuppressants, and anticancer drugs (20). In the last 2 decades there has been a shift in pharmaceutical research that has emphasized high-throughput screening of large libraries of synthetically produced compounds. This shift in focus away from natural products occurred in part because of the difficulty of finding new novel natural products. For example, members of the aspergilli are known to be rich sources of bioactive metabolites, but there remains a discontinuity between the vast number of SM gene clusters and the relatively few compounds described for the genus, leaving the true SM potential of these fungi unknown (7, 12, 14).

The disproportionate cluster to SM ratio of *Aspergillus* species is due largely to the inability to promote the expression of SM gene clusters in the laboratory. However, recent genomic mining of *A. nidulans* has uncovered several techniques to stimulate formerly silent clusters (4, 5, 6, 9, 11, 12, 22, 30, 32, 34). Epigenetic tools, in particular (e.g., inactivation of genes encoding histone-modifying enzymes or use of histone deacetylase inhibitors), have tremendous power as they activate tracks of chromatin otherwise subject to regulation by unknown environmental ligands (5, 15, 33).

We suggest that at least one microbial activation pathway proceeds through H3K4 modification. The *A. nidulans cclA*Δ mutant, defective in H3K4 methylation (5), inhibited growth of wild-type *A. nidulans* and the pathogen *A. fumigatus*. We hypothesized that this inhibitory property might be related to altered SM production in *cclA*Δ. This was confirmed when crude extracts from *A. nidulans cclA*Δ strains, but not extracts from wild-type strains, exhibited antifungal activities against *A. flavus* and *A. fumigatus*, fungi with soil niches similar to that of *A. nidulans*. This activity appeared quite broad as crude extract and also showed antibiosis against *C. albicans*, as well as the bacteria *P. aeruginosa*, *B. cereus*, and *M. luteus*. Based on our studies with the purified compounds from two of the *cclA*Δ-induced clusters, the inhibitory activity of crude extracts is mediated primarily by 2-OH. This was surprising, because neither emodin nor 2-aminoemodin exhibited any detectable antimicrobial activity at low molarities in our assays, despite

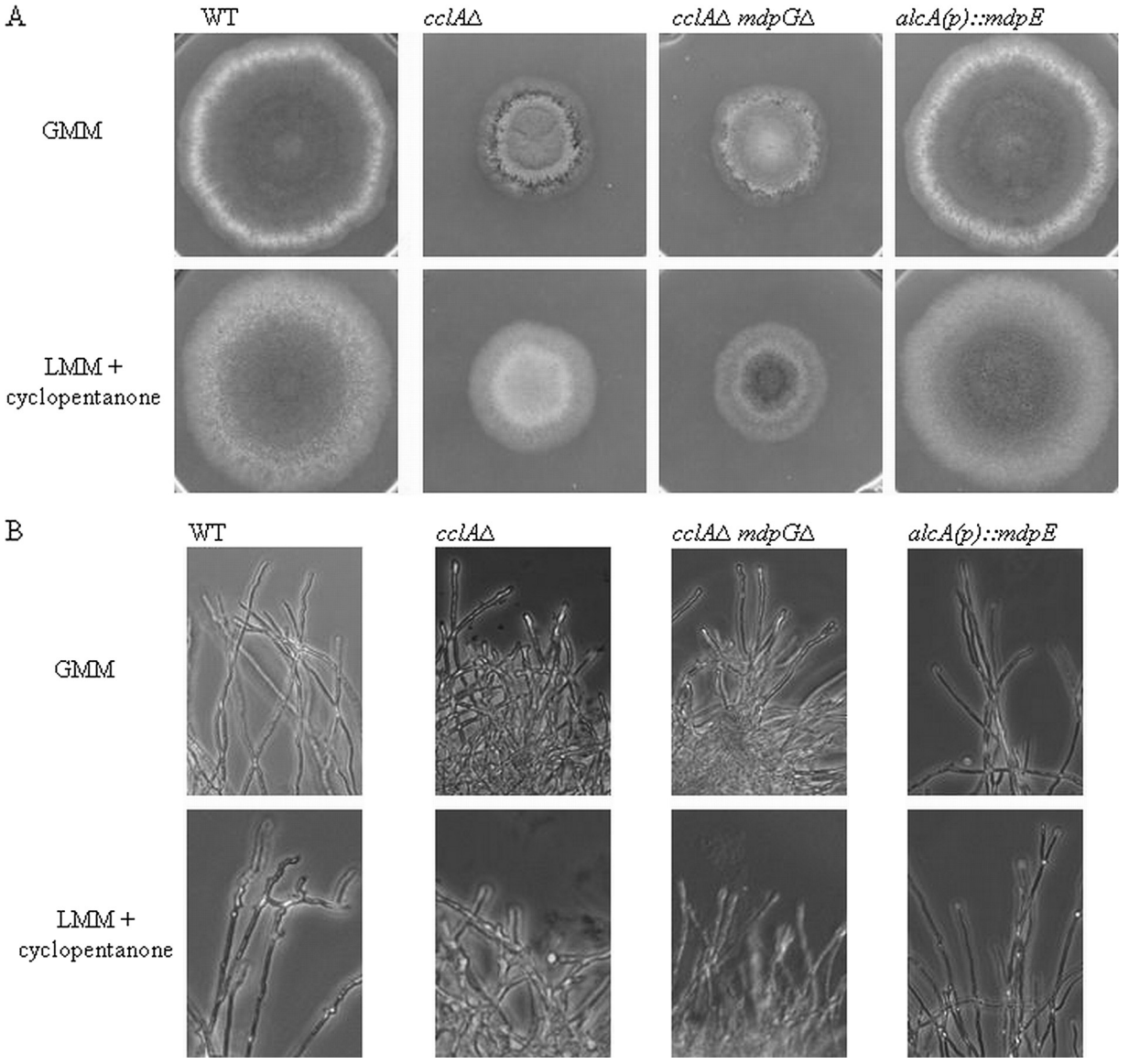

FIG. 3. Morphology abberancies in *A. nidulans* mutants. (A) Colony morphology varies between strains. *cclA*Δ leads to a decrease in colony diameter, likely independent of production of emodin derivatives, as the *cclA*Δ *mdpG*Δ strain devoid of emodin derivative production also was inhibited in diameter. However, the decreased conidiation observed in both the *cclA*Δ and *alcA(p)::mdpE* strains appeared to be associated with the increased expression of the monodictyophenone cluster. (B) Microscopic examination of hyphal morphology differs among strains but is not dependent upon production of emodin derivatives.

other investigators' reports of antibacterial properties of emodin (2, 35).

We utilized strains of *A. fumigatus* with known drug resistance genotypes in an effort to identify molecular pathways susceptible to 2-OH activity. This pathogenic fungus in particular is increasingly resistant to common antifungals, with some scientists citing possible resistance acquisition due to overuse of agricultural fungicides (23). Since all of the *A. fumigatus* drug-resistant strains exhibited equivalent susceptibilities, we conclude that the mechanism of action is independent of ergosterol biosynthesis and drug efflux pump activity. The ability of catalase to abrogate 2-OH activity points toward the production of reactive oxygen intermediates as the primary mechanism of inhibitory activity. This is consistent with previous studies that have reported 2-OH is a free radical generator (18). The absence of emodin-mediated inhibitory activity suggests that the level of reactive oxygen species produced may be below that threshold required for antifungal activity. However, the addition of the hydroxyl group to emodin is likely to result in a compound with increased potential of producing reactive oxygen species. This may account for why 2-OH is more inhibitory than emodin. Previous results support an increased reactivity of 2-OH over emodin (13).

Microbes often exhibit self-protection from their own antimicrobials. As mentioned earlier, the gliotoxin-producing species *A. fumigatus* is tolerant of gliotoxin but shows enhanced sensitivity to this antifungal when one of the genes in the gliotoxin gene cluster, *gliT*, is deleted (29, 31). GliT encodes a

gliotoxin reductase that reduces the oxidized form of gliotoxin, thus inhibiting glutathione depletion in the fungal cell. An examination of the monodictyophenone gene cluster did not identify a protein with possible 2-OH protection activity (10), and our examination of sensitivity of the *cclA*Δ, *cclA*Δ *mdpG*Δ, and *alcA(p)*::*mdpE* strains supported a lack of specific 2-OH protection as encoded by the cluster. However, morphological examination of the three mutants did suggest that one or more of the monodictyophenone cluster-generated SMs negatively impacts conidiophore formation in the producing organisms.

Here, we have presented 2-OH as an example of the antimicrobial possibilities possessed within silent SM gene clusters. We suggest that pathways activated during niche securement and microbial interactions can be turned on in the absence of these interactions through chromatin remodeling. The procedures outlined in this paper present a methodology that can be used to activate these clusters with subsequent production of novel compounds that can be explored for their ecological niche, antimicrobial activity, and new drug therapies.

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