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Aspergillus Volatiles Regulate Aflatoxin Synthesis and Asexual Sporulation in *Aspergillus parasiticus*▽

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***Aspergillus parasiticus* is one primary source of aflatoxin contamination in economically important crops. To prevent the potential health and economic impacts of aflatoxin contamination, our goal is to develop practical strategies to reduce aflatoxin synthesis on susceptible crops. One focus is to identify biological and environmental factors that regulate aflatoxin synthesis and to manipulate these factors to control aflatoxin biosynthesis in the field or during crop storage. In the current study, we analyzed the effects of aspergillus volatiles on growth, development, aflatoxin biosynthesis, and promoter activity in the filamentous fungus *A. parasiticus*. When colonies of *Aspergillus nidulans* and *A. parasiticus* were incubated in the same growth chamber, we observed a significant reduction in aflatoxin synthesis and asexual sporulation by *A. parasiticus*. Analysis of the headspace gases demonstrated that *A. nidulans* produced much larger quantities of 2-buten-1-ol (CA) and 2-ethyl-1-hexanol (EH) than *A. parasiticus*. In its pure form, EH inhibited growth and increased aflatoxin accumulation in *A. parasiticus* at all doses tested; EH also stimulated aflatoxin transcript accumulation. In contrast, CA exerted dose-dependent up-regulatory or down-regulatory effects on aflatoxin accumulation, conidiation, and aflatoxin transcript accumulation. Experiments with reporter strains carrying *nor-1* promoter deletions and mutations suggested that the differential effects of CA were mediated through separate regulatory regions in the *nor-1* promoter. The potential efficacy of CA as a tool for analysis of transcriptional regulation of aflatoxin biosynthesis is discussed. We also identify a novel, rapid, and reliable method to assess norsolorinic acid accumulation in solid culture using a Chroma Meter CR-300 apparatus.**

Aflatoxins are environmental carcinogens and mutagens produced by several aspergilli. The entrance of these compounds into the food chain occurs through the contamination of economically important crops (corn, peanuts, tree nuts, dried fruits and vegetables, and medicinal herbs) predominantly by the aflatoxigenic fungi *Aspergillus parasiticus* and *Aspergillus flavus* (1, 9, 10).

One goal of our laboratory is to identify biological and environmental factors that control aflatoxin synthesis in aspergillus; these factors could then be manipulated in the field or during crop storage to reduce aflatoxin contamination. Our laboratory recently focused on the identification of fungal volatiles that regulate aflatoxin synthesis; others have studied the correlation between the pattern of specific fungal volatiles and the ability to synthesize ochratoxin (12) or aflatoxin (26) in attempts to use these compounds to identify toxigenic isolates.

We previously analyzed the effects of ethylene and CO_2, gases produced naturally by *A. parasiticus* and *Aspergillus nidulans* in culture, on development and toxin synthesis (15); these filamentous fungi produce aflatoxin and sterigmatocystin, respectively. Sterigmatocystin is a carcinogenic late pathway precursor of aflatoxin and is generated by a biosynthetic pathway analogous to that used in aflatoxin biosynthesis. Ethylene and CO_2, alone or in combination, influenced fungal growth and development and inhibited aflatoxin biosynthesis in laboratory culture and on peanuts. The detailed mechanisms remain to be elucidated; however, we showed that ethylene blocks aflatoxin gene expression, at least in part, at the transcriptional level (15).

In the current study, we sought to identify additional fungal metabolites that control aflatoxin synthesis; we screened *A. nidulans* and *A. parasiticus* for volatiles that negatively impact aflatoxin synthesis. Colonies of *A. parasiticus* were incubated alone or in the same growth chamber as colonies of *A. nidulans*. The presence of *A. nidulans* reduced the accumulation of norsolorinic acid by *A. parasiticus* B62, a *nor-1* disruption mutant. *nor-1* encodes norsolorinic acid reductase that catalyzes the conversion of the first stable aflatoxin pathway intermediate, norsolorinic acid, to averantin. This observation suggested that *A. nidulans* could communicate with *A. parasiticus* by means of gases or volatile compounds. We then identified several volatile compounds produced by both *A. parasiticus* and *A. nidulans* in laboratory culture and demonstrated that one of these, 2-buten-1-ol (or crotyl alcohol [CA]), exerts dose-dependent up-regulatory and down-regulatory effects on aflatoxin accumulation, conidiation, *nor-1* transcriptional activity, and aflatoxin transcript accumulation in *A. parasiticus*. The data also suggested that the up-regulatory and down-regula-

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tory effects on *nor-1* transcriptional activity are mediated through separate regulatory regions in the *nor-1* promoter.

MATERIALS AND METHODS

Strains, media, and growth conditions. Isogenic *A. parasiticus* strains used in this study were derived from the parent strain SU-1 (ATCC 56775), a wild-type aflatoxin producer. *A. parasiticus* D8D3 contains the GUS (*uidA*) (encodes β-D-glucuronidase) reporter fused to the *nor-1* promoter and has been used previously as a *nor-1* reporter strain (7). Strain B62 (ATCC 24690) (*niaD nor-1 br-1*) accumulates the first stable aflatoxin pathway intermediate, norsolorinic acid, due to a mutation in *nor-1* (20); because other activities assist Nor-1 in this conversion step, a small quantity of aflatoxin still accumulates in this mutant strain. Accumulation of this brightly colored red pigment in *A. parasiticus* B62 can be detected visually on the bottom surface of colonies grown on glucose minimal salts (GMS) solid medium (see below) as a red line along the colony margin. *A. nidulans* FGSC4 (wild-type sterigmatocystin producer) was obtained from the Fungal Genetics Stock Center (Kansas City, KS).

Chemically defined GMS medium supplemented with 5 μM Zn^{2+} was used as the base growth medium for *A. parasiticus* (3). Glucose minimal agar medium (GMM) was used for the growth of *A. nidulans* (5). Yeast extract sucrose agar medium (6) was used as a growth medium for *Stachybotrys chartarum*, *Mucor racemosus*, and *Penicillium expansum*. Media were allowed to solidify in the lids of sterile 60- by 15-mm petri dishes.

Conidiospores (3×10^3 spores/plate) of *A. parasiticus* D8D3 and B62 and *A. nidulans* FGSC4 were center inoculated onto appropriate agar media. The small petri dish lids carrying inoculated media were placed inside a larger, 150- by 15-mm petri dish that was then covered. This system allowed free gas or volatile exchange between colonies inside the large dish while preventing direct colony contact. A 150- by 15-mm control dish contained three lids carrying *A. parasiticus* D8D3 or *A. parasiticus* B62 only. Treatments contained one lid carrying *A. parasiticus* D8D3 or B62 placed together with two lids inoculated with *A. nidulans* (2× treatment) or two lids carrying *A. parasiticus* D8D3 or B62 placed together with one lid inoculated with *A. nidulans* (1× treatment). Cultures were incubated at 30°C and in 99.8% relative humidity in the dark for the appropriate time periods (see Results).

To evaluate the effects of 2-ethyl-1-hexanol (EH) and CA on growth, asexual sporulation (conidiation), and aflatoxin synthesis, a designated quantity of the test compound was dispensed into the bottom of a sterile 0.5-ml microcentrifuge tube with a small round opening in its lid made by a sterile 18-gauge needle. This tube was placed in the center of the large petri dish between the small petri dish lids carrying the fungal colonies. Control plates contained an empty tube. The cultures were grown at 30°C in the dark for appropriate periods of time.

Measurements of CO_2, O_2, and ethylene concentration. Headspace gases were sampled through a port equipped with a Teflon-lined rubber septum installed in the lid of the 150- by 15-mm petri dish and analyzed for carbon dioxide, oxygen, and ethylene levels by means of gas chromatography (GC) as described previously (15).

Volatile analysis. Volatiles produced by *A. parasiticus* and *A. nidulans* were sampled and analyzed as described previously by Song et al. (17). Briefly, headspace gases in large petri plates containing three colonies of either *A. nidulans* or *A. parasiticus* were sampled by means of a solid-phase microextraction (SPME) device (Supelco, Bellefonte, PA) coated with polydimethylsiloxane-divinylbenzene fibers. The SPME was inserted into the large petri dish, the cover was replaced, and the volatiles were absorbed for 4 min. The SPME was then removed from the petri dish and desorbed in the injection port of a gas chromatograph (model 3400; Varian). Detection of volatiles was performed by time-of-flight mass spectrometry using an instrument equipped with an electronic ionization source (FCD-650; LECO Corp., St. Joseph, MI). The volatiles were identified by comparison of mass spectra to a mass spectrum library at the National Institute for Standard Technology (version 1.0). Measurements were performed on two to three replicates. Appropriate controls for medium, plastic ware, and accidental contaminants were conducted.

Assessment of EH and CA concentrations in headspace gases. Analysis of volatiles was performed essentially as described previously by Song et al. for hexanal (17), with the following modifications. A 0.5-ml plastic microcentrifuge tube containing a designated volume of EH or CA was placed between the lids of three small petri dishes (60 by 15 mm) containing GMS agar medium; the lids were positioned in a large petri dish (150 by 15 mm). The large plate was covered, sealed with Parafilm, and incubated at 30°C for 24 h. Samples of headspace gases were withdrawn by a gas-tight Hamilton syringe through a small, premade, sealed opening in the lid and injected into the gas chromatograph (Carle GC series). Evaporation of 0.1 and 1 μl of EH into a specially made 4.4-liter jar fitted with a Mininert gas-tight sampling valve generated EH standards. The concentration of CA was estimated using a 2.68 nmol/liter CA standard generated by placing 1 μl of the compound into a similar 4.4-liter jar.

Detection of aflatoxins B_1, B_2, G_1, and G_2 and norsolorinic acid. Aflatoxins were extracted from the agar medium and mycelium three times with 5 ml chloroform each. The extracts were combined, dried under a stream of N_2, and redissolved in 70% methanol. Aflatoxins were detected by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography (TLC) as described previously by Roze et al. (14). ELISA provided a measure of primarily aflatoxin B_1 levels, whereas TLC enabled one to measure aflatoxin B_1, B_2, G_1, and G_2 levels. Norsolorinic acid was extracted from the agar and mycelium twice with chloroform and once with acetone and then analyzed by TLC (4). The quantity of norsolorinic acid in the agar was also estimated directly by scanning the bottom surface of petri dishes carrying fungal colonies with a Minolta CR-300 Chroma Meter (Konica Minolta, Osaka, Japan); intensity readings from the red-green axis ("*a*" value) were recorded.

Evaluation of conidiation. *A. parasiticus* conidia were harvested and their number per colony was estimated using a hematocytometer as described previously by Roze et al. (14).

Assessment of GUS reporter activity in *A. parasiticus* D8D3 and in the *nor-1* promoter deletion mutants. A fluorimetric assay, as described previously, was used to determine GUS activity (14).

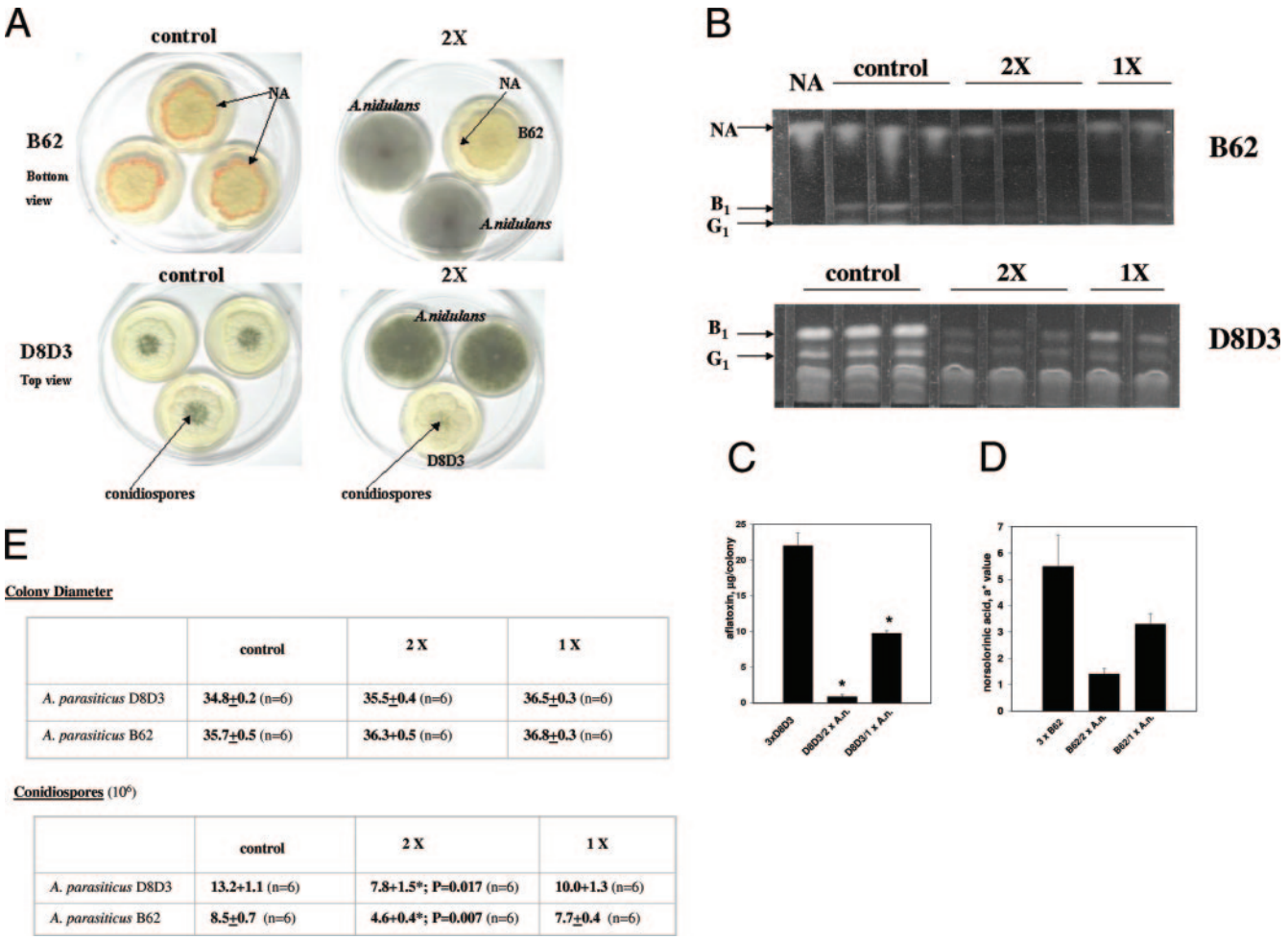
Total RNA isolation and quantification of transcript levels. *A. parasiticus* D8D3 was grown on GMS agar for 3 days at 30°C in the dark. Total RNA was isolated from duplicate colonies by the TRIzol method (TRIzol reagent; Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (QIAGEN, Valencia, CA), and the RNA quality was examined by an Agilent 2100 bioanalyzer (Agilent Technologies). First-strand cDNA was synthesized using 1 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two microliters of cDNA was used as a template in the subsequent PCR using the following thermocycler (Perkin-Elmer GeneAmp PCR system 2400) parameters: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with a final extension step at 72°C for 10 min. Pairs of gene-specific primers for PCR are shown in Fig. 6C. PCR products were separated by electrophoresis on a 1% agarose gel. Band intensities for aflatoxin gene transcripts were compared to the intensity of the transcript of the housekeeping gene (β-tubulin) under the same treatment. Transcript levels as detected by reverse transcription (RT)-PCR for β-tubulin did not change with any treatment, suggesting that the changes in band intensities for aflatoxin gene transcripts reflect real changes in transcript levels in the samples.

Analysis of intracellular cAMP levels in *A. parasiticus*. *A. parasiticus* was grown in the dark for 2 or 3 days on GMS agar medium overlaid with cellophane, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. The frozen mycelium was ground in liquid nitrogen using a mortar with a pestle, resuspended in 0.1 N HCl, and used for quantitative determination of cyclic AMP (cAMP) by a competitive immunoassay according to instructions provided by the manufacturer (Direct cAMP enzyme immunoassay kit; Assay Designs, Inc., Ann Arbor, MI).

Statistical analysis. Statistical analyses were performed using SigmaStat one-way analysis of variance scientific statistical software, version 1.0, from the Jandel Corporation.

RESULTS

Effect of *A. nidulans* on aflatoxin accumulation, conidiation, and colony diameter in *A. parasiticus*. Growth of one colony of *A. parasiticus* B62 in the presence of two colonies of *A. nidulans* (2× treatment) for 7 days caused a visible reduction of norsolorinic acid accumulation compared with a control containing three colonies of *A. parasiticus* B62 (Fig. 1A). We observed a "dose response"; two colonies of *A. nidulans* produced a larger effect on B62 than one colony of *A. nidulans*. TLC (Fig. 1B), ELISA (Fig. 1C), and a Chroma Meter-based assay (Fig. 1D) quantified the magnitude of the effect. ELISA demonstrated that 2× treatment caused up to a 20-fold reduction in aflatoxin B_1 in strain D8D3, while 1× treatment resulted in only a twofold decrease in this toxin. TLC analysis confirmed these data and demonstrated that the effect ex-



E

Colony Diameter

	control	2 X	1 X
A. parasiticus D8D3	**34.8±0.2** (n=6)	**35.5±0.4** (n=6)	**36.5±0.3** (n=6)
A. parasiticus B62	**35.7±0.5** (n=6)	**36.3+0.5** (n=6)	**36.8±0.3** (n=6)

Conidiospores (10^6)

	control	2 X	1 X
A. parasiticus D8D3	**13.2+1.1** (n=6)	**7.8+1.5*; P=0.017** (n=6)	**10.0+1.3** (n=6)
A. parasiticus B62	**8.5±0.7** (n=6)	**4.6+0.4*; P=0.007** (n=6)	**7.7±0.4** (n=6)

FIG. 1. *A. nidulans* affects toxin accumulation and conidiospore production in *A. parasiticus*. (A) Growth on solid media. *A. parasiticus* D8D3 and B62 and *A. nidulans* FGSC4 were center inoculated onto GMS agar medium and GMM, respectively, and incubated at 30°C in the dark for 7 days. The control contained three petri dish lids (60 by 15 mm) with either *A. parasiticus* B62 or D8D3. The 2× treatment contained one petri dish lid with *A. parasiticus* B62 or D8D3 together with two lids carrying *A. nidulans*. (B) TLC analysis. Aflatoxins were extracted from colonies and agar and analyzed by TLC (see Materials and Methods). NA, norsolorinic acid; B_1 and G_1, aflatoxin B_1 and aflatoxin G_1 respectively. (C) ELISA. Aflatoxin accumulation in *A. parasiticus* D8D3 was analyzed by ELISA. Three colonies per treatment were analyzed for each experiment. Representative data (means ± SE) from one of two experiments (with similar trends) are shown. *, statistically significant difference compared with the control (Student's *t* test). (D) Norsolorinic acid accumulation in *A. parasiticus* B62 was analyzed by using a Minolta Chromo Meter. Representative data (means ± SE of three to four measurements per colony) from one of two experiments (with similar trends) are shown. Three colonies per treatment were analyzed for each experiment. *, statistically significant difference compared with the control (Student's *t* test). A.n., *A. nidulans*. (E) Colony diameter and conidiospore production. Three colonies per treatment (numbers in parentheses) were analyzed for each experiment. Representative data (means ± SE) from one of two experiments (with similar trends) are shown. *, statistically significant difference compared with the control (Student's *t* test).

tended to aflatoxins B_2, G_1, and G_2. TLC and the Chroma Meter-based assay provided additional evidence that 2× and 1× treatments resulted in similar effects on norsolorinic acid accumulation in strain B62. The Chroma Meter assay proved to be a reliable and rapid screening tool because it does not require the extraction of the compounds from the growth medium.

The 2× treatment generated up to a twofold reduction in conidiospores by *A. parasiticus* D8D3 and B62, whereas the 1× treatment did not generate a statistically significant effect (Fig. 1E). Colony growth (estimated by colony diameter) was not affected by any treatment (Fig. 1E).

Effect on aflatoxin accumulation may be unique to *Aspergillus* volatiles. *M. racemosus* 1216B, *S. chartarum*, and *P. expansum* 28830 were tested in place of *A. nidulans* in experiments similar to those described above. No changes in norsolorinic acid accumulation in *A. parasiticus* B62 were observed using either 2× or 1× treatments (data not shown) after 7 days. However, 2× treatment with *M. racemosus* resulted in an approximately threefold reduction in conidiospores produced by *A. parasiticus* B62 (not shown); none of these treatments affected colony growth.

Volatiles other than CO_2, O_2, and ethylene are responsible for effects on *A. parasiticus*. We hypothesized that *A. nidulans*

TABLE 1. CO_2 and O_2 concentrations in headspace gases[a]

Condition	% CO_2 ± SE	% O_2 ± SE
Room air	0.05 ± 0.0	24.5 ± 0.35
3 GMM, medium only	0.05 ± 0.0	23.0 ± 0.15
2 GMM/1 GMS, medium only	0.05 ± 0.0	23.3 ± 0.17
3 GMS with *A. parasiticus*	0.23 ± 0.01	22.4 ± 0.17
3 GMM with *A. nidulans*	0.06 ± 0.003	22.5 ± 0.86
2 GMM with *A. nidulans*/1 GMS with *A. parasiticus*	0.14 ± 0.004	22.7 ± 0.32

[a] Three 60- by 15-mm petri dish lids with growth medium only or with growth medium inoculated with *A. parasiticus* or *A. nidulans* were placed into a large 150- by 15-mm plate. The plates were incubated at 30°C. Headspace gas samples were taken through the septum in the lid of the large plate at day 7 of growth and analyzed by GC. Numbers represent means ± standard errors (SE) of two to four measurements. GMS or GMM, one small petri dish lid with solidified GMS or GMM agar medium, respectively.

TABLE 2. Initial concentrations of EH and CA in headspace gases[a]

Treatment (μl/plate)	EH concn (pM)	CA concn (nM)
10	0.75	3.15
25	0.96	ND
50	3.50	ND
100	10.26	14.67

[a] A 0.5-ml plastic tube with a hole in the lid and containing a designated volume of EH or CA was placed into a large petri dish containing a sampling port in the side for gas sampling. The dish was sealed with Parafilm, and gas samples were obtained and analyzed after 6 to 24 h. A 0.5-ml gas sample was removed through the opening using a gas-tight Hamilton syringe. A gas chromatograph (Carle GC series) was used to measure the volatile concentration. A volatile standard was generated by evaporating 0.1 and 1 μl of EH or CA into a 4.4-liter jar fitted with a Mininert gas-tight sampling valve. ND, not done.

produces volatiles that mediate the observed effects on aflatoxin and norsolorinic acid accumulation. CO_2 is a potent regulator of fungal morphogenesis (18). We demonstrated previously that CO_2 and ethylene are synthesized by *A. nidulans* and inhibit aflatoxin accumulation (15). Oxygen also affects aflatoxin production (8, 12). To rule out possible effects of CO_2, O_2, and ethylene on data interpretation in the experiments described above, we measured concentrations of these gases in the headspace of large petri dishes carrying fungal colonies. Gas samples were obtained at day 7 of growth through an F-145 sterile rubber septum plug (Alltech Associates, Inc., Deerfield, IL) installed in the center of the lid of the large petri dish. We observed no significant changes in O_2 levels in petri dishes containing any combination of *A. parasiticus* and/or *A. nidulans* colonies. We were unable to detect ethylene in the headspace, likely because the concentration was too low under these growth conditions. In contrast, three *A. parasiticus* colonies produced the highest CO_2 levels (0.23%). Three *A. nidulans* colonies produced the lowest CO_2 levels (0.06%), and one *A. parasiticus* colony with two *A. nidulans* colonies produced intermediate CO_2 levels (0.14%) (Table 1); these data were not consistent with O_2, ethylene, or CO_2 being primary contributors to the observed effects of *A. nidulans* on aflatoxin and conidiospore production in *A. parasiticus*.

Volatile analysis of headspace gases. Headspace gases obtained from large petri dishes carrying three colonies of *A. parasiticus* or *A. nidulans* were subjected to GC/mass spectrum analyses to identify compounds uniquely produced or produced in different quantities by *A. nidulans* and *A. parasiticus* in culture.

In initial experiments, gas samples were obtained directly from large petri dishes carrying fungal colonies incubated for 6 days. Using this procedure, we identified two volatile alcohols that were produced in significantly larger quantities by *A. nidulans* than by *A. parasiticus* (not shown); these included EH (up to 10-fold-greater quantities) and CA (up to 1,000-fold-greater quantities). Ethanol was produced in significantly higher quantities by *A. parasiticus* than by *A. nidulans* (trace) under these growth conditions (not shown).

In follow-up experiments, large petri dishes were incubated for 6 days and placed into 1-liter Teflon containers. The containers were sealed and incubated at 30°C for an additional 20 h, and headspace gases were obtained. Under these growth conditions, *A. nidulans* produced one additional alcohol (1-penten-3-ol) in higher quantities than *A. parasiticus*; two additional compounds were detected at low levels that we suspect were contaminants derived from the Teflon container (not shown).

Effects of CA and EH on aflatoxin biosynthesis in *A. parasiticus*. We focused attention on CA and EH because of their structural similarity to ethylene and hexanal, biologically active compounds with strong effects on fungi (15, 17, 22). To generate different concentrations of these volatiles in the headspace of a petri dish, microcentrifuge tubes containing different volumes (1 to 100 μl) of these compounds were placed into large petri dishes as described in Materials and Methods. To determine the initial concentration of each volatile in the headspace, large petri dishes (no fungal colonies) were sealed with Parafilm, samples of headspace gases were taken after 6 h, and GC analysis was performed (Table 2). We observed a roughly linear correlation between the initial volume of the compound in the microcentrifuge tube and the concentration of the volatilized compound detected in the headspace of the sealed plates.

A. parasiticus B62 and D8D3 were then grown on GMS agar medium in the presence of different concentrations of CA or EH for 6 days at 30°C. We measured norsolorinic acid, aflatoxins B_1 and G_1, and colony diameter as a function of dose. EH inhibited growth by approximately 10% (not shown) and increased the production of norsolorinic acid, aflatoxin B_1, and aflatoxin G_1 at doses ranging from 10 μl to 100 μl (Fig. 2). Treatment with EH also resulted in the accumulation of norsolorinic acid on the entire bottom surface of the colony com-

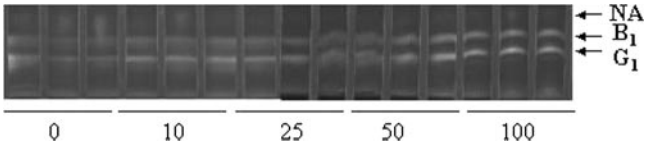


FIG. 2. TLC analysis of toxin accumulation under EH exposure. *A. parasiticus* B62 conidiospores were center inoculated onto GMS agar medium and incubated for 6 days at 30°C in the dark. Fungal colonies were treated with EH (see Materials and Methods); treatments included 0 μl (control), 10 μl, 25 μl, 50 μl, or 100 μl. Aflatoxins were extracted from the colony plus agar and analyzed by TLC (see Materials and Methods). Each lane represents an extract from one colony plus agar.

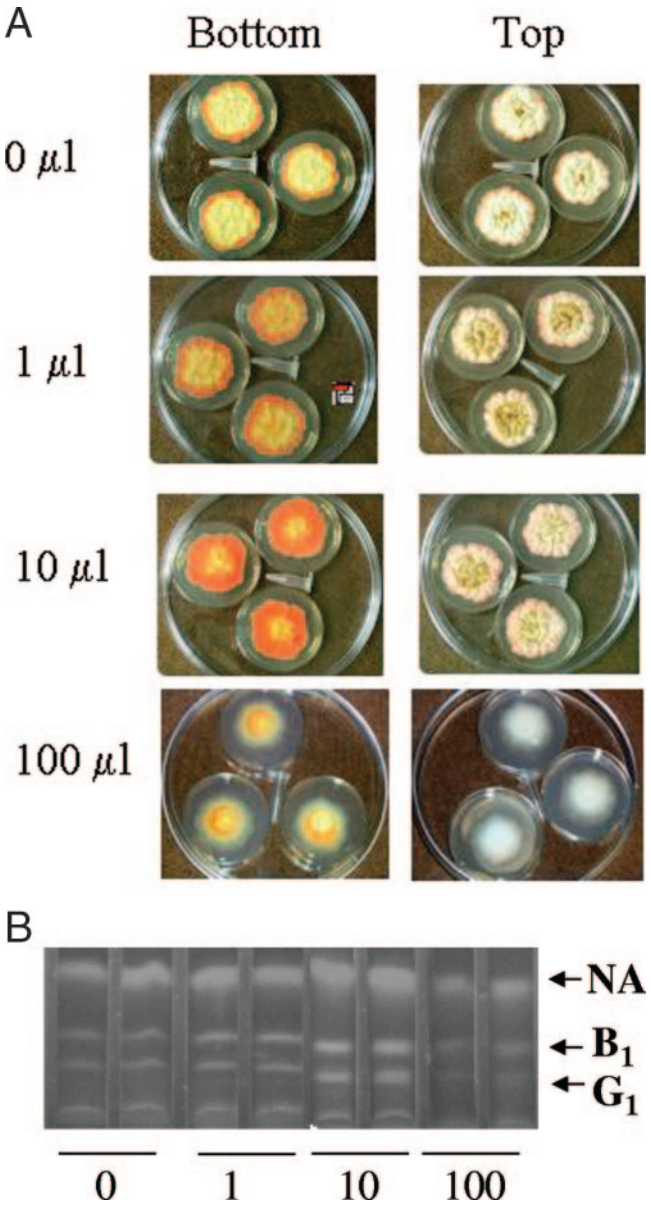


FIG. 3. Dose-response effect of CA on toxin accumulation in *A. parasiticus* B62. *A. parasiticus* B62 conidiospores were center inoculated onto GMS agar and incubated for 6 days at 30°C in the dark (see Materials and Methods). Fungal colonies were treated with CA (see Materials and Methods); treatments included 0 μl (control), 1 μl, 10 μl, or 100 μl. Aflatoxins were extracted from the medium and mycelium and analyzed by TLC (see Materials and Methods). (A) Bottom and top view of colonies. (B) TLC analysis of aflatoxin and norsolorinic acid (NA) accumulation. Each lane represents an extract from one colony plus agar.

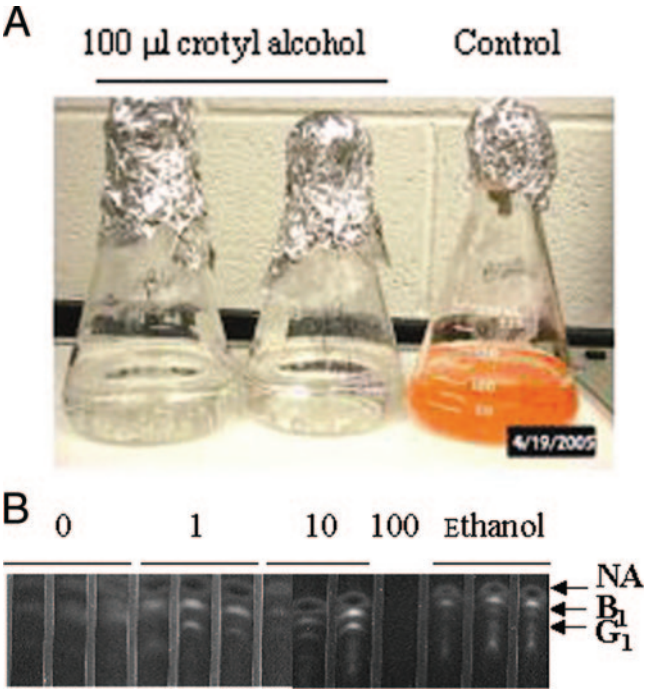


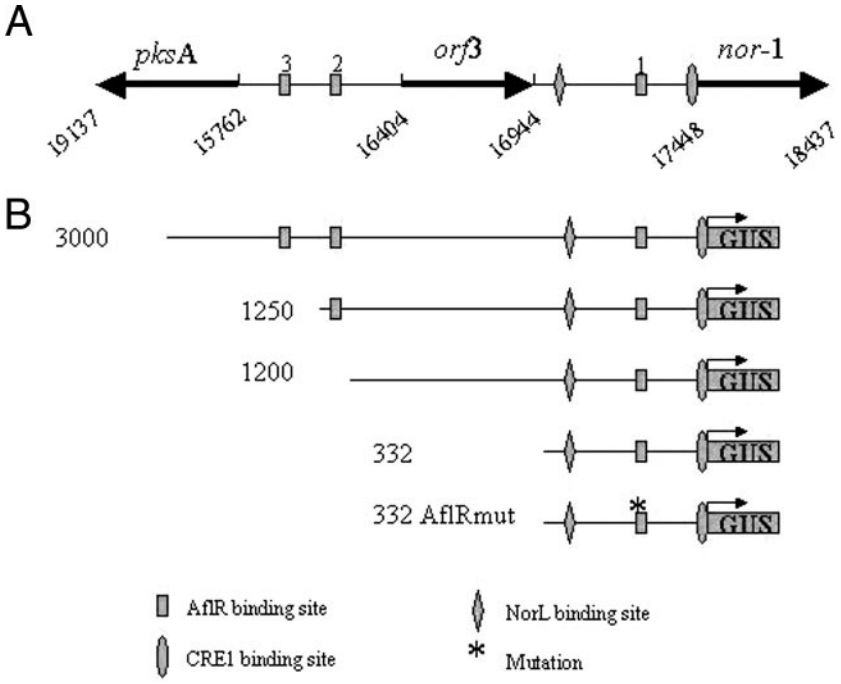
FIG. 4. Effect of CA on toxin accumulation of *A. parasiticus* B62 in liquid medium. *A. parasiticus* B62 conidiospores were inoculated into 250-ml flasks containing 100 ml liquid GMS medium and incubated for 4 days under standard conditions (see Materials and Methods); treatments included 0 μl (control), 1 μl, 10 μl, or 100 μl CA or 1 ml ethanol (dispensed directly into the medium). (A) Visible accumulation of norsolorinic acid in the growth medium in the presence of CA. (B) TLC analysis of toxin accumulation for both treatments. Each lane represents an extract from one flask. NA, norsolorinic acid.

pared to untreated controls in which NA accumulated along the margin of the colony. The effect of CA on visible norsolorinic acid accumulation depended on dose (Fig. 3). One hundred microliters inhibited norsolorinic acid accumulation; in contrast, lower doses (1 μl to 25 μl) strongly increased norsolorinic acid accumulation but did not produce an effect on growth (Fig. 3A). By day 6 of growth on a control plate, we observed norsolorinic acid accumulation along the margin of a colony. Low doses (1 μl and 10 μl) of CA resulted in norsolorinic acid accumulation over almost the entire bottom surface of a colony (Fig. 3A), similar to treatment with EH (not shown). TLC data confirmed data from visual analysis (Fig. 3B): low doses of CA (1 μl and 10 μl) stimulated toxin accumulation, while a high dose greatly reduced toxin accumulation (Fig. 3B).

CA was also added to 100 ml of liquid GMS medium, and B62 was inoculated and incubated under standard shake culture conditions (30°C, shaking at 150 rpm, and in the dark) (Fig. 4A and B). Similar to effects on solid media, low doses (1 μl or 10 μl) strongly stimulated the accumulation of norsolorinic acid and aflatoxins B_1, B_2, G_1, and G_2 (Fig. 4B). The addition of 100 μl of CA in liquid culture completely blocked detectable norsolorinic acid (Fig. 4A) and aflatoxin (Fig. 4B) accumulation; these effects were accompanied by a clearly observable reduction in mycelial growth.

Ethanol is one volatile produced by *A. parasiticus* in significantly higher quantities than by *A. nidulans*. The addition of 1% ethanol to liquid GMS medium produced a strong positive effect on toxin accumulation (Fig. 4B).

CA affects transcriptional activity of the *nor-1* promoter. To determine whether CA exerts an influence on aflatoxin biosynthesis at the level of gene transcription, *A. parasiticus* D8D3, a *nor-1*::GUS reporter strain carrying a 3,000-bp *nor-1* promoter fragment integrated at 3′ end of *nor-1* in the *A. parasiticus* genome, was center

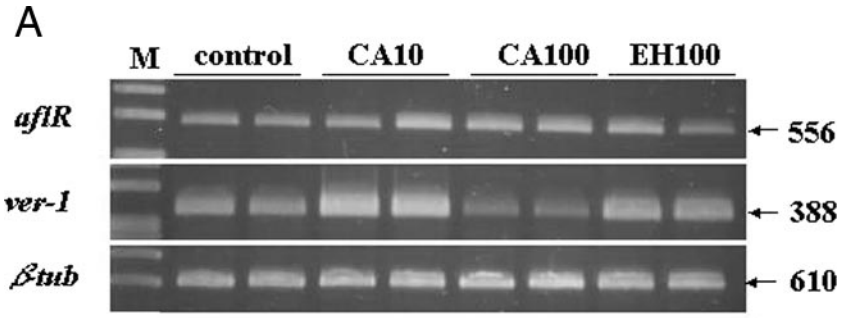


C GUS activity in reporter strains treated with crotyl alcohol (CA)

CA µl / Reporter	0	10	100
D8D3, 3000	447.3±4.5	2197.6±105* P<0.0001	10.2±0.2* P<0.0001
1250: 14A 1250: 77A	5.8±1.6 25.4±0.2	33.0±0.8* P=0.0003 61.9±0.8* P=0.0003	6.0±1.6 4.0±0.1* P=0.0002
1200: 21A 1200: 16B	11.4±1.6 9.7±0.4	20.5±1.7 95.2±1.1* P<0.001	1.2±0.7* P=0.004 1.3±0.4* P<0.001
332: 10A 332: 12A	29.5±3.8 11.9±4.3	18.3±2.4 11.2±1.8	3.7±3.7* P<0.01 5.3±2.8
332AflR*: 50A 332AflR*: 108A	5.1±1.8 7.8±0.2	2.7±0.2 10.3±0.6	6.7±3.0 4.6±3.2

FIG. 5. Separate regulatory elements mediate activation and inhibition of *nor-1* promoter activity by CA. (A) Schematic of the intergenic region between *nor-1* and *pksA* in the *A. parasiticus* aflatoxin gene cluster. Positions of the AflR, CRE1, and NorL transcription factor binding sites are indicated. The numbers below the schematic indicate nucleotide positions in the gene cluster (NCBI accession number AY371490). Three consensus AflR binding sites are labeled 1, 2, and 3. (B) *nor-1* promoter deletion constructs used in the *nor-1*/GUS reporter strains. The numbers on the left indicate lengths of the promoter region with the respect to the *nor-1* translational start site. (C) GUS activity in *A. parasiticus* reporter strains 3000, 1250, 1200, and 332 treated with CA. GUS activity was measured in two single-spore isolates of each reporter strain as described in Materials and Methods. GUS activity (means ± SE) was measured in three replicates and expressed as pmol 4-methylumbelliferone/min/mg total protein. 332AflR*, AflR mutant in context of the 332-bp promoter; 14A, 77A, 21A, 16B, 10A, 12A, 50A, and 108A, single-spore isolates of the GUS reporter strains.

inoculated onto GMS agar and grown in the presence of 0 µl, 10 µl, or 100 µl of CA for 72 h at 30°C. This time point was empirically determined; analysis at 48 h did not generate detectable GUS activity, while at 72 h, we did detect reproducible levels of GUS activity. GUS activity, which provides a useful indicator of *nor-1* promoter activity (13), increased up to fourfold with 10 µl of CA but decreased over 30-fold with 100 µl of this compound (Fig. 5). These are representative data from two independent experiments.



C

Gene	Primers	PCR product bp	Intron
aflR	F 5'TGA GAA CGA TAA GGA CGA C	556	none
	R 5'CAT CCT CAA TCG AAT CAA C		
ver-1	F 5'TTG TAT CGT TCG GTC ACC	388	2
	R 5'GGT TCA AAG GAG AGA GCC		
β-tubulin	F 5'TTC CGT CCC GAC AAC TTC	610	none
	R 5'TCA GGA ACG GAG ACG GC		

FIG. 6. Aflatoxin transcript accumulation in *A. parasiticus* D8D3 under volatile exposure. *A. parasiticus* D8D3 conidiospores were inoculated onto GMS agar medium and incubated for 3 days at 30°C in the dark. Fungal colonies were treated with CA (10 μl or 100 μl) or EH (100 μl); control plates were not treated with volatiles. Total RNA was extracted from frozen mycelium from two independent colonies as described in Materials and Methods. (A) Semiquantitative RT-PCR performed on total RNA (see Materials and Methods). PCR products were separated by electrophoresis on a 1% agarose gel. Transcript accumulation was analyzed for the following aflatoxin genes: *aflR* (556 bp) and *ver-1* (388 bp). Transcript accumulation of β-tubulin (β-*tub*), a constitutively expressed housekeeping gene, was analyzed for comparison. M, HaeIII digestion of ΦX174 RF DNA. (B) PCR products obtained using genomic DNA (g) and cDNA (c) as templates. Expected sizes of PCR products were as follows: for *aflR*, 556 bp (g) and 556 bp (c); for *ver-1*, 501 bp (g) and 388 bp (c); and for β-tubulin, 610 bp (g) and 610 bp (c). (C) Gene-specific primers used in PCRs.

CA and EH affect transcript accumulation of aflatoxin genes. To determine if CA and EH affect the expression of aflatoxin genes in addition to *nor-1*, we analyzed transcript accumulation for *ver-1*, *aflR* (a transcription factor which activates aflatoxin gene expression), and β-tubulin (a housekeeping gene) by semiquantitative RT-PCR (Fig. 6). *A. parasiticus* D8D3 was incubated for 72 h on GMS agar medium and grown in the presence of 10 or 100 μl CA, 100 μl EH, or no volatile (control). Purified RNA from duplicate colonies was analyzed by RT-PCR (see Materials and Methods). Volatile treatment had little detectable effect on *aflR* and β-tubulin transcript accumulation; CA (10 μl) and EH increased *ver-1* transcript accumulation, while CA (100 μl) reduced *ver-1* transcript accumulation.

Differential effects of CA on *nor-1* promoter activity are mediated through different regulatory regions. We previously identified three transcription factors (AflR, NorL, and CRE1bp) required for maximum levels of *nor-1* transcription and characterized their DNA binding sites (13, 16). Each of these *cis*-acting sites was located within a 332-bp *nor-1* promoter fragment situated between the *nor-1* transcriptional start site and the stop codon of the upstream gene (*orf3*) (Fig. 5A). Reporter strains carrying a 3,000-bp genomic region including the 332-bp promoter, *orf3*, and a small portion of the coding region of *pksA* (Fig. 5A) had 31-fold-higher levels of GUS activity than reporter strains carrying the 332-bp *nor-1* promoter fragment. These data suggested that additional regulatory sites lie upstream from the 332-bp promoter fragment and influence *nor-1* transcriptional activity.

We therefore analyzed *nor-1*::GUS reporter strains carrying 3,000-bp, 1,250-bp, 1,200-bp, and 332-bp promoter fragments upstream of the *nor-1* transcriptional start site (Fig. 5B) to identify regions that mediate the CA regulatory response. In addition, we analyzed *nor-1*::GUS reporter strains carrying the 332-bp fragment in which the consensus AflR *cis*-acting site was replaced by a restriction endonuclease site (Fig. 5B) to determine if this site was necessary for the observed regulatory

response. All reporter strains contained the reporter constructs integrated at the 3′ end of *nor-1* in the *A. parasiticus* genome; we demonstrated previously that this integration site generated normal patterns of promoter activity (13).

Two single-spore isolates of each reporter strain were grown on GMS agar medium in the presence of 0 μl, 10 μl, or 100 μl of CA for 72 h, and GUS activity was measured. Reporter strains carrying the 332-bp promoter fragment showed approximately 15-fold-lower GUS activity, and strains carrying the 1,200-bp and 1,250-bp promoter fragments showed approximately 30-fold-lower overall GUS activity than strain D8D3 carrying the 3,000-bp promoter fragment. Nevertheless, strains carrying the 3,000-bp, 1,250-bp, and 1,200-bp promoter fragments showed clear up-regulatory and down-regulatory responses to 10 μl and 100 μl of CA, respectively (only one of two strains of carrying the 1,250-bp promoter fragment showed consistent down-regulatory effects). However, only the up-regulatory (and not the down-regulatory) effect was detected in reporter strains carrying the 332-bp promoter fragment. Mutation in the AflR *cis*-acting site in the background of the 332-bp promoter resulted in low GUS activity with or without CA treatments. Neither up-regulatory nor down-regulatory effects could be observed in the AflR mutant (Fig. 5C).

Steady-state cAMP levels in *A. parasiticus* under treatment with CA. A cAMP/PKA-signaling pathway is involved in the regulation of aflatoxin biosynthesis and conidiation in *A. parasiticus* (15). We previously demonstrated that ethylene blocked the cAMP-mediated stimulation of aflatoxin biosynthesis and decreased the accumulation of aflatoxin in *A. parasiticus* (16), suggesting that ethylene could influence the cAMP/PKA signaling pathway downstream from PKA. To evaluate the possible influence of CA on cAMP/PKA signaling, three independent experiments were performed to estimate cAMP levels in *A. parasiticus* D8D3 mycelium treated with 0 μl, 10 μl, or 100 μl CA for 48 h and 72 h at 30°C. We consistently observed an approximately twofold decrease in steady-state cAMP levels with the 100-μl CA treatment for 72 h. No other treatment provided consistent results.

DISCUSSION

Living organisms have evolved sensitive and efficient mechanisms to utilize gases as signals to communicate and to adapt to the changing environment. Carbon dioxide, oxygen, and ethylene are common gases that serve as regulators of gene expression in a diverse group of organisms including bacteria, fungi, and plants (18). Signaling by gases is conserved in higher vertebrates (21). Directly relevant to our line of research, several cotton leaf volatiles, neem leaf volatiles, and volatile aldehydes were previously shown to produce effects on fungal growth (22) and aflatoxin biosynthesis (11, 22, 24, 25, 27).

Our data clearly demonstrate that the *A. nidulans* volatiles EH, CA, and ethanol have strong regulatory influences on asexual development and aflatoxin synthesis in *A. parasiticus.* Because these volatiles are also synthesized by *A. parasiticus* in culture, albeit at much different levels, we speculate that they represent part of the normal control circuitry, together with CO_2 and ethylene, that the mold utilizes to control the timing and level of aflatoxin synthesis in the soil and on plant material. The fact that *Mucor*, *Stachybotrys*, and *Penicillium* do not mimic this biological effect suggests that the regulatory effect is specific to aspergilli.

In the current study, we observed that CA and EH produce differential effects on *A. parasiticus* in culture when added in pure form. In nature, the regulatory effects of these volatiles might require modification to the active derivative by cell metabolism, may result from combined actions of several biologically active molecules, and may depend on dose. For example, the biological effects of EH may be based on its structural similarity to hexanal; this compound inhibits growth (17) and aflatoxin production in aspergilli (22). Alternatively, hexanal could be metabolized to hexanol (17) and, possibly, to EH by these filamentous fungi. The down-regulatory effects of high doses of CA (in contrast to low doses) on aflatoxin synthesis may be due to an adaptive response to nonphysiologically high levels of CA. In support of this idea, we demonstrated previously that *A. parasiticus* adapts to nonphysiologically high exogenous cAMP levels by down-regulating PKA activity (14). Follow-up studies are required to clarify these possibilities.

To begin to understand the mechanistic basis for the regulatory effects of CA and EH, we analyzed *aflR*, *ver-1*, and β-tubulin transcript accumulation under volatile treatment. Effects of volatile treatment on transcript accumulation of the aflatoxin structural gene paralleled effects on aflatoxin accumulation; β-tubulin transcript accumulation was unaffected. These data suggest that EH and CA affect aflatoxin synthesis in a relatively specific manner. Negligible effects on *aflR* transcript levels were observed, strongly suggesting that CA and EH regulatory effects were mediated via changes in AflR activity or by an independent mechanism.

The specific effects of CA on *nor-1* promoter activity were analyzed using a set of promoter deletions in a GUS reporter construct. The data suggested that the region from positions −1200 to −332 mediated *nor-1* activation, whereas the region from positions −332 to −1 mediated *nor-1* inhibition. As a confounding factor, we recognize that the highest dose of CA used (100 μl) in these studies may influence *nor-1* promoter activity, at least in part, due to growth inhibition.

We compared the nucleotide sequence of the 868-bp activation region with that of the 332-bp inhibition region using ClustalW (version 1.83); the analysis revealed 42% identity between these regions. The analysis also identified a GC box, CCGCCC, in both regions. It is of interest that the GC box within the 332-bp region is adjacent to the 5′ end of the AflR consensus DNA binding site. These two elements together with the TATA box and cAMP response element (CRE1) are located within 110 bp of the transcriptional start site of *nor-1* (16). Others previously demonstrated that a group of transcription factors (including Sp factors) selectively bind GC-rich consensus sequences (including the GC box) in many promoters and are capable of either activating or repressing transcription (2, 19). In addition, Sp1 can form complexes with the cAMP response element binding protein (23). Based on these observations, we hypothesize that CA affects *nor-1* transcription via these specific GC-rich response elements (GC boxes) located in the activation and inhibition regulatory regions of the *nor-1* promoter.

We previously presented a model in which parallel signal transduction pathways control aflatoxin gene transcription and biosynthesis in response to a preferred carbon source (glucose

or sucrose) (16). Recent work in our laboratory (L. V. Roze, A. E. Arthur, S. Y. Hong, A. Chanda, and J. E. Linz, unpublished data) allowed us to provide new insights into the sequence of events that govern transcriptional activation of the entire gene aflatoxin cluster. In the expanded model, activation of CRE1bp results in the recruitment of histone acetyltransferase, generating a bidirectional wave of histone H4 acetylation and, subsequently, in gene activation within the aflatoxin cluster in the order and at the specific times required by the biochemical pathway. The proposed role of the GC box in the regulation of the *nor-1* transcription introduces an additional player to the set of *cis*-regulatory elements (CRE1 and AflR), which together may mediate the initiation of histone H4 acetylation followed by the activation of gene transcription. However, due to inconclusive cAMP data, the role of the cAMP/PKA pathway in mediating CA activity is unclear; future experiments are required to identify the signal transduction pathway(s) that mediates the CA response and its relationship to the regulatory model presented above.

Studying natural gaseous compounds and the mechanisms of their action on aflatoxin biosynthesis has practical applications; these metabolites offer great promise in the development of safe, practical, and inexpensive compounds that block aflatoxin synthesis in the field or during storage of plant materials without adverse effects on food quality. To this end, we clearly demonstrated that treatment of peanuts with an ethylene-CO_2 gas mixture could provide an effective antiaflatoxin postharvest measure (15). Although the chemical and biological properties of CA (an irritant harmful to the respiratory system, eyes, and skin) restrict its use as an antiaflatoxin agent, further analysis of the effects of CA on aflatoxin gene transcription may have broader implications for understanding the role of signaling pathways and transcriptional mechanisms on aflatoxin gene activation; this in turn may facilitate the identification of novel and effective means to control aflatoxin contamination.

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REFERENCES

1. **Bhatnagar, D., J. W. Cary, K. Ehrlich, J. Yu, T. E. Cleveland, M. S. Price, W. C. Nierman, H. S. Kim, B. Pritchard, C. A. Jacobus, and G. A. Payne.** 2006. Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. Mycopathologia **162:**155–166.
2. **Braun, H., R. Koop, A. Ertmer, S. Nacht, and G. Suske.** 2001. Transcription factor Sp3 is regulated by acetylation. Nucleic Acids Res. **29:**4994–5000.
3. **Buchanan, R. L., and D. F. Lewis.** 1984. Regulation of aflatoxin biosynthesis: effect of glucose on activities of various glycolytic enzymes. Appl. Environ. Microbiol. **48:**306–310.
4. **Butchko, R. A. E., T. H. Adams, and N. P. Keller.** 1999. Aspergillus nidulans mutants defective in *stc* gene cluster regulation. Genetics **153:**715–720.
5. **Calvo, A. M., H. W. Gardner, and N. P. Keller.** 2001. Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. J. Biol. Chem. **276:**25766–25774.
6. **Cary, J. W., J. M. Dyer, K. C. Ehrlich, M. S. Wright, S. H. Liang, and J. E. Linz.** 2002. Molecular and functional characterization of a second copy of the aflatoxin regulatory gene, aflR-2, from *Aspergillus parasiticus*. Biochim. Biophys. Acta **1576:**316–323.
7. **Chiou, C. H., M. Miller, D. L. Wilson, F. Trail, and J. E. Linz.** 2002. Chromosomal location plays a role in regulation of aflatoxin gene expression in *Aspergillus parasiticus*. Appl. Environ. Microbiol. **68:**306–315.
8. **Clevstrom, G., H. Ljunggren, S. Tegelstrom, and K. Tideman.** 1983. Production of aflatoxin by an *Aspergillus flavus* isolate cultured under a limited oxygen supply. Appl. Environ. Microbiol. **46:**400–405.
9. **Council for Agricultural Science and Technology.** 2003. Mycotoxins: risks in plant, animal, and human systems. CAST Taskforce report 139. Council for Agricultural Science and Technology, Ames, IA.
10. **Eaton, D. L., and J. D Groopman (ed.).** 1994. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. Academic Press, San Diego, CA.
11. **Greene-McDowelle, D. M., B. Ingber, M. S. Wright, H. J. Zeringue, Jr., D. Bhatnagar, and T. E. Cleveland.** 1999. The effects of selected cotton-leaf volatiles on growth, development and aflatoxin production of *Aspergillus parasiticus*. Toxicon **37:**883–893.
12. **Jelen, H. H., and J. Grabarkiewicz-Szczesna.** 2005. Volatile compounds of *Aspergillus* strains with different abilities to produce ochratoxin A. J. Agric. Food Chem. **53:**1678–1683.
13. **Miller, M. J., L. V. Roze, F. Trail, and J. E. Linz.** 2005. Role of *cis*-acting sites NorL, a TATA box, and AflR1 in *nor-1* transcriptional activation in *Aspergillus parasiticus*. Appl. Environ. Microbiol. **71:**1539–1545.
14. **Roze, L. V., R. M. Beaudry, N. P. Keller, and J. E. Linz.** 2004. Regulation of aflatoxin synthesis by FadA/cAMP/protein kinase A signaling in *Aspergillus parasiticus* Mycopathologia **158:**219–232.
15. **Roze, L. V., A. M. Calvo, A. Gunterus, R. Beaudry, M. Kall, and J. E. Linz.** 2004. Ethylene modulates development and toxin biosynthesis in *Aspergillus* possibly via an ethylene sensor-mediated signaling pathway. J. Food Prot. **67:**438–447.
16. **Roze, L. V., M. J. Miller, M. Rarick, N. Mahanti, and J. E. Linz.** 2004. A novel cAMP-response element, CRE1, modulates expression of *nor-1* in *Aspergillus parasiticus*. J. Biol. Chem. **279:**27428–27439.
17. **Song, J., R. Leepipattanawit, W. M. Deng, and R. M. Beaudry.** 1996. Hexanal vapor is a natural, metabolizable fungicide: inhibition of fungal activity and enhancement of aroma biosynthesis in apple slices. J. Am. Soc. Hortic. Sci. **121:**937–942.
18. **Stretton, S., and A. E. Goodman.** 1998. Carbon dioxide as a regulator of gene expression in microorganisms. Antonie Leeuwenhoek Int. J. Gen. Mol. Microbiol. **73:**79–85.
19. **Suske, G.** 1999. The Sp-family of transcription factors. Gene **238:**291–300.
20. **Trail, F., P. K. Chang, J. Cary, and J. E. Linz.** 1994. Structural and functional analysis of the *nor-1* gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus*. Appl. Environ. Microbiol. **60:**4078–4085.
21. **Wang, R.** 2002. Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? FASEB J. **16:**1792–1798.
22. **Wright, M. S., D. M. Greene-McDowelle, H. J. Zeringue, D. Bhatnagar, and T. E. Cleveland.** 2000. Effects of volatile aldehydes from *Aspergillus*-resistant varieties of corn on *Aspergillus parasiticus* growth and aflatoxin biosynthesis. Toxicon **38:**1215–1223.
23. **Yao, J., C. Grant, E. Harhaj, M. Nonnemacher, T. Alefantis, J. Martin, P. Jain, and B. Wigdahl.** 2006. Regulation of human T-cell leukemia virus type 1 gene expression by Sp1 and Sp3 interaction with TRE-1 repeat III DNA. Cell Biol. **25:**262–276.
24. **Zeringue, H. J.** 2000. Identification and effects of maize silk volatiles on cultures of *Aspergillus flavus*. J. Agric. Food Chem. **48:**921–925.
25. **Zeringue, H. J., and D. Bhatnagar.** 1994. Effects of neem leaf volatiles on submerged cultures of aflatoxigenic *Aspergillus parasiticus*. Appl. Environ. Microbiol. **60:**3543–3547.
26. **Zeringue, H. J., D. Bhatnagar, and T. E. Cleveland.** 1993. $C_{15}H_{24}$ volatile compounds unique to aflatoxigenic strains of *Aspergillus flavus*. Appl. Environ. Microbiol. **59:**2264–2270.
27. **Zeringue, H. J., and S. P. McCormick.** 1990. Aflatoxin production in cultures of *Aspergillus flavus* incubated in atmospheres containing selected cotton leaf-derived volatiles. Toxicon **28:**445–448.