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## Association of ergot alkaloids with conidiation in *Aspergillus fumigatus*

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**Abstract:** Ergot alkaloids are mycotoxins that affect the nervous and reproductive systems of exposed individuals through interactions with monoamine receptors. They have been studied more widely in ergot fungi and grass endophytes but also are found in *Aspergillus fumigatus*, an opportunistic human pathogen that reproduces and disseminates exclusively through conidia. The ergot alkaloids festuclavine and fumigaclavines A, B and C are present in or on conidia of *A. fumigatus*. Cultures of the fungus that are free of conidia are difficult to obtain, obscuring comparisons of conidia versus vegetative hyphae as sources of the ergot alkaloids. To create conidiation-deficient strains of *A. fumigatus* we manipulated the bristle A gene (*brlA*), which controls vesicle formation or budding growth necessary for conidiation in *Aspergillus* spp. Disruption of *brlA* in *A. fumigatus*, via homologous recombination, resulted in a nonconidiating mutant that produced bristle-like structures instead of conidiophores and conidia. Moreover the disrupted strain failed to produce ergot alkaloids as verified by HPLC analyses. Complementation with a wild-type allele restored conidiation and ergot alkaloid production. These results suggest that ergot alkaloids are not produced within the vegetative mycelium of the fungus and are associated directly with conidiation.

**Key words:** aspergillosis, bristle A gene, clavines, mycotoxins

### INTRODUCTION

Ergot alkaloids are a complex family of indole-derived secondary metabolites produced by several fungi (Flieger et al 1997, Gröger and Floss 1998, Panaccione 2005, Schardl et al 2006). The ecological roles of ergot alkaloids are poorly understood; however

they have activities against mammals, insects, nematodes and bacteria (Schwarz and Eich 1983, Clay and Cheplick 1989, Ball et al 1997, Clay and Schardl 2002, Panaccione 2005, Panaccione et al 2006). Their effects on humans are thought to be mediated by their agonistic and/or antagonistic interactions with monoamine neurotransmitter receptors (Pertz 1996, Gröger and Floss 1998, Pertz and Eich 1999).

*Aspergillus fumigatus* Fres., a common saprophyte and opportunistic pathogen, produces several ergot alkaloids, including festuclavine and fumigaclavines A, B and C (Spilsbury and Wilkinson 1961, Cole et al 1977). We recently reported a high performance liquid chromatography (HPLC) procedure for identification and quantification of these alkaloids and demonstrated them to be present in or on conidia of *A. fumigatus* in quantities that collectively total approximately 1% of the dry mass of the conidium (Panaccione and Coyle 2005). Fumigaclavine C is the end product of the *A. fumigatus* pathway, with festuclavine, fumigaclavine B and fumigaclavine A (in that sequence) acting as the final three intermediates in its biosynthesis (Panaccione 2005, Schardl et al 2006, Unsöld and Li 2006). Differences in abundance and activity of the enzymes responsible for converting one intermediate to the next presumably account for the accumulation of festuclavine and fumigaclavine A to relatively high concentrations whereas fumigaclavine B is typically present in much lower concentrations (Panaccione and Coyle 2005, Panaccione 2005).

*A. fumigatus* in vitro sporulates prolifically, and cultures free of conidia are difficult to obtain. Our unpublished observations of ergot alkaloid yields from cultures of *A. fumigatus* on agar-based media (primarily conidia and limited vegetative hyphae) versus submerged, broth-based media (consisting mainly of hyphae and fewer conidia) suggested an apparent association between conidiation and ergot alkaloids.

Research on *Aspergillus* spp. has associated secondary metabolism and sporulation (Calvo et al 2002, Bok and Keller 2004). For example sporulation and sterigmatocystin production have been shown to be regulated by a FadA G $\alpha$  protein-dependent signaling pathway in *Aspergillus nidulans* Eidam (Winter) (Hicks et al 1997). More specifically sterigmatocystin production and conidiation both are regulated negatively by a cAMP-dependent protein kinase catalytic subunit (*pkaA*) (Shimizu and Keller 2001).

Conidiation has been studied extensively in *A.*

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*nidulans*. Conidiophore development is contingent on differentiation of the fungus from polarized apical extension to budding growth (Cole 1986). One gene in particular, *brlA*, has been determined to control conidiophore development and encodes a nucleic acid binding protein (BrlA) (Boylan et al 1987, Adams et al 1988, Adams et al 1998). BrlA is an early protein in a signaling cascade that controls the expression of many genes, including some that encode for other regulatory proteins by binding to *brlA* response elements (BRE) within the promoter regions of these genes (Chang and Timberlake 1992). In *A. nidulans* transcription of *brlA* may initiate at different sites resulting in two overlapping transcription units, *brlA $\alpha$*  and *brlA $\beta$*  (Prade and Timberlake 1993). The products of these two forms are interchangeable functionally, translated in the same reading frame and differ only in that the product of *brlA $\beta$*  has an additional 23 amino acids at the amino terminus (Prade and Timberlake 1993). In this paper we report mutational analyses of the *brlA* gene of *A. fumigatus* to produce nonconidiating mutants for the purpose of examining whether ergot alkaloids are produced during vegetative growth or conidiation.

#### MATERIALS AND METHODS

*Fungi and culture conditions.*—*Aspergillus fumigatus* isolate FGSC A1141 (Panaccione and Coyle 2005) and derivatives were cultured routinely on potato-dextrose agar (PDA) prepared from dehydrated potato flakes (Pillsbury, Minneapolis, Minnesota) (20 g/L), D-glucose (20 g/L) and agar (15 g/L). Cultures intended for preparation of protoplasts or DNA were grown overnight in 15 mL of malt-extract medium (50 g malt extract, 50 g lactose, 5 g asparagine and 0.4 g K<sub>2</sub>HPO<sub>4</sub> per L) in a Petri dish (20 mm deep, 100 mm diam; Fisher Scientific, Pittsburgh, Pennsylvania) on an orbital shaker set at 37 C and 150 rpm. Nonconidiating mutants were cultured similarly except pieces of agar-based cultures containing hyphal fragments were used in place of conidia as inoculum.

*Disruption and complementation of brlA.*—The presence of a *brlA* orthologue in the *A. fumigatus* genome (Nierman et al 2005) was determined by tblastn with protein sequence deduced from *A. nidulans brlA* (Adams et al 1988) as a query sequence. A 640-bp fragment internal to the coding sequence of the putative *brlA* orthologue was amplified by PCR primed with oligonucleotides brlAF (5'-GCCACGCT-TACGACAGAGGT-3') and brlAR (5'-CGGTAGATAGGC-GACTGGTG-3'). The 50  $\mu$ L PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% (v/v) Triton X-100, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each deoxyribonucleotide triphosphate, 1  $\mu$ M of each primer and 2.5 units of *Taq* DNA polymerase (Promega, Madison, Wisconsin), which was added once the thermocycler reached 95 C in the initial denaturing period. The reaction began with an initial denaturing step of 2 min 30 s at 95 C, followed by 35 cycles

of 1 min at 94 C, 1 min at 57 C and 1 min at 72 C, with a final extension step of 72 C for 7 min. A 4.6 kb disruption construct, pBRLA1, was generated by ligating the PCR product into the T/A overlap vector pCR2.1 (Invitrogen, Carlsbad, California). The construct was linearized at the unique *Nde*I site located within the *brlA* fragment before transformation of the fungus. Protoplasts were prepared and transformed according to the protocol described by Murray et al (1992) and with modifications noted by Coyle and Panaccione (2005). Protoplasts were cotransformed with the linearized disruption construct, pBRLA1, and a hygromycin resistance plasmid, pMocosX (Orbach 1994), linearized at a unique *Not*I site (Coyle and Panaccione 2005). Nonconidiating transformants were identified by their altered colony morphology and color and were confirmed by light microscopy. Nonconidiating mutants were purified to nuclear homogeneity through repetitive subculturing from hyphal tips (because nuclear purification via single conidium isolation was not possible). Homologous recombination between pBRLA1 and the native *brlA* locus within the transformants was verified by three PCR assays similar to the PCR reaction described above but with different primers and annealing temperatures. The 5' border of the recombination event was confirmed by PCR (annealing temperature at 57 C) primed with oligonucleotides UF (5'-TGTAACGACGGCCAGT-GAAT-3'), which anneals to vector sequences near the universal primer annealing site in pCR2.1) and brlAFscrn (5'-CTCCAACGAATGTCCGTCTATG-3', complementary to *brlA* sequences near the 5'-end of the *brlA* gene and flanking the intended site of integration) (FIG. 1a). The juxtaposition of sequences near the 3' border of the integration event was verified by PCR (annealing temp 55 C) primed from oligonucleotides UR (5'-AGCTATGAC-CATGATTACGCCA-3', complementary to vector sequences near the reverse primer annealing site of pCR2.1), and brlARscrn (5'-TAGTGACAAGCTCTGCTTGGGA -3', which anneals to *brlA* sequences near the 3'-end of the gene and flanking the intended site of integration) (FIG. 1a). PCR across the entire *brlA* locus was conducted by priming with oligonucleotides brlAFscrn and brlARscrn (both described above) at an annealing temperature of 55 C.

Integration of the transformation construct also was analyzed by Southern hybridization as described by Coyle and Panaccione (2005), except for substitution of a *brlA*-specific probe. The *brlA* probe was made by amplifying wild-type *A. fumigatus* genomic DNA via PCR from primers brlAF and brlAR, according to the PCR conditions described above, substituting 1 $\times$  DIG DNA Labeling Mix (Roche, Mannheim, Germany) for unlabeled dNTP and increasing the annealing temperature to 57 C.

The *brlA* ko strain was complemented with a 2.9 kb fragment containing a full-length copy of the *brlA* coding sequences along with approximately 1 kb of 5'-flanking sequences and 0.6 kb of 3'-flanking sequences. The complementing fragment was amplified by PCR primed from WbrlAF (5'-CTCTCCAGGAACCTTGAACAGGTTCTC-3') and WbrlAR (5'-CCACATAGGACACTCGAGGATG-GAGACA-3') in a 50  $\mu$ L PCR reaction containing 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM Tris-HCl (pH 9.0), 0.1% (v/v) Triton

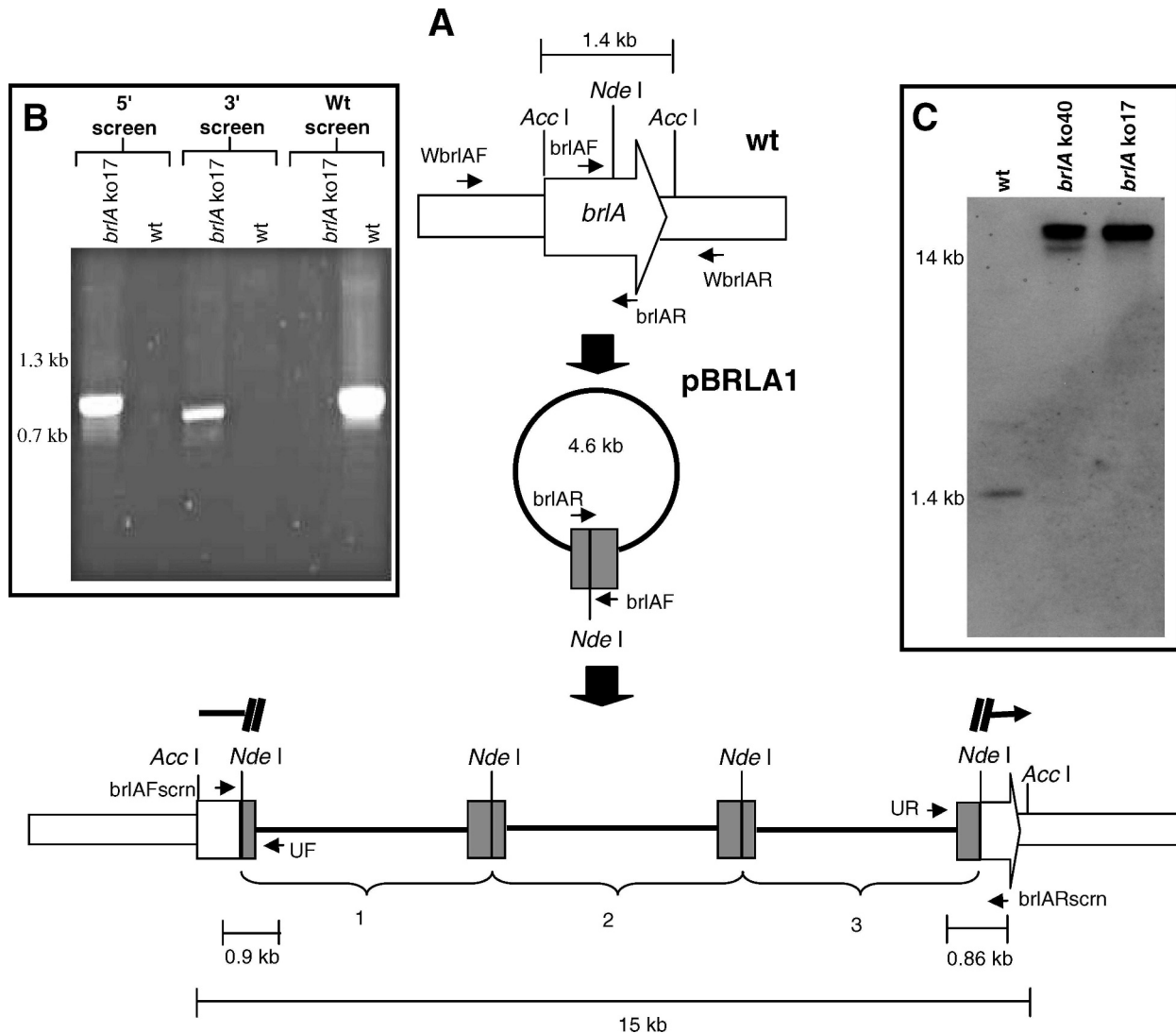


FIG. 1. Homologous recombination at *brlA*. a. Primers *brlAF* and *brlAR* prime amplification of a 640-bp internal fragment from *brlA*. Gene disruption construct *pBRLA1* contains the internal fragment of *brlA* and was linearized at a unique *Nde*I site before transformation. The bottom panel shows integration of three copies of the disruption construct within *brlA* and the positions of primers used in PCR experiments to demonstrate integration at this locus. b. Homologous recombination of the transforming DNA at *brlA* was demonstrated by PCR with primer set *brlAFscrn* and *UF* confirming integration at the 5'-end of the gene and primer set *UR* and *brlARscrn* confirming integration at the 3'-end. Amplification across the 5' and 3' junctions of integration resulted in 899 bp and 863 bp fragments respectively. Primer positions are shown near the bottom of part A. The wild-type (wt) screen was a PCR primed from *brlAFscrn* and *brlARscrn*, and amplification does not occur from DNA that has disruption constructs within the intended site of integration due to the increase in template length. PCR of nontransformed (wt) DNA gives a product of 910 bp. c. Integration of transforming DNA at the targeted site was confirmed by Southern blot hybridization. Fungal DNA extracts were digested with *Acc*I and hybridized with a digoxigenin-dUTP labeled *brlA* probe. The large size of the *brlA*-containing fragments in strains *brlA ko17* and *brlA ko40* indicate three or more copies of *pBRLA1* are present in the integration site. The relative mobilities of relevant fragments of *Bst*EII-digested bacteriophage lambda DNA are indicated on the left in panels B and C.

X-100, 1.5 mM MgCl<sub>2</sub>, 200 μM of each dNTP, 1 μM of each primer and 1 unit of DyNAzyme DNA polymerase (New England Biolabs, Beverly, Massachusetts) added during the initial denaturing step. The thermocycler program was as described above; however the annealing temperature was 62 C and the extension step was 3 min 20 s. The PCR product was cotransformed into a *brlA*-disrupted mutant

along with a phleomycin resistance plasmid, *pBC-phleo* (Fungal Genetics Stock Center, University of Missouri at Kansas City, Kansas City, Missouri). Transformants were selected on complete regeneration medium (Panaccione et al 2001) containing 100 μg/mL phleomycin and incubated at 37 C. Complemented transformants were cultured from individual germinated conidia to achieve a homokaryotic

culture. To test for the presence of the original disruption construct PCR reactions were initiated from primers brlAFscrn and UF (for the 5' flank of the integration) and UR and brlARscrn (for the 3' flank). An additional PCR screen was performed with primers WbrlAF and WbrlAR (described above) to test for the presence of the introduced wild-type allele in the complemented transformants. PCR conditions are provided above with descriptions of the primers.

*Analysis of ergot alkaloids and conidiation.*—For routine analysis of ergot alkaloids small cubes of cultures grown on PDA (containing approximately 8 mm × 8 mm of colony surface area) were submerged in 0.5 mL of 80% methanol in a 1.9 mL microcentrifuge tube and pulverized with a plastic pestle. The samples were rotated end over end (44 rpm) 2 h and spin filtered through a 0.2 μM pore nylon filter. Ergot alkaloids were detected and quantified from 20 μL of the prepared sample by reverse-phase HPLC on a C18 column with fluorescence detection based on excitation and emission wavelengths of 272 nm and 372 nm respectively (Panaccione and Coyle 2005). The identities of peaks corresponding to festuclavine and fumigaclavines A, B and C were established previously by mass spectral analyses of native and de-esterified fractions (Panaccione and Coyle 2005) and confirmed by elimination of these peaks from extracts of strains in which the first gene in the ergot alkaloid pathway (*dmaW*) was inactivated by gene disruption (Coyle and Panaccione 2005).

To quantify ergot alkaloids and conidiation from the same samples cultures were grown on PDA 5 d at 37 C and an additional 9 d at room temperature. Samples containing 50.3 mm<sup>2</sup> of colony surface area were extracted from cultures with the broader end of a disposable 1 mL pipette tip (Fisher Scientific, catalog No. 02-681-172). Samples were submerged in 1 mL 80% methanol, agitated by vortexing and rotated at 44 rpm 2 h. Separate aliquots were removed for HPLC analyses and for quantification of conidia. Concentrations of ergot alkaloids were based on peak area relative to an external standard of agroclavine (Sigma, St Louis, Missouri) as described by Panaccione and Coyle (2005). Conidia were enumerated in dilutions of the methanol extracts with a hemacytometer and light microscope. Six samples were analyzed for each treatment. Data from wild type and a complemented *brlA* disruptant were compared by Student's *t*-test.

## RESULTS

*Disruption and complementation of brlA.*—The *A. fumigatus* genome contains an orthologue of *brlA* (GenBank accession No. XM\_747933). The sequence located on chromosome 1 between nucleotides 2258000 and 2259280 was predicted to encode a protein that is 68% identical to BrlA $\alpha$  from *A. nidulans* (Mah and Yu 2006). Based on sequence analysis there is no evidence for capacity to encode the alternate BrlA $\beta$  product that has been observed in *A. nidulans* (Prade and Timberlake 1993). BrlA $\beta$  of

*A. nidulans* is transcribed from the same locus as BrlA $\alpha$  and is identical to BrlA $\alpha$  except for containing an additional 23 amino acids at the amino terminus. In *A. fumigatus* only two of 19 amino acid residues preceding the initiator methionine of BrlA $\alpha$  are identical to those in the analogous positions of BrlA $\beta$  of *A. nidulans*. A fragment of *A. fumigatus* DNA corresponding to nucleotides 191–830 of the BrlA $\alpha$  coding sequence of *A. nidulans* was used to direct homologous recombination of the gene disruption construct pBRLA1 at the *brlA* locus of *A. fumigatus* (FIG. 1a). Homologous recombination of pBRLA1 at *brlA* would interrupt coding sequences common to both *brlA $\alpha$*  and *brlA $\beta$*  (if it were to occur) in *A. fumigatus*.

Cotransformation of pBRLA1 and *NotI*-linearized pMOCosX into *A. fumigatus* resulted in five nonsporulating colonies out of a total of 93 hygromycin-resistant transformants. Agar-based cultures of the putative *brlA* disruption strains were white and fluffy, unlike the dense, sooty, dark-green appearance of wild-type cultures (FIG. 2a). Observed by light microscopy the nonconidiating cultures had extensive, bristle-like, aerial hyphae that lacked the swollen apical vesicles, phialides and conidia of mature conidiophores (FIG. 2b). Putative disruptants that had been subcultured from hyphal tips six times in an effort to achieve a homokaryotic culture were analyzed by PCR and Southern blot hybridization. Amplification of transformant DNA annealed to primer sets UF and brlAFscrn resulted in an 899-bp product (FIG. 1). Similarly PCR from UR and brlARscrn produced an 863-bp product. Products of these sizes from transformants but not from the wild type indicated integration of the disruption construct into the native copy of *brlA* (FIG. 1). The lack of a PCR product from these transformants when amplification was primed from brlAFscrn and brlARscrn indicated the absence of a wild-type copy of *brlA*. Integration of one or more copies of pBRLA1 at *brlA* results in a large distance between priming sites, whereas in the wild type the primer annealing sites are separated by only 910 bp (FIG. 1). Moreover the absence of a product in the *brlA* disrupted strains (FIG. 1b) indicated nuclear homogeneity of these strains because any wild-type nuclei in the colony would provide template for amplification of the 910-bp product. Southern blot analyses confirmed the presence of multiple disruption constructs within *brlA*, which is evident by a large shift in the size of the *AccI* fragment containing the targeted gene region (FIG. 1c). The lack of multiple bands hybridizing to transformant *brlA* ko17 on the Southern blot verified that random integration of constructs elsewhere in the genome had not occurred (FIG. 1c).

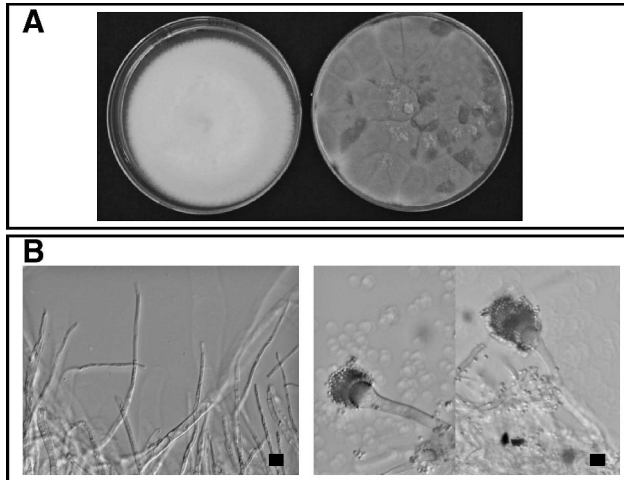


FIG. 2. Morphology of *brlA* mutants. a. Cultures of *A. fumigatus* wild type, right, and *brlA* ko, left, on agar-based media. b. Light microscopy of the same cultures allows for comparisons of wild-type conidiophore development and spore production, right, versus bristle-like structures in *brlA* ko, left. Bar = 10  $\mu$ m.

Disrupted strain *brlA* ko17 was cotransformed with a 2.9 kb PCR product containing the wild-type *brlA* allele and *NotI*-linearized pBCphleo. Six conidiating, phleomycin-resistant transformants were analyzed by PCR. The presence of a wild-type copy of *brlA* in the conidiating transformants was demonstrated by the presence of a 2.9 kb product in reactions primed with WbrlAF and WbrlAR (FIG. 3). Additional PCR analyses of the *brlA* locus in the complemented strains indicated that pBRLA1 was still present at the previously disrupted *brlA* locus because products of the predicted size were amplified from primer pairs flanking the site of integration of pBRLA1 (FIG. 3). Thus the complementing wild-type *brlA* fragment had integrated at a locus other than the native *brlA* and the recipient strain was derived from a *brlA* disruptant. Similar quantities of conidia were produced by a *brlA*-complemented disruptant and the wild type (TABLE I).

*Analysis of ergot alkaloid production in brlA-disrupted mutants and complemented derivatives.*—Mycelia of *brlA*-disrupted mutants contained no detectable ergot alkaloids (FIG. 4, TABLE I). In contrast conidiating colonies of wild-type and the *brlA*-complemented strain accumulated festuclavine and fumigaclavines A, B and C in quantities that were similar by strain (FIG. 4, TABLE I). The relatively large peak eluting at 53 min in the *brlA* ko17 profile represents a metabolite associated with hyphae. It is present in low levels in wild-type and complemented extracts (FIG. 4), which come from colonies that contain abundant conidiophores and conidia but limited hyphae. Its

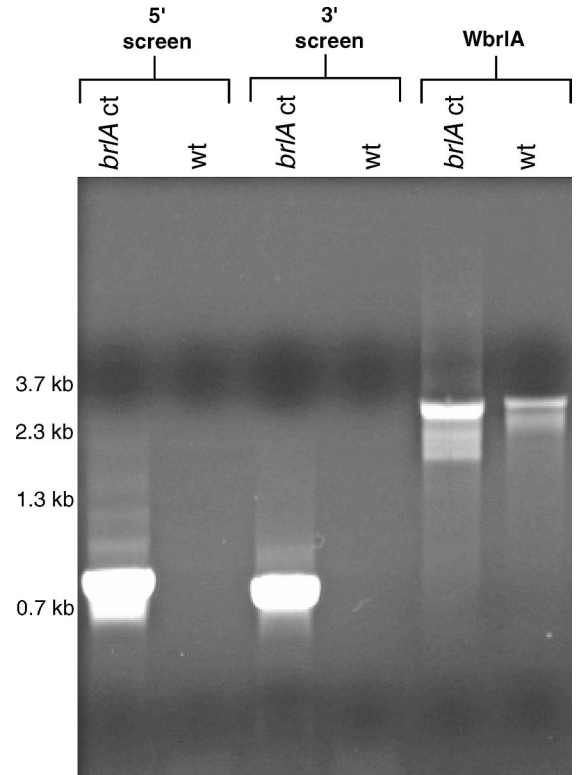


FIG. 3. PCR analyses of complemented transformant. The presence of an introduced wild-type allele of *brlA* in the complemented strain (*brlA* ct) was shown by PCR with primer set WbrlAF and WbrlAR. Retention of the disruption construct previously integrated at the original *brlA* locus was detected by PCR with primers flanking the 5'-end of the integration site (*brlAF*scrn and UF) and primers flanking the 3'-end of the integration site (*brlAR*scrn and UR). (Primer positions are shown in FIG. 1.) The relative mobilities of relevant fragments of *BstEII*-digested bacteriophage lambda DNA are indicated to the left of the photograph.

presence in ergot alkaloid-deficient strains derived by knocking out the gene *dmaW* (Coyle and Panaccione 2005) indicates that it is unrelated to ergot alkaloids.

*Examination of the promoter region of ergot alkaloid cluster genes for bristle A response element (BRE) sequences.*—Because ergot alkaloids were produced only in cultures expressing a functional *brlA* the chromosomal region containing 11 genes from *easA* to *easF* of the ergot alkaloid gene cluster (Coyle and Panaccione 2005, Schardl et al 2006) was examined for the BRE sequence, 5'-(C/A)(A/G)AGGG(G/A)-3' (Chang and Timberlake 1992), and its 26 occurrences were mapped (FIG. 5). Given the degenerate nature of the BRE sequence and its ability to function in both orientations it will occur by chance once in every 1024 bp of random sequence. BRE occurred once every 1404 bp in the coding sequences, whereas in

TABLE I. Mean conidiation and ergot alkaloid production by strain ( $n = 6$ ) for the wild-type (wt), the complemented *brlA* knockout (*brlA* ct), and the *brlA* knockout (*brlA* ko17) with associated confidence intervals ( $\alpha = 0.05$ )

Strain	Conidia $\times 10^6/\text{mm}^2$	Ergot alkaloid concentration (fg per conidium)			
		Festuclavine	Fumigaclavine A	Fumigaclavine B	Fumigaclavine C
wt	1.3 ( $\pm 0.6$ ) <sup>a</sup>	10.2 ( $\pm 2.7$ )	1.1 ( $\pm 0.3$ )	0.2 ( $\pm 0.0$ )	6.7 ( $\pm 1.6$ )
<i>brlA</i> ct	0.9 ( $\pm 0.4$ )	15.2 ( $\pm 6.3$ )	1.1 ( $\pm 0.5$ )	0.3 ( $\pm 0.2$ )	8.8 ( $\pm 4.4$ )
<i>brlA</i> ko17	ND <sup>b</sup>	ND	ND	ND	ND

<sup>a</sup> Comparison of wt and *brlA* ct data within each column of table by *t*-test indicates no significant difference at  $P < 0.05$ .

<sup>b</sup> Not detected.

the 5' noncoding regions these elements occurred once every 409 bp, making BRE about 3.4 times more common in the 5' noncoding regions. As assessed in a paired *t*-test BRE occurred at a higher frequency per bp in 5' noncoding sequences than in the corresponding coding sequences ( $P = 0.03$ ). However

there were no occurrences of the BRE sequences upstream of three of 11 genes including *dmaW*, the gene controlling the first step in the ergot alkaloid pathway (FIG. 5). Six other genes had less than three BRE within their upstream noncoding sequences. One 5' noncoding region contained four BRE sequences, and due to divergent orientation of genes this noncoding sequence was part of the promoter region of two ergot alkaloid cluster genes.

#### DISCUSSION

Disruption of *brlA* in *A. fumigatus* resulted in a nonconidiating mutant lacking any recognizable conidiophores. A similar phenotype has been reported by Mah and Yu (2006) for *brlA* mutants of *A. fumigatus* constructed by a different knockout strategy. The absence of ergot alkaloids in our nonconidiating mutants indicates that ergot alkaloids are not produced during vegetative growth and are solely associated with conidiation in *A. fumigatus*.

The phenotype of the *A. fumigatus brlA* mutants indicates that *brlA* functions similarly in *A. fumigatus* as it does in *A. nidulans*. *brlA* first was discovered as the Mendelian locus defective in mutants that failed to conidiate but instead grew bristles reaching 20–30 times the normal conidiophore length (Clutterbuck 1969). The wild-type allele was cloned by complementation of the *brlA* mutant via transformation and recovery of the transforming DNA (Timberlake et al 1985). Adams et al (1988) demonstrated that expression of *brlA* under conditions that typically repress conidiation in *A. nidulans* (submerged broth culture) resulted in production of structures that produced conidium-like cells. Two developmentally regulated genes downstream of *brlA*, *abaA* and *wetA* were not expressed in the nonfunctional *brlA* mutant but were transcribed in the modified strains expressing *brlA*. Furthermore disruption of Cys<sub>2</sub>-His<sub>2</sub>-Zn(II) coordination sites resulted in the inability of BrlA to induce the conidiation pathway thus supporting the functionality of BrlA as a transcription factor that requires two zinc fingers to bind DNA (Adams et al 1990).

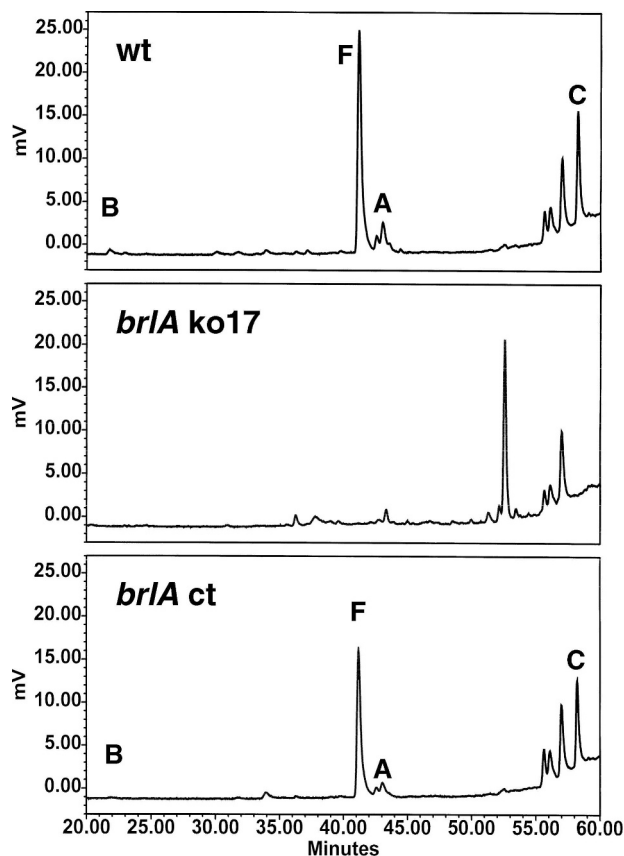


FIG. 4. HPLC traces of wild-type *A. fumigatus* (wt), a *brlA* disrupted strain (*brlA* ko17) and a complemented strain of *brlA* ko17 (*brlA* ct) to which a functional copy of *brlA* has been added. Ergot alkaloids were extracted with 80% methanol and analyzed by reverse phase HPLC with fluorescence detection (excitation and emission wavelengths of 272 nm and 372 nm respectively). B = fumigaclavine B; F = festuclavine; A = fumigaclavine A; C = fumigaclavine C.

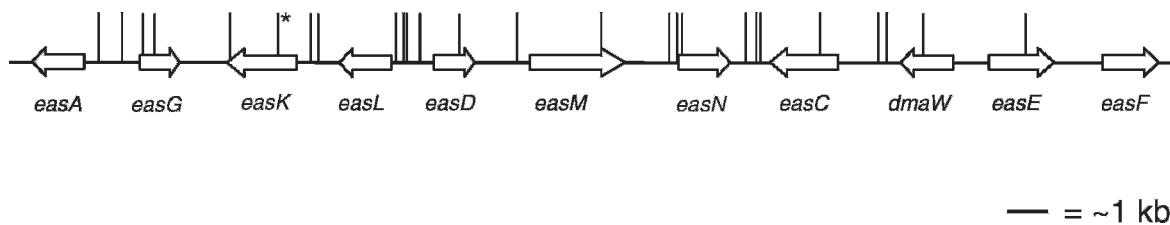


FIG. 5. Occurrence of BRE sequences in the ergot alkaloid gene cluster. This map shows the locations of the BRE sequence within 5' noncoding regions (13), coding regions (9), 3' noncoding regions (3), and within an intron (1, marked with asterisk) of these genes.

Links between sporulation and secondary metabolite production in *Aspergillus* spp. have been demonstrated by mutational analyses of other genes that regulate sporulation (Calvo et al 2002, Bok and Keller 2004). Relevant studies in *A. fumigatus* include work done on the regulatory genes *stuA* (Sheppard et al 2005) and *laeA* (Bok et al 2005). Strains mutated at *stuA* produced conidiophores that were stunted and appeared as vesicles that lacked phialides and arose directly from septate hyphae (Sheppard et al 2005). In microarray analysis *stuA* mutants showed changes in accumulation of transcripts from genes in two separate gene clusters that are hypothesized to control production of unidentified secondary metabolites. Numerous other genes were affected including those encoding genes and proteins involved in development and morphogenesis, such as the cell wall protein, PhiA, and a sexual development repressor protein, Pro1. Mutation of *laeA* impaired conidiophore (and conidium) development in broth cultures of *A. fumigatus* but *laeA* mutants conidiated normally on agar-based media. *LaeA* regulates expression of genes involved in conidiophore and rodlet formation as well as genes involved in production of secondary metabolites such as gliotoxin (Bok et al 2005).

Whether *BrlA* directly regulates the ergot cluster genes or whether those genes are controlled by a factor or factors whose expression depends upon *brlA* has not been established. Overall BRE were more abundant in the 5' noncoding sequences compared to their corresponding coding sequence. However the *brlA* response element was absent from the 5' noncoding sequences of three ergot alkaloid cluster genes (including *dmaW*) and was present in only one or two copies upstream of six other genes. Chang and Timberlake (1992) found that at least three BRE must be present in the upstream region to increase transcript accumulation from that gene. The occasional absence and relatively low copy number of the BRE associated with ergot cluster genes indicates that factors controlled by or in addition to *BrlA* regulate the ergot cluster genes. Previous work demonstrates

that among the genes controlled by *BrlA* are genes encoding other regulatory factors such as *AbaA* and *WetA* (Boylan et al 1987, Adams et al 1988). A global analysis of gene expression in a *brlA* mutant compared to wild type, revealing the genes and secondary metabolite gene clusters controlled by *BrlA*, is already in progress (D.C. Sheppard, McGill University, pers comm).

Association of ergot alkaloids with conidiation might provide insight into the ecological significance and utility of these toxins to the fungus. When *A. fumigatus* grows pathogenically its hyphae colonize host tissue and conidia are not produced (Fraser 1993). Because ergot alkaloids are not produced in hyphae it is unlikely that they contribute to the invasive phase of aspergillosis. Conidia are produced during saprophytic growth and serve primarily for dissemination. Due to their exceptionally small size and low specific gravity (Panaccione and Coyle 2005) conidia can be inhaled deeply into the lungs of humans and animals. To initiate infection conidia must survive the initial host-defense response. The possibility that ergot alkaloids contribute to increasing the survival of inhaled conidia remains to be investigated.

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#### LITERATURE CITED

- Adams TH, Boylan MT, Timberlake WE. 1988. *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. *Cell* 54:353–362.
- , Deising H, ———. 1990. *brlA* requires both zinc fingers to induce development. *Mol Cell Biol* 10:1815–1817.
- , Wieser JK, Yu J-H. 1998. Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* 62:35–54.



- Ball OJ-P, Miles CO, Prestidge RA. 1997. Ergopeptine alkaloids and *Neotyphodium lolii*-mediated resistance in perennial ryegrass against adult *Heteronychus arator* (Coleoptera, Scarabaeidae). *J Econ Entomol* 90:1382–1391.
- Bok JW, Keller NP. 2004. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* 3:527–535.
- , Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Keller NP. 2005. LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryot Cell* 4:1574–1582.
- Boylan MT, Mirabito PM, Willett CE, Zimmerman CR, Timberlake WT. 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. *Mol Cell Biol* 7:3113–3118.
- Calvo AM, Wilson RA, Bok JW, Keller NP. 2002. Relationship between secondary metabolism and fungal development. *Microbiol Mol Biol Rev* 66:447–459.
- Chang YC, Timberlake WE. 1992. Identification of *Aspergillus brlA* response elements selection in yeast. *Genetics* 133:29–38.
- Clay K, Cheplick GP. 1989. Effect of ergot alkaloids from fungal endophyte-infected grasses on fall armyworm (*Spodoptera frugiperda*). *J Chem Ecol* 15:169–182.
- , Schardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am Nat* 160:S99–S127.
- Clutterbuck AJ. 1969. A mutational analysis of conidial development in *Aspergillus nidulans*. *Genetics* 63:317–327.
- Cole GT. 1986. Models of cell differentiation in conidial fungi. *Microbiol Rev* 96:95–132.
- Cole RJ, Kirksey JW, Dorner JW, Wilson DM, Johnson JC Jr, Johnson AN, Bedell DM, Springer JP, Chexal KK, Clardy JC, Cox RH. 1977. Mycotoxins produced by *Aspergillus fumigatus* species isolated from molded silage. *J Agric Food Chem* 25:826–830.
- Coyle CM, Panaccione DG. 2005. An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. *Appl Environ Microbiol* 71:3112–3118.
- Flieger M, Wurst M, Shelby R. 1997. Ergot alkaloids—sources, structures and analytical methods. *Folia Microbiol* 42:3–30.
- Fraser RS. 1993. Pulmonary aspergillosis: pathologic and pathogenetic features. *Pathol An* 28:231–277.
- Gröger D, Floss HG. 1998. Biochemistry of ergot alkaloids—achievements and challenges. *Alkaloids* 50:171–218.
- Hicks JK, Yu J-H, Keller NP, Adams TH. 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G $\alpha$  protein-dependent signaling pathway. *EMBO J* 16:4916–4923.
- Mah J-H, ———. 2006. Upstream and downstream regulation of asexual development in *Aspergillus fumigatus*. *Euk Cell* 5:1585–1595.
- Murray FR, Latch GCM, Scott DB. 1992. Surrogate transformation of perennial ryegrass, *Lolium perenne*, using genetically modified *Acremonium* endophyte. *Mol Gen Genet* 233:1–9.
- Nierman WC, Pain A, Anderson MJ, et al. 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* 438:1151–1156.
- Orbach MJ. 1994. A cosmid with a HyR marker for fungal library construction and screening. *Gene* 150:159–162.
- Panaccione DG. 2005. Origins and significance of ergot alkaloid diversity in fungi. *FEMS Microbiol Lett* 25:9–17.
- , Coyle CM. 2005. Abundant respirable ergot alkaloids from the common airborne fungus *Aspergillus fumigatus*. *Appl Environ Microbiol* 71:3106–3111.
- , Johnson RD, Wang J, Young CA, Damrongkool P, Scott B, Schardl CL. 2001. Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proc Nat Acad Sci USA* 98:12820–12825.
- , Cipoletti JR, Sedlock AB, Blemings KP, Schardl CL, Machado C, Seidel GE. 2006. Effects of ergot alkaloids on food preference and satiety in rabbits, as assessed with gene knockout endophytes in perennial ryegrass (*Lolium perenne*). *J Agric Food Chem* 54:4582–4587.
- Pertz H. 1996. Naturally occurring clavines: antagonism/partial agonism at 5-HT<sub>2A</sub> receptors and antagonism at  $\alpha$ 1-adrenoceptors in blood vessels. *Planta Med* 62:387–392.
- , Eich E. 1999. Ergot alkaloids and their derivatives as ligands for serotonergic, dopaminergic, and adrenergic receptors. In: Kren V, Cvak L, eds. *Ergot: the genus Claviceps*. Amsterdam, The Netherlands: Harwood Academic Publishers. p 411–440.
- Prade RA, Timberlake WE. 1993. The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiphore development. *EMBO J* 12:2439–2447.
- Schardl CL, Panaccione DG, Tudzynski P. 2006. Ergot alkaloids—biology and molecular biology. *Alkaloids* 62:45–86.
- Schwarz G, Eich E. 1983. Influence of ergot alkaloids on growth of *Streptomyces purpurascens* and production of its secondary metabolites. *Planta Med* 47:212–214.
- Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, Nierman WC, Filler SG. 2005. The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. *Mol Biol Cell* 16:5866–5879.
- Shimizu K, Keller NP. 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.
- Spilsbury JF, Wilkinson S. 1961. The isolation of festuclavine and two new clavine alkaloids from *Aspergillus fumigatus* Fres. *J Chem Soc* 5:2085–2091.
- Timberlake WE, Boylan MT, Cooley MB, Mirabito PM, O'Hara EB, Willett CE. 1985. Rapid identification of mutation-complementing restriction fragments from *Aspergillus nidulans* cosmids. *Exp Mycol* 9:351–355.
- Unsöld IA, Li S-M. 2006. Reverse prenylation in the biosynthesis of fumigaclavine C in *Aspergillus fumigatus*: gene expression, purification, and characterization of the fumigaclavine C synthase FGAPT1. *ChemBioChem* 7:158–164.