Aspergillus nidulans Mutants Defective in *stc* Gene Cluster Regulation

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ABSTRACT

The genes involved in the biosynthesis of sterigmatocystin (ST), a toxic secondary metabolite produced by *Aspergillus nidulans* and an aflatoxin (AF) precursor in other *Aspergillus* spp., are clustered on chromosome IV of *A. nidulans*. The sterigmatocystin gene cluster (*stc* gene cluster) is regulated by the pathway-specific transcription factor *aflR*. The function of *aflR* appears to be conserved between ST- and AF-producing aspergilli, as are most of the other genes in the cluster. We describe a novel screen for detecting mutants defective in *stc* gene cluster activity by use of a genetic block early in the ST biosynthetic pathway that results in the accumulation of the first stable intermediate, norsolorinic acid (NOR), an orange-colored compound visible with the unaided eye. We have mutagenized this NOR-accumulating strain and have isolated 176 Nor$^-$ mutants, 83 of which appear to be wild type in growth and development. Sixty of these 83 mutations are linked to the *stc* gene cluster and are likely defects in *aflR* or known *stc* biosynthetic genes. Of the 23 mutations not linked to the *stc* gene cluster, 3 prevent accumulation of NOR due to the loss of *aflR* expression.

THE filamentous fungus *Aspergillus nidulans* produces a carcinogenic polyketide called sterigmatocystin (ST), the penultimate precursor in the aflatoxin (AF) biosynthetic pathway found in the related species *A. parasiticus*, *A. flavus*, and *A. nomius* (Cole and Cox 1981). Both polyketides cause mammalian hepatocarcinomas (Bressac *et al.* 1991) and animal toxicities (Anderson *et al.* 1990), and both are thought to immunologically impair susceptible populations such as infants and the elderly (Cardwell and Miller 1996). *A. parasiticus* and *A. flavus* are common contaminants of a variety of agricultural products such as corn, peanuts, and cottonseed and the health risks associated with ingestion of AF have led to strict national and international guidelines regarding the acceptable levels of AF in food and feed.

The ST biosynthetic genes (*stc* genes) are clustered on chromosome IV of *A. nidulans* (Brown *et al.* 1996b). The cluster contains 24 coregulated genes (*stcA* through *stcX*), most of which encode ST biosynthetic enzymes (Kelkar *et al.* 1997; Keller and Hohn 1997), and one pathway-specific regulatory gene, *aflR*, which encodes a 433-amino acid C6 zinc binuclear cluster DNA-binding protein (Chang *et al.* 1993; Payne *et al.* 1993; Yu *et al.* 1996; Fernandes *et al.* 1998). Several lines of evidence indicate that AflR is a primary regulator of the other *stc* genes. *aflR* mRNA is detectable only after primary growth has ceased and *stc* gene expression follows shortly thereafter (Yu *et al.* 1996). No *stc* gene transcripts can be detected in *aflR* deletion mutants (Yu *et al.* 1996). AflR regulates *stc* gene expression by binding to the palindromic sequence 5′-TCG(N_5)CGA-3′ found in the promoter regions of several *stc* genes (Fernandes *et al.* 1998). As this same motif is found in AF cluster genes, and the *A. flavus* and *A. nidulans aflR* genes are functionally conserved (Yu *et al.* 1996), it is likely that AflR regulation of AF cluster genes will be similar in other *Aspergillus* spp. Given that AflR has been shown to be a key regulator of ST production in Aspergillus species, novel strategies to control ST biosynthesis may be discovered from an understanding of the mechanisms that regulate the expression of *aflR*.

Although we have identified two members of a G protein signal transduction pathway that are required for normal *aflR* expression and activity and hence ST biosynthesis (Hicks *et al.* 1997), most of the genetic requirements for AflR function and *stc* cluster activation are unknown. In this article we present a screening method to rapidly identify pertinent ST mutants by taking advantage of the fact that the first stable intermediate in the ST/AF biosynthetic pathway, norsolorinic acid (NOR), is a visible orange pigment. NOR-accumulating mutants of *A. parasiticus* and *A. flavus* identified through mutagenesis techniques (Lee *et al.* 1971; Papa 1982) have previously been used to visually identify AF-inducing and -inhibiting factors (Keller *et al.* 1994, 1997) and AF deposition in living corn seed (Keller *et al.* 1994). The production of NOR in *A. nidulans* requires at least four characterized *stc* genes; *aflR*, *stcA*, *stcJ*, and *stcK*. *stcA* encodes a polyketide synthase (Yu and Leonard

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TABLE 1

***Aspergillus nidulans* strains used in this study**

Strain name	Genotype	Source
FGSC237	*pabaA1 yA2;trpC801 veA1*	FGSC[a]
FGSC410	*pabaA1 biA1;riboB2 chaA1 veA1*	FGSC
FGSC89	*biA1;argB2;veA1*	FGSC
RJH003	*biA1;wA3;argB2;methG1;veA1*	This study
TJH3.40	*biA1;wA3;methG1 stcE::argB;veA1*	This study
RJH007	*pabaA1 yA2;stcE::argB;veA1*	This study
MRB strains	*biA1;wA3;methG1 stcE::argB;veA1*	This study

[a] Fungal Genetics Stock Center.

1995) and *stcJ* and *stcK* encode the α and β subunits, respectively, of a fatty acid synthase (Brown *et al.* 1996a). The fifth gene in the cluster, *stcE*, encodes a dehydrogenase that converts NOR to the next stable intermediate, averantin. By disrupting *stcE*, we created a NOR-accumulating strain which produces visible amounts of NOR when grown on oatmeal agar. Subsequent mutagenesis of this strain resulted in the visual identification of 176 mutants unable to accumulate NOR (Nor^- mutants). We describe the isolation and the genetic characterization of these mutants.

MATERIALS AND METHODS

Fungal strains and growth conditions: All fungal strains are listed in Table 1. Fungal strains were maintained on appropriately supplemented minimal medium (Käfer 1977) and stored as silica stocks. Standard genetic techniques were used for sexual crosses and diploid formation (Pontecorvo *et al.* 1953). Mutants were isolated on 1.0% oatmeal agar containing 0.25% Tween, 1.0% glucose, 0.5% yeast extract, and 1.6% agar plus appropriate supplements. For subsequent NOR assays, 1.0% oatmeal medium with 1.6% agar and appropriate supplements was used. All strains were incubated at 37°.

Nucleic acid manipulations: *stcE* was disrupted by homologous integration of plasmid pJH3. A 3.98-kb *Eco*RV/*Hin*dIII fragment from pAHK25 (Brown *et al.* 1996b) was first subcloned into pK19 to create pJH1. pJH2 was created by inserting a polylinker containing a *Bam*HI site into the *Pst*I site within the coding region of *stcE* in pJH1. pJH3 was created by inserting a 1.8-kb *Bam*HI fragment from pSalargB (Burse *et al.* 1983) containing *argB* into the *Bam*HI site in pJH2. pJH3 was used to transform *A. nidulans* strain RJH003 to arginine prototrophy. Subsequent transformants were visually screened for the ability to accumulate NOR and genomic DNA from such transformants was analyzed for the replacement of *stcE* with *argB* in the *stcE* locus. One transformant satisfying these requirements, TJH3.40, was used for the mutagenesis experiment. Additionally, TJH3.40 was sexually crossed to FGSC237 to introduce additional auxotrophies into the Δ*stcE* background. One para-aminobenzoic acid auxotrophic progeny, RJH007 (Table 1) was used for diploid analysis. Disruption of *stcE* in RJH007 was confirmed by visualization of NOR and Southern analysis.

For mRNA extraction, wild-type and mutant strains were grown in 5 ml of liquid stationary complete medium (minimal medium with 2.0% dextrose, 0.2% peptone, 0.1% yeast extract, and 0.1% casein hydrolysate) in a 20-ml screw-cap vial. Cultures were set at a 45° angle to increase surface area and then incubated at 37° for 3 days after which fungal mycelium was harvested and frozen in liquid nitrogen. RNA was extracted with Trizol (GIBCO, Grand Island, NY) following the manufacturer's instructions. Total RNA (20 μg) was run on a 1.2% agarose gel containing 4% formaldehyde (v/v) and transferred to Hybond-N+ (Amersham, Arlington Heights, IL) nylon membrane following the manufacturer's instructions. DNA fragments from pAHK25 (a 1.29-kb *Xba*I/*Eco*RV fragment containing the *aflR* gene) and from pAHK27 (a 2.27-kb *Kpn*I fragment containing the *stcF*, *stcG*, and *stcH* genes) were labeled with ^{32}P and hybridized to the nylon membranes at 60° for 16 hr. Membranes were washed for 30 min each in 2× SSC 0.1% SDS, 1× SSC 0.1% SDS, and 0.1× SSC 0.1% SDS at 60° and X-ray film was exposed overnight at −80°.

Mutagenesis: Conidia of TJH3.40 were mutagenized with 4-nitro-1-quinoline oxide (NQO) as previously described by Wieser *et al.* (1994). A total of 100,000 surviving colonies were screened on oatmeal agar (as described in *Fungal strains and growth conditions*) and 176 Nor^- mutants—hereafter referred to as MRB strains—were identified. The survival rate of the mutagenesis was between 1 and 10% (data not shown).

Genetic techniques: Linkage of NQO-generated mutations to the *stc* cluster was determined by crossing each MRB strain to a *stcE* wild-type strain (FGSC89) and analyzing the meiotic progeny for accumulation of NOR. Linkage of the new mutation to the *stc* gene cluster would be indicated by an absence or low percentage of NOR-accumulating progeny due to a low frequency of recombination between the mutated gene and the *stcE* deletion. Mutations were determined to be unlinked to the *stc* gene cluster if 25% of the progeny were able to accumulate NOR. Approximately 200 meiotic progeny were analyzed from each cross.

The number of linkage groups or loci represented in the mutations unlinked to the *stc* cluster was determined by crossing each mutant in every pairwise combination. Each mutant was first crossed to FGSC410 to introduce a new genetic marker, in this case a mutation in the *riboB* gene, into the mutant background. Progeny from crosses between the original mutants and the recombinants were scored for NOR production. Two mutations were considered to be in the same or closely linked genes if none or a low percentage (<6%) of progeny accumulated NOR. If ~25% of the progeny accumulated NOR, the mutations were considered to be in different genes.

Diploids were generated between MRB strains and RJH007 to determine the dominant or recessive nature of each mutation. Diploids were tested on solid oatmeal medium for the accumulation of NOR and haploidization was induced by incorporation of 9 μl benomyl (2 mg/ml) into solid media.

NOR assays: NOR production was assessed by growing all strains at 37° in liquid and solid media. All mutants were visually screened on solid oatmeal medium containing 1.0% oatmeal and appropriate supplements. The mutants not linked to the *stc* gene cluster were further assessed by growing them on solid oatmeal media at 30°, 37°, and 42° and on pH 4 and pH 8 media at 37°. This same subset of mutants was cultured on three different liquid media: (1) complete medium, (2) complete medium plus oatmeal, and (3) oatmeal. Mycelium was harvested after 3 days of stationary culture, frozen, lyophilized, crushed in a 1.7-ml centrifuge tube, and extracted with 750 μl chloroform for 10 min. An equal volume of water was added and the samples were vortexed and spun at 13,000 rpm for 10 min to separate the aqueous and organic phases. The organic phase was removed, dried, and resuspended in 50 μl chloroform. Twenty-five microliters of each

extract was separated on a silica gel TLC plate in toluene:ethyl acetate:acetic acid (80:10:10) and visualized with long-wave UV light.

RESULTS

Isolation of Nor⁻ mutants: We employed a visual screen for the identification of mutants defective in *stc* gene cluster activity. The mutants identified by this screen for NOR accumulation fell into two broad classes; 93 showed gross morphological defects and 83 exhibited growth and development resembling the original TJH3.40 strain. The 83 mutants whose growth and development resembled that of the original TJH3.40 strain were chosen for further analysis as we know that morphological mutants in *A. nidulans* are also defective in *stc* gene cluster activity (Hicks *et al.* 1997), and we wished to identify mutants in pathways more directly related to *stc* gene expression.

Linkage of mutations to the *stc* gene cluster: To date it is known that four *stc* genes are required for NOR production, *aflR*, *stcA*, *stcJ*, and *stcK*. Mutations in any one of these four genes would lead to the loss of NOR production. Also, other genes have been found in the *A. parasiticus* and *A. flavus* aflatoxin clusters that are involved in aflatoxin production in these species and we believe it is possible that homologous genes that may be required for NOR production in *A. nidulans* exist in the region of the *stc* cluster. Therefore, MRB strains containing mutations linked to the cluster could have mutations in one of the four known *stc* cluster genes or in unidentified genes such as homologs of genes in the aflatoxin gene cluster. Linkage to the *stc* gene cluster was determined by crossing each MRB strain to a *stcE* wild-type strain (ST accumulating) and analyzing meiotic progeny for accumulation of NOR. MRB strains containing *aflR*, *stcA*, *stcJ*, and *stcK* mutations should produce a low percentage of NOR-accumulating progeny as such mutations would be closely linked to the *stcE::argB* locus. MRB strains containing mutations unlinked to the *stc* gene cluster would produce 25% NOR-accumulating progeny when crossed to a wild-type strain. Examination of 100–200 ascospore progeny of 60 of the 83 mutants showed between 0.99 and 6.2% recombination, suggesting that these 60 are linked to the *stc* gene cluster and represent candidate mutations in *aflR*, *stcA*, *stcJ*, *stcK*, or another linked locus.

Determination of linkage groups among the 23 unlinked mutants: To determine the number of genes or loci represented in the 23 unlinked mutations, the mutants were crossed in every pairwise combination. This required first crossing each MRB strain to FGSC410 to introduce another genetic marker, a riboflavin mutation, into the mutant strains, thus generating RRB1 through RRB23. Each RRB strain, containing the *stcE::argB2* disruption, the new mutation, and *riboB2*, was crossed to the 23 MRB mutants, and progeny were analyzed for the accumulation of NOR. As in the above test, NOR accumulation in 25% of the MRB × RRB progeny indicated that the mutations were in two separate linkage groups. If none of the progeny accumulated NOR, this suggested that the mutations were in the same or closely linked loci. From this analysis we determined that 5 mutants, MRB246, 278, 288, 308, and 379, contain mutations that are closely linked (and are therefore possibly allelic), whereas the remaining 18 mutants represented unique loci (Figure 1). In certain cases (indicated by an asterisk) viable ascospores were never generated after multiple attempts to cross two strains. We are unable to determine the genetic interaction in such cases.

278	288	308	379	230	634	665	683	685	698	700	701	720	726	727	733	746	757	765	769	
S	S	S	S	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	246
	S	S	S	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	278
		S	S	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	288
			S	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	308
				D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	379
					D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	230
						D	D	D	*	D	*	D	D	*	D	*	D	D	*	D	234
							D	D	D	D	D	D	D	D	D	D	D	D	*	D	263
								D	D	D	D	D	D	D	D	D	D	D	D	D	265
									D	D	*	D	D	*	D	*	D	D	*	D	283
										D	D	D	D	D	D	D	D	D	D	D	285
											D	D	D	D	D	D	D	D	D	D	298
												D	D	D	D	D	D	D	D	D	300
													D	D	D	D	D	D	D	D	303
														D	D	D	D	D	D	D	311
															D	*	D	*	D	*	320
																D	D	D	D	D	326
																	D	*	D	*	327
																		D	D	D	333
																			D	*	346
																				D	357
																				D	365

Figure 1.—Linkage groups among the 23 unlinked mutations causing defects in NOR production. S indicates that the mutations in the two mutants are either in the same or in a closely linked gene. D indicates that the two mutations acted as two different, unlinked genes. An asterisk indicates that crosses between those two mutants never yielded viable ascospores.

mRNA and NOR analysis: To determine whether the 23 unlinked mutations could define potential regulators/activators of *aflR* expression or AflR activity, we grew the mutants in three different media and analyzed culture extracts for NOR production and mRNA for *aflR* and *stc* gene transcripts. Cultures grown on liquid oatmeal in this manner did not produce enough mycelium for mRNA extraction, but organic extraction of NOR was possible. For 20 of the strains, transcript accu-

TABLE 2

Summary of NOR analysis and *aflR* transcription

Mutant name	Liquid media: CM NOR[a]	CM *aflR*	CM + oatmeal NOR	CM + oatmeal *aflR*	Oatmeal: NOR
MRB246[b]	+	+	+	+	+
MRB278[b]	+	+	+	+	−
MRB288[b]	+	+	+	+	−
MRB308[b]	+	−	+	+	−
MRB379[b]	+	+	+	+	−
MRB230	+	−	+	−	−
MRB234	−	−	−	−	−
MRB263	+	+	−	+	+
MRB265	+	+	+	+	−
MRB283	+	+	+	+	+
MRB285	+	+	+	+	−
MRB298	+	+	+	+	+
MRB300	−	−	−	−	−
MRB303	+	−	+	+	+
MRB311	+	+	+	+	+
MRB320	−	−	−	+	−
MRB326	+	−	+	+	+
MRB327	+	−	+	+	−
MRB333	+	−	−	+	−
MRB346	+	+	−	+	−
MRB357	+	+	+	+	−
MRB365	−	−	−	+	+
MRB369	+	−	+	+	+
TJH3.40	++	+	++	+	++

[a] +, relative amount of NOR or presence of *aflR* transcript; −, no NOR and no *aflR* transcript.
[b] The first five mutants listed represent one linkage group.

mulation varied depending on growth medium. TLC analysis showed that all of these 20 mutants produced trace amounts of NOR that was visually undetectable in the oatmeal plate assay (Table 2). Only 3 mutants, MRB230, MRB234, and MRB300, consistently failed to produce NOR on 1.0% oatmeal plates or detectable *aflR* and *stc* transcripts (Figure 2). These 3 mutants represent the best candidates for nonconditional regulators of *aflR*.

In addition, all 23 mutants were tested for temperature and pH sensitivity. ST and AF biosynthesis has been shown to be pH regulated (Keller *et al.* 1997) and AF biosynthesis is temperature regulated (Schroeder and Hein 1967). The results from these experiments (Table 3) showed that several mutants were conditional for NOR production and/or had pleiotropic effects. For example, MRB326, MRB311, and MRB298 produced visible NOR at 29° but not 37° (the temperature used for the screen; Figure 3). This was in contrast to wild-type TJH3.40, which produced NOR at both 29° and 37° (Figure 3). Interestingly, NOR production is temperature sensitive in the wild type as it is not produced at 42° in TJH3.40 (Figure 3). Two mutants were pH sensitive;

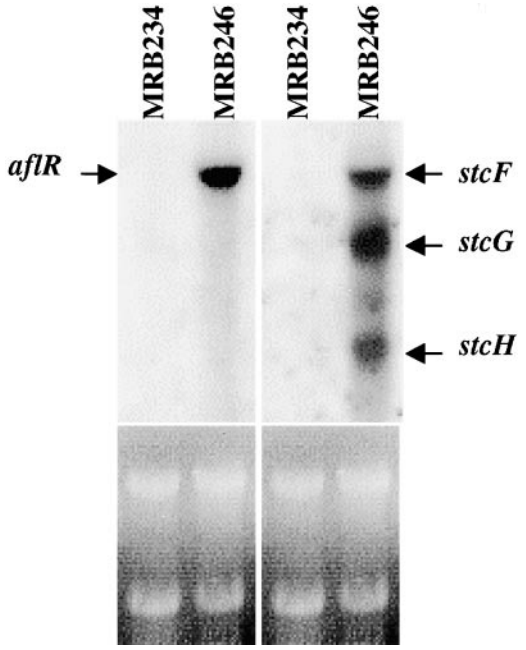

Figure 2.—(A) RNA analysis of MRB mutants. DNA fragments used as probes are as described in materials and methods. (B) Ethidium bromide-stained gel prior to transfer. Transcript sizes are as follows: *aflR*, 1.6 kb; *stcF*, 1.6 kb; *stcG*, 1.0 kb; and *stcH*, 0.6 kb.

MRB234 could not grow at either pH extreme and MRB311 did not grow at pH 4 and at pH 8 had a fluffy phenotype and produced NOR.

Dominant/recessive nature of mutations: Diploids between MRB strains and another *stcE* disruption strain, RJH007, were created to determine the dominant or recessive nature of each mutation. For the 23 unlinked mutations, only one diploid (MRB265 × RJH007) did not produce NOR, indicating that MRB265 contains a dominant mutation. *aflR* and *stc* gene cluster transcripts were detectable in MRB265 (data not shown).

DISCUSSION

In an effort to reduce or eliminate AF contamination from food and feed supplies we are concentrating on understanding the genetic regulation of the Aspergillus gene cluster required for mycotoxin biosynthesis. A positively acting transcription factor present in the gene cluster, AflR (Chang *et al.* 1993; Payne *et al.* 1993), is required for *stc* gene transcription (Yu *et al.* 1996) and at least one AflR binding motif in the *stc* promoter (Fernandes *et al.* 1998) is required for expression of the *stc* gene cluster. Other genes required for normal AF/ST production and *aflR* expression include *flbA* and *fadA*, genes involved in a G protein-signaling pathway required for Aspergillus developmental processes (Hicks *et al.* 1997). Obviously a more precise understanding of how *aflR* expression is regulated would

TABLE 3

Relative temperature and pH effects on growth characteristics and NOR production on solid media

Mutant name	Oatmeal medium				
	29°	37°	42°	pH 4[a]	pH 8[a]
MRB246[b]	++	++	−	+	+
MRB278[b]	++	++	+	+	±
MRB288[b]	++	++	+	+	+
MRB308[b]	++	++	+	+	+
MRB379[b]	++	++	+	+	+
MRB230	++	++	+[c]	+	±
MRB234	++	++	+[d]	−	−
MRB263	++	++	+	+	+
MRB265	++	++	+	+	+
MRB283	++	++	+	+	+
MRB285	++	++	+	+	+
MRB298	++N	++	+	+	+
MRB300	++	++	+	+	±
MRB303	++	++	+	+	+
MRB311	++N	++	+	−	+NN[d]
MRB320	++	++	+	+	+
MRB326	++NN	++	+	+	±
MRB327	++	++	+	+	+
MRB333	++	++	+	+	+
MRB346	++	++	+	+	+
MRB357	++	++	+	+	+
MRB365	++	++	+	+	±
MRB369	++	++	+	+	+
TJH3.40	++NN	++NN	+	+	+

+ indicates relative growth rate; N indicates relative NOR accumulation as assessed visually.

[a] pH assay was conducted at 37°.

[b] The first five mutants listed represent one linkage group.

[c] Aconidial, flat growth.

[d] Aconidial, fluffy growth.

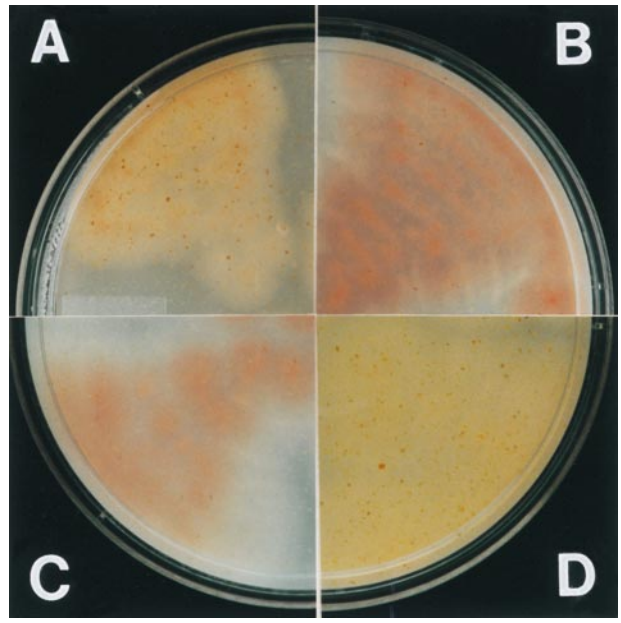

Figure 3.—Temperature effects on NOR accumulation. A and B show strain TJH3.40 grown at 42° and 37°, respectively. C and D show strain MRB326 grown at 29° and 37°, respectively.

greatly aid in designing strategies to reduce AF/ST production in agricultural commodities.

The design of the screen developed here ensured that we would obtain several different categories of Nor$^-$ mutants, some of which would be defective in genes already found to be required for NOR production, including *flbA*, *fadA*, *aflR*, and three ST biosynthetic genes, *stcA* (encoding a polyketide synthase) and *stcJ* and *stcK* (encoding the α and β subunits, respectively, of a fatty acid synthase). Through genetic analysis, the mutations were determined to be linked or unlinked to the *stc* gene cluster. We are interested in characterizing those that are unlinked and thus represent the best possibilities for novel *trans*-acting regulators of ST/AF biosynthesis.

To assess how many loci are represented in the group of 23 unlinked mutants, meiotic progeny from each pairwise combination of mutants were analyzed for NOR production. This analysis revealed one linkage group that contained 5 mutants (MRB246, 278, 288, 308, and 379). MRB246, in contrast to the other four strains, was not able to grow at 42°, MRB308 did not express *aflR* in complete medium, and MRB278 grew poorly at pH 8. These differences could reflect distinct types of mutations in one gene or mutations in different, tightly linked genes. Interestingly, the remaining 18 mutants each fell into unique linkage groups, thus suggesting that our screen was not saturated. The high number of linkage groups may reflect the fact that a vast number of factors have been reported to influence AF production in *A. flavus* and *A. parasiticus* (Zaika and Buchanan 1987). Two of these factors are pH (Keller *et al.* 1997) and temperature (Schroeder and Hein 1967). An examination of the 23 mutants showed that several were pH (MRB230, MRB234, MRB278, MRB300, MRB311, MRB326, and MRB365) and temperature (MRB230, MRB234, MR246, MRB298, MRB311, and MRB326) sensitive with respect to growth and/or NOR production. Several mutants showed both pH and temperature sensitivity. We also found NOR production to be temperature sensitive in TJH3.40. Complementation of these 9 mutants may reveal some insight into pH and temperature control of ST/AF biosynthesis.

Finally, to further characterize the unlinked mutants in terms of the regulation of expression of the *stc* gene cluster, we looked for *aflR* and other *stc* gene cluster transcripts in these mutants. Only 3 of the 23 mutants lacked *aflR* and *stc* gene expression in all conditions tested. These 3 mutants fall into different linkage groups and appear to be specific regulators of *aflR*. The other 20 mutations likely affect some other aspect of ST biosynthesis, as *aflR* and other *stc* cluster genes were transcribed in one or more of the three media tested (Table

2). Perhaps these 20 mutations represent unlinked genes that are necessary for allocation of resources (*e.g.*, acetyl CoAs) for polyketide formation or represent independent regulators of *aflR*. The mutations may block the availability of resources for the ST pathway or the localization of *stc* gene products to subcellular sites as is described for the penicillin biosynthetic pathway (Muller *et al.* 1992). With the exception of the dominant mutation (MRB265), the genes denoted by the MRB mutations can be identified through classic complementation analysis with a wild-type cosmid library using the restoration of NOR production as the screen.

Aflatoxin contamination remains a problem worldwide and we believe that logical AF control strategies will be developed only through understanding the genetic regulation of the biosynthesis of this mycotoxin. To date we have found that all aspects of ST regulation in *A. nidulans* have been conserved with regard to AF regulation in the aflatoxigenic *Aspergillus* spp. including *aflR* function. Therefore it is likely that factors involved in the regulation of *aflR* should also be conserved in all species. If a novel approach to regulating ST production by regulating the expression of *aflR* exists, this might be applicable to controlling AF contamination in agricultural products.

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LITERATURE CITED

Anderson, J. A., C. H. Chung, S. H. Cho and R. H. Adamson, 1990 Versicolorin A hemiacetal, hydroxydihydrosterigmatocystin, and aflatoxin G_2 alpha reductase activity in extracts from *Aspergillus parasiticus*. Induction of hepatocellular carcinoma in nonhuman primates by chemical carcinogens. Mycopathologia **111:** 39–45.

Bressac, B., M. Kew, J. Wands and M. Ozturk, 1991 Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. Nature **350:** 429–431.

Brown, D. W., T. H. Adams and N. P. Keller, 1996a *Aspergillus* has distinct fatty acid synthases for primary and secondary metabolism. Proc. Natl. Acad. Sci. USA **93:** 14873–14877.

Brown, D. W., J.-H. Yu, H. S. Kelkar, M. Fernandes, T. C. Nesbitt *et al.*, 1996b Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA **93:** 1418–1422.

Burse, B., A. Dmochowska, M. Skrzypek, P. Weglenski, M. A. Bates *et al.*, 1983 Cloning and characterization of the ornithine carbamoyltransferase gene from *Aspergillus nidulans*. Gene **25:** 109–117.

Cardwell, K., and J. D. Miller, 1996 Mycotoxins in foods in Africa. Natural Toxins **4:** 103–107.

Chang, P.-K., J. W. Cary, D. Bhatnagar, T. E. Cleveland, J. W. Bennett *et al.*, 1993 Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. **59:** 3273–3279.

Cole, R. J., and R. H. Cox, 1981 *Handbook of Toxic Fungal Metabolites*, pp. 67–72. Academic Press, New York.

Fernandes, M., N. P. Keller and T. H. Adams, 1998 Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. Mol. Microbiol. **28:** 1355–1365.

Hicks, J. K., J.-H. Yu, N. P. Keller and T. H. Adams, 1997 *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G-alpha protein-dependent signaling pathway. EMBO J. **16:** 4916–4923.

Käfer, E., 1977 Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations, pp. 33–67 in *Advances in Genetics*, edited by E. W. Caspari. Academic Press, New York.

Kelkar, H. S., T. W. Skloss, J. F. Haw, N. P. Keller and T. H. Adams, 1997 *Aspergillus nidulans stcL* encodes a putative *P*-450 monooxygenase required for bisfuran desaturation during aflatoxin/sterigmatocystin biosynthesis. J. Biol. Chem. **272:** 1589–1594.

Keller, N. P., and T. M. Hohn, 1997 Metabolic pathway gene clusters in filamentous fungi. Fungal Genet. Biol. **21:** 17–29.

Keller, N. P., R. A. E. Butchko, B. Sarr and T. D. Phillips, 1994 A visual pattern of mycotoxin production in maize kernels by *Aspergillus* spp. Phytopathology **84:** 483–488.

Keller, N. P., T. C. Nesbitt, B. Sarr, T. D. Phillips and G. B. Burow, 1997 pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. Phytopathology **87:** 643–648.

Lee, L. S., J. W. Bennett, L. A. Goldblatt and R. E. Lundin, 1971 Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. J. Am. Oil Chem. Soc. **48:** 93–94.

Muller, W. H., R. A. L. Bovenberg, M. H. Groothuis, F. Kattevilder, E. B. Smaal *et al.*, 1992 Involvement of microbodies in penicillin biosynthesis. Biochim. Biophys. Acta **1116:** 210–213.

Papa, K. E., 1982 Norsolorinic acid mutant of *Aspergillus flavus*. J. Gen. Microbiol. **128:** 1345–1348.

Payne, G. A., G. J. Nystrom, D. Bhatnagar, T. E. Cleveland and C. P. Woloshuk, 1993 Cloning of the *afl*-2 gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. Appl. Environ. Microbiol. **59:** 156–162.

Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. MacDonald and A. W. J. Bufton, 1953 The genetics of *Aspergillus nidulans*. Adv. Genet. **5:** 141–238.

Schroeder, H. W., and H. Hein, 1967 Aflatoxins: production of the toxins *in vitro* in relation to temperature. Appl. Microbiol. **15:** 441–445.

Wieser, J., B. N. Lee, J. W. Fondon and T. H. Adams, 1994 Genetic requirements for initiating asexual development in *Aspergillus nidulans*. Curr. Genet. **27:** 62–69.

Yu, J.-H., and T. J. Leonard, 1995 Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. J. Bacteriol. **177:** 4792–4800.

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

Zaika, L. L., and R. L. Buchanan, 1987 Review of compounds affecting the biosynthesis or bio-regulation of aflatoxins. J. Food Prot. **50:** 691–708.

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