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Genetics of *Aspergillus flavus*: complementation and mapping of aflatoxin mutants

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SUMMARY

Mutants of *Aspergillus flavus* impaired in aflatoxin production were induced with *N*-methyl-*N'*-nitrosoguanidine and analysed by means of the parasexual cycle. The gene symbol *afl* was assigned to this type of mutation. Diploid complementation tests revealed that most of 14 *afl* mutants belonged to different complementation groups. One mutant (*afl*-1) failed to complement or only partially complemented all other mutants. Haploidization of one diploid revealed the independent segregation of two *afl* mutants. Heterozygous diploids were synthesized between two *afl* mutants and tester strains genetically labelled on eight linkage groups. Haploidization of the diploids led to the assignment of two *afl* mutants to linkage groups. Linkage of *afl*-4 to w^+ and *afl*-1 to *leu* on linkage groups II and VII, respectively, was demonstrated.

1. INTRODUCTION

The demonstration of a parasexual cycle with the identification of linkage groups in *Aspergillus flavus* now makes genetic investigations of aflatoxin production in this fungus feasible (Papa, 1973, 1976). Aflatoxins, toxic secondary metabolites of strains of *A. flavus* and *A. parasiticus*, have attracted much attention. Although much is known about their biosynthesis and biological effects, little is known about their genetic control.

A genetic locus involved in the interconversion of aflatoxins B_1 and B_2 in *A. flavus* was shown to be linked to the histidine locus on linkage group VIII (Papa, 1977). Gussack *et al.* (1977) reported parasexuality in an aflatoxigenic strain of *A. flavus* containing virus-like particles. No evidence of a correlation between aflatoxin production or non-production and the presence of virus-like particles was obtained in their studies. More recently, two independently segregating aflatoxin mutants of *A. parasiticus* were assigned to linkage groups (Papa, 1978).

Several mutants of *A. parasiticus* have been shown to accumulate anthraquinone intermediates of the aflatoxin pathway (Donkersloot *et al.* 1972; Lee *et al.* 1971, 1975). These accumulants were converted to aflatoxin in *in vivo* conversion experiments (Singh & Hsieh, 1977). Heathcote, Dutton & Hibbert

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(1976) also examined the order of biosynthesis by feeding labelled aflatoxins and related metabolites to actively growing mutant and wild-type cultures of *A. flavus*. The present study includes diploid complementation and linkage detection of aflatoxin mutants of *A. flavus*.

2. MATERIALS AND METHODS

(i) *Strains*

The induction and recovery of the mutant strains used in this study have been described previously (Leaich & Papa, 1974; Papa, 1976). All strains produced aflatoxin B_1, low levels of B_2, and no detectable G toxins. Two tester strains were synthesized through a series of parasexual crosses. One tester carried gene markers proline-*pro*, white-*w*, methionine-*met*-3, tan-*t*, p-aminobenzoic acid-*pab*, and histidine-*his* on linkage groups I, II, III, IV, V and VIII, respectively. The other tester carried gene markers *w*, *t*, and leucine-*leu* on linkage groups II, IV and VII, respectively.

(ii) *Media*

Cultures were maintained on a complete medium (CM) consisting of Czapek-Dox broth, 0·75% malt extract, 0·25% yeast extract, and 1·5% agar. The minimal medium (MM) was CM without the malt and yeast extracts. Diploids were plated on CM containing 0·007% *p*-fluorophenylalanine (PFA) to induce haploidization. The medium (YES) for production of aflatoxin consisted of 2% yeast extract and 20% sucrose. Aflatoxin mutants were detected on modified MM containing corn steep liquor and the appropriate supplements to meet the requirement of auxotrophs (Hara, Fennell & Hesseltine, 1974). The corn steep liquor was kindly provided by CPC International, Inc., Argo, Ill.

(iii) *Induction and recovery of aflatoxin mutants*

Mutants impaired in aflatoxin production were induced in seven auxotrophic spore-colour mutants by exposing conidia to *N*-methyl-*N'*-nitrosoguanidine (NG). Conidia were suspended in a 0·02% solution of NG for 1 h and agitated by means of a magnetic stirrer. A dilute suspension of treated spores was added to 1 l of cooled (45 °C) modified MM containing corn steep liquor, mixed thoroughly, and poured into glass Petri plates. Following a 10-day incubation period in the dark at 28 °C, plates were examined under UV (366 nm) illumination for the absence of fluorescence in the agar surrounding the colonies. Non-fluorescing colonies were subcultured and further tested for their inability to produce aflatoxin by thin-layer chromatography. The symbol *afl* was assigned to this type of mutation. The tan-spored mutant *afl*-4 was mutagenized again with NG in order to recover a white-spored variant.

(iv) *Assay for aflatoxin*

For aflatoxin assays, Erlenmeyer flasks (250 ml) containing 50 ml of YES medium were inoculated with spores from cultures 7–10 days old, incubated at 27 °C for 7 days, and stored frozen. The extraction and assay procedures were

similar to those described by Lillard, Hanlin & Lillard (1970). Thin-layer chromatographic plates (pre-coated, silica gel 60) from EM Laboratories, Inc., Elmsford, N.Y., were spotted with standard and appropriately diluted extract. The aflatoxin standard was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Quantification of aflatoxin was by direct measurement of fluorescence using a Turner Model 111 fluorometer. The extracted mycelial mats were filtered, dried overnight at 70 °C, and weighed. Aflatoxin levels were calculated and expressed as μg/g mycelial dry wt. Only aflatoxin B_1 was included in this study.

(v) *Genetic analysis*

Techniques involved in forcing heterokaryons, recovering diploids, and subsequently haploidizing diploids were the same as those reported by Papa (1976). The *afl* mutants were paired in heterokaryons to yield diploids for complementation tests. The similarity of spore colours and/or nutritional requirements of some mutants precluded the establishment of all possible heterokaryons. Three to four diploids were recovered from most heterokaryons and tested for aflatoxin production. One diploid formed between two complementing *afl* mutants was haploidized and segregation data obtained. Parasexual crosses were made between two *afl* mutants and tester strains for linkage detection. The independent or joint segregation of pairs of gene markers was determined from 2×2 contingency tests (Snedecor, 1956).

3. RESULTS

Fourteen aflatoxinless mutants (*afl*-1 to *afl*-14) were recovered from six auxotrophic spore-colour mutants after treatment of conidia with NG (Table 1). Although many aflatoxin variants were recovered, only those lacking the ability to produce aflatoxin were retained. None of the *afl* mutants had additional nutritional requirements and only one (*afl*-5) acquired a different spore colour.

Table 1. *Aflatoxinless mutants of* Aspergillus flavus *and the parental strains from which they were recovered*

Parents				Aflatoxinless (*afl*) mutants†
Genotype	Spore colour	Requirement	Aflatoxin B_1*	
t leu	Tan	Leucine	324	1, 2, 3
t pdx	Tan	Pyridoxin	2908	4, 5
ylo pab	Yellow	*p*-Aminobenzoic acid	3729	6, 7
w met-3	White	Methionine	2123	8, 9, 10
w nic	White	Nicotinamide	127	11, 12, 13
fwn arom	Fawn	Aromatic amino acids	3062	14

* Aflatoxin B_1 was recorded in μg/g mycelial dry wt.

† All *afl* mutants have the same spore colour and requirement as the parents from which they were derived except *afl*-5 which has yellow spores. A white-spored variant was also induced in *afl*-4.

Table 2. *Complementation of* afl *mutants (1–14) in diploids*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	.	—*	—	53†	86	172	22	8	0	19	66	3	146	40
2		.	—	1704	X*	5393	3392	1272	X	5025	X	1730	6564	4487
3			.	4632	3987	X	X	30	371	1826	X	X	6023	3372
4				.	—	1162	2775	98	268	X	2108	590	1589	1367
5					.	X	2496	559	514	1647	2038	134	3045	1219
6						.	—	1119	610	1840	5830	X	5113	4362
7							.	5668	4449	1186	3984	6326	98	4254
8								.	—	—	—	—	—	X
9									.	—	—	—	—	X
10										.	—	—	—	X
11											.	—	—	X
12												.	—	X
13													.	X
14														.

* —, No heterokaryons; X, heterokaryons but no diploids.
† Aflatoxin B_1 recorded in $\mu g/g$ mycelial dry wt.

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Seventy-one heterokaryons were formed among pairs of *afl* mutants and diploids were recovered from 55.

Complementation analyses of diploids revealed that most of the 14 aflatoxin mutants complemented in diploids (Table 2). Mutant *afl*-1 failed to complement or only partially complemented 11 other mutants. The average aflatoxin B_1 level for these 11 diploids was 56 μg/g mycelial dry wt, whereas the average for other mutants in all diploid combinations ranged from 1050 to 4325 μg/g. Aflatoxin mutants *afl*-3 and *afl*-4 complemented well in diploids but both only partially complemented *afl*-8. Also, very little complementation was detected between *afl*-5 and *afl*-12. No apparent relationship was observed between aflatoxin B_1 levels of diploids and the strains from which the *afl* mutants were recovered.

Haploidization of a diploid synthesized between *afl*-1 and a tester strain provided evidence for linkage between *afl*-1 and *leu* on linkage group VII (Table 3). Of 178 haploid segregants, only four had nonparental gene combinations for *leu* and *afl*-1. To be sure that these four had not been mistakenly classified, they were tested twice for nutritional requirements and aflatoxin. In addition, they grew well on CM + PFA and did not sector, thus indicating that they were indeed

Table 3. *Linkage detection of aflatoxin mutant* afl-*1*

(*a*) Cross

afl-1 (*afl t leu*) X Tester (*pro w met*-3 *t pab his*)

(*b*) Diploid

Linkage groups

I	II	III	IV	V	VII	VIII	?
+ / *pro*	+ / *w*	+ / *met*-3	*t* / *t*	+ / *pab*	*leu* / +	+ / *his*	*afl*-1 / +

(*c*) Haploid segregants

Linkage group marker	Allele	Aflatoxin* +	Aflatoxin* −	Total	χ^2
pro	−	49	50	99	1·02
	+	46	33	79	
met-3	−	0	0	0	—
	+	95	83	178	
w	−	46	44	90	0·21
	+	49	39	88	
pab	−	16	2	18	8·62†
	+	79	81	160	
leu	+	93	2	95	158·90†
	−	2	81	83	
his	−	46	23	69	7·16†
	+	49	60	109	
Total		95	83	178	0·81

* The upper left and lower right genotypes for each gene pair are the parental combinations.

† Chi-square values are significant at the 0·01 level, thus indicating a lack of independence. Chi-square test of 1 : 1 on the total was non-significant.

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haploid. Non-random assortment was also noted between *afl*-1 and the loci for p-aminobenzoic acid, histidine, and methionine-3. In fact, no segregation occurred at the latter locus. Apparently selection against the *pab*,*afl*-1 and *his*,*afl*-1 genotypes rather than linkage accounted for the highly significant chi-square values.

Considerable variability in aflatoxin B_1 levels existed among the 95 aflatoxin-producing segregants. The mean and standard deviation was $963 \pm 719\ \mu g/g$ mycelial dry wt.

Haploidization of two diploids synthesized between *afl*-4 and tester strains led to the establishment of linkage between *afl*-4 and the white locus on linkage group II (Table 4). All other gene markers except *his* segregated independently;

Table 4. *Linkage detection of aflatoxin mutant* afl-*4*

(*a*) Crosses

(1) *afl*-4 (*afl t pdx*) X Tester (*pro w met*-3 *t pab his*)
(2) *afl*-4 (*afl t pdx*) X Tester (*w t leu*)

(*b*) Diploids

	Linkage groups								
	I	II	III	IV	V	VI	VII	VIII	?
(1)	+ / *pro*	+ / *w*	+ / *met*-3	*t* / *t*	+ / *pab*	*pdx* / +		+ / *his*	*afl*-4 / +
(2)		+ / *w*		*t* / *t*		*pdx* / +	+ / *leu*		*afl*-4 / +

(*c*) Haploid segregants

	Linkage group marker	Allele	Aflatoxin† +	Aflatoxin† −	Total	χ^2
(1)	*pro*	−	61	85	146	0·02
		+	17	24	41	
	w	−	78	0	78	183·00**
		+	0	109	109	
	met-3	−	13	28	41	1·67
		+	65	81	146	
	pab	−	37	61	98	1·00
		+	41	48	89	
	pdx	+	41	72	113	2·92
		−	37	37	74	
	his	−	31	26	57	4·69*
		+	47	83	130	
	Total		78	109	187	5·14*
(2)	*w*	−	43	0	43	84·04**
		+	0	45	45	
	pdx	+	25	22	47	0·43
		−	18	23	41	
	leu	−	41	44	85	0·00
		+	2	1	3	
	Total		43	45	88	0·04

† The upper left and lower right genotypes for each gene pair are the parental combinations.

*, ** Chi-square values are significant at the 0·05 and 0·01 levels, respectively. The totals were tested for a 1:1 ratio.

however, *his* was not considered to be linked to *afl*-4. All of the 275 haploid segregants from the two diploids had parental gene combinations for *w* and *afl*-4. The aflatoxin$^+$ segregants from diploids 1 and 2 produced mean aflatoxin B_1 levels of 2151 ± 2105 and 2076 ± 1189 μg/g, respectively.

The analysis of a parasexual cross between two complementing *afl* mutants (*afl*-4 and *afl*-12) is given in Table 5. Independent segregation of *afl*-4 and *afl*-12 was concluded on the basis of the 1:3 ratio of aflatoxin$^+$ to aflatoxin$^-$ segregants. Linkage of *afl*-4 to the white locus, as determined previously, resulted in all coloured progeny being aflatoxin$^-$. Mutant *afl*-12 segregated independently of *t*, *w*, *nic* and *pdx*.

Table 5. *Parasexual cross between two complementing aflatoxin mutants*

(*a*) Cross

afl-4 (*afl t pdx*) X *afl*-12 (*afl t w nic*)

(*b*) Diploid

Linkage groups					
II		IV		VI	?
+	*afl*-4	*t*	+	*pdx*	+
w	+	*t*	*nic*	+	*afl*-12

(*c*) Haploid segregants

Linkage group marker	Allele	Aflatoxin +	Aflatoxin −	Total	χ^2
w	−	13	19	32	16·77*
	+	0	39	39	
nic	−	4	30	34	1·12
	+	9	28	37	
pdx	−	8	29	37	0·20
	+	5	29	34	
Total		13	58	71	1·69

* Chi-square value is significant at the 0·01 level. Chi-square test of 1:3 on the total was non-significant.

4. DISCUSSION

Aflatoxin variants were easily induced and recovered in *A. flavus* following the treatment of conidia with NG. Although considerable variability in aflatoxin levels was created, only those mutants (*afl*) incapable of accumulating aflatoxin were studied. Rapid screening for *afl* mutants was greatly facilitated by the methods of Hara *et al.* (1974) employing corn steep liquor. Many of the colonies growing on the test medium exhibited no fluorescence, yet when subjected to analysis by thin-layer chromatography, low levels of aflatoxin B_1 were detected. Nevertheless, large numbers of *afl* mutants could be recovered quite easily in a relatively short time.

Diploid complementation tests revealed that most of the *afl* mutants in this study are non-allelic. Before all possible tests of allelism can be made among these mutants, however, additional spore colour and/or nutritional requirements

need to be incorporated so that all necessary heterokaryons can be forced between pairs of mutants. Thus, it is not possible to adequately assign *afl* mutants to complementation groups at this time. Diploids of non-complementing or only weakly complementing *afl* mutants generally produced less than 150 μg of aflatoxin B_1 per g mycelial dry weight whereas diploids of complementing mutants usually produced more than 1000 μg. The most significant finding was that *afl*-1 failed to complement or only weakly complemented other *afl* mutants tested. Although the other mutants were recessive in diploids, *afl*-1 appeared to be dominant. In order to test the possible dominance of *afl*-1, four diploids were synthesized between *afl*-1 and each of four high aflatoxin B_1-producing strains. The aflatoxin$^+$ strains accumulated an average of 2500 μg aflatoxin B_1 per g mycelial dry weight, whereas the diploids exhibited an 87% average reduction in aflatoxin B_1. Consequently, *afl*-1 was acting in a dominant fashion.

The failure of *afl*-1 to complement other *afl* mutants is not due to allelism but rather to some other mechanism. Holt, Edwards & Macdonald (1976), in examining penicillinless mutants, suggested that penicillinless loci may be structural genes coding for enzymes in the pathway, genes altering permeability, or genes with regulatory functions. Possibly *afl*-1 alters the permeability in both haploids and diploids so that aflatoxin is not excreted, or it may have a regulatory role. It is also possible that *afl*-1 may promote rapid metabolism of aflatoxin to undetectable products and therefore never accumulate in large quantities.

If some of the aflatoxinless loci of this study involve structural genes coding for enzymes in the aflatoxin pathway, they are not likely to be found in the final steps of the pathway since no pigmented accumulants (anthraquinone intermediates) were seen in the cultures. In *A. parasiticus*, the immediate precursors of aflatoxin, norsolorinic acid, averufin and versicolorin A, could be detected in the mycelial mats by their orange, red or yellow pigments (Donkersloot, Mateles & Yang, 1972; Lee *et al.* 1971, 1975). Biochemical analyses of *afl* mutants from different complementation groups should aid in elucidating the biosynthetic pathway of aflatoxin.

Parasexual crosses of *afl*-1 and *afl*-4 to tester strains provided good evidence of linkage between *afl*-1 and *leu* and between *afl*-4 and w^+. For *afl*-1 and *leu*, only four of the 178 haploid segregants had nonparental genotypes. Additional genetic markers on linkage group VII will be required before the nonparental types can be attributed specifically to mitotic recombination or to mutation. In crosses involving *afl*-4 and w^+, all of the 275 progeny possessed parental genotypes.

Most unlinked genes did not depart appreciably from an approximate 1:1 expectation. The most notable exception was the *met*-3 marker, as it was not recovered in one cross and was recovered in only 22% of the progeny in another. In addition, the *pab* marker was recovered in low frequency in one cross but not in the other, while the *his* marker was slightly reduced in two crosses. In each of the above, the frequency of a parental and a nonparental type was either reduced or, as with *met*-3, zero. Early chromosomal non-disjunction or specific selection against an allele can lead to this pattern. Kohli *et al.* (1977) also pointed out that

early mitotic crossing over between two linked mutants resulting in homozygosity for one of them could also create the same pattern. The likelihood of this occurrence, however, would be extremely small.

Parasexual crosses are useful in determining linkage between complementing *afl* mutants. For unlinked recessive mutants blocked in aflatoxin synthesis, the haploid progeny should segregate in a ratio of 1 afl^+:3 afl^-. For linked recessive mutants, however, only recombinant segregants would produce aflatoxin. From the data of Table 5 it can be seen that *afl*-4 and *afl*-12 are unlinked and, as previously noted, *afl*-4 is linked to w^+. Consequently, all w^+ t progeny were expected to be afl^- and the w progeny were expected to be segregating in a ratio of 1 afl^+:1 afl^-.

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