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Norsolorinic Acid Mutant of *Aspergillus flavus*

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A mutant of *Aspergillus flavus* accumulating norsolorinic acid and approximately 50% less aflatoxin than the parent strain was recovered after treatment of conidia with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The gene *nor* controlling this mutant phenotype was recessive and linked to *afl-1* and *leu* on linkage group VII. Diploids homozygous for *nor* were similar to haploids in norsolorinic acid accumulation. The analysis of recombinant diploids and haploids showed that *afl*-1 and *nor* were both distal to *leu* on the same chromosome arm.

INTRODUCTION

Aflatoxins, secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*, have attracted considerable attention, and much has been reported about their biosynthesis and biological effects. Several intermediates in the aflatoxin biosynthetic pathway have been established through studies of induced mutants (Hsieh *et al.*, 1976). Lee *et al.* (1971) recovered a mutant of *A. parasiticus* which accumulated norsolorinic acid and less aflatoxin than the wild-type. This mutant was recessive in diploids (Bennett, 1979). Several aflatoxin mutants having no detectable pigmented intermediates have been recovered in *A. flavus* (Papa, 1979).

This paper describes the genetic analysis of a norsolorinic acid mutant of *A. flavus*, including linkage detection and mapping.

METHODS

Strains. Five auxotrophic strains of *A. flavus* having either white (*w*) or tan (*t*) spore colour were used in this study. Their individual genotypes were as follows: 1. *w lys* (requiring lysine); 2. *t pdx leu* (pyridoxine, leucine); 3. *t leu*; 4. *afl-1 t leu*; and 5. *t arg* (arginine). Strain 4 was also impaired in aflatoxin production (*afl-1*). The induction of auxotrophy, spore colour variation, and aflatoxin mutants has been described previously (Leaich & Papa, 1974; Papa, 1976, 1979).

Following the exposure of conidia from strain 1 to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), a mutant, designated 1-N and having an orange-red mycelium, was recovered. The gene controlling the production of this orange-red pigment (norsolorinic acid) has been designated *nor* (Bennett, 1979).

Media. The complete medium (CM) consisted of Czapek–Dox broth, 0·75% (w/v) malt extract, 0·25% (w/v) yeast extract and 1·5% (w/v) agar. The minimal medium (MM) was CM without the malt and yeast extracts. Diploids were plated on CM containing 0·007% (w/v) *p*-fluorophenylalanine to induce haploidization. Appropriately supplemented MM was used for the identification of nutritional requirements of haploid segregants. The medium for production of aflatoxin consisted of 2% (w/v) yeast extract and 20% (w/v) sucrose.

Aflatoxin and norsolorinic acid determinations. Aflatoxin and norsolorinic acid were extracted according to procedures described by Lillard *et al.* (1970) and Papa (1976). Extracts were suspended in chloroform, appropriately diluted, and spotted on thin-layer chromatographic plates (pre-coated silica gel) from EM Laboratories, Elmsford, N.Y., U.S.A. Chromatograms for aflatoxin and norsolorinic acid separations were developed in chloroform/acetone (88 : 12, v/v) and benzene/acetic acid (95 : 5 v/v), respectively. Initially, amounts of aflatoxin were quantified using a Turner model 111 fluorometer. Later, only visual estimates were made by observing the intensity of fluorescence of extracts on thin-layer plates. In some cases extracts were merely scored

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as aflatoxin positive or negative. The aflatoxin standard was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Norsolorinic acid could usually be detected in chloroform extracts; however, when total extraction was desired, acetone extraction procedures described by Bennett (1979) were followed. Extracts were usually scored as norsolorinic acid positive or negative. A standard was prepared from a norsolorinic acid-producing mutant of *A. parasiticus* (ATCC 24690) isolated by Bennett & Goldblatt (1973). The authenticity of norsolorinic acid from our *A. flavus* mutant was checked by comparing extracts with the standard on a DuPont 860 high-pressure liquid chromatograph. Several mutants impaired in aflatoxin production and not accumulating norsolorinic acid served as controls.

Genetic analysis. Techniques involved in forcing heterokaryons, recovering diploids, and subsequently haploidizing diploids were the same as those described by Papa (1976). For linkage detection, parasexual crosses were made between mutant 1-N and haploid strains 2, 3, 4, and 5. A total of 418 haploid segregants (117, 103, 115, and 83 from diploids involving strains 2, 3, 4, and 5, respectively) were recovered and the segregation of *nor* and other gene markers was determined.

Diploids homozygous for *nor*, resulting from mitotic recombination, were identified from the appearance of orange-red sectors on plates of heterozygous diploids. Sectors were selected and subcultured on CM until they were uniformly pigmented. Twenty diploids and 10–15 haploid segregants from each of 17 of the diploids were tested for nutritional requirements. Each diploid and all haploids auxotrophic for leucine were also tested for aflatoxin production. Based on the distribution of genes within the same linkage group, the sequence of the genes in relation to the centromere was determined.

RESULTS AND DISCUSSION

A mutant accumulating an orange-red pigment was recovered following NG treatment of conidia from a white-spored lysine auxotroph of *A. flavus*. By comparing extracts from this mutant to a standard, it was concluded that the accumulating pigment was norsolorinic acid. Identical R_F values were obtained for the orange-red pigments of this *nor* mutant and the standard in both chromatography systems (thin-layer and high-pressure liquid). The *nor* mutant accumulated approximately 50% less aflatoxin than the parental strain. A significant reduction in aflatoxin accumulation was also noted in a *nor* mutant of *A. parasiticus* (Lee *et al.*, 1971).

Diploids (1-N)/2, (1-N)/3, and (1-N)/5 formed between 1-N (*nor w lys*) and haploid strains 2, 3, and 5 were aflatoxin positive. The (1-N)/4 diploid, heterozygous for the dominant aflatoxin mutant *afl-1* (Papa, 1980), however, did not accumulate aflatoxin. None of the diploids accumulated norsolorinic acid, thus indicating that *nor* was recessive. Similarly, *nor-1* in *A. parasiticus* was recessive in diploids (Bennett, 1979).

Haploidization of diploids provided evidence for linkage of *nor* to *leu* and *afl-1* (Table 1). Linkage between *leu* and *afl-1* on linkage group VII was previously reported (Papa, 1979). No recombinants were obtained between *nor* and *leu* among 335 haploid segregants or between *nor* and *afl-1* among 115 segregants. The other gene markers (*w*, *arg*, *lys*, and *pdx*) segregated independently of *nor*. Mutant *nor* and its wild-type allele did not necessarily segregate in 1:1 ratio among the haploids recovered from each diploid; however, overall, the ratio (191:227) was not significantly different from 1:1 at the 0·05 level of probability. Leucine prototrophs apparently had a selective advantage among segregants from diploid (1-N)/4. Consequently, only 10/115 segregants were *nor*.

Twenty diploids homozygous for *nor* were recovered following mitotic recombination in diploid (1-N)/4. Each diploid was aflatoxin positive and prototrophic for leucine. Since *afl-1* is dominant over its wild-type allele, each *nor/nor* diploid was obviously also homozygous for the wild-type allele of *afl-1* (Table 2). Of 17 diploids haploidized, eight (Type A) yielded 118 prototrophs and nine (Type B) yielded 50 prototrophs and 47 auxotrophs. Type A diploids were apparently homozygous and type B diploids heterozygous for the leucine locus. All haploid segregants were *nor* and those auxotrophic for leucine were all aflatoxin positive. Leucine prototrophs were not tested for aflatoxin.

Table 1. *Linkage detection of norsolorinic acid mutant nor among haploid segregants from four diploids*

Diploid	No. of haploid segregants			Frequency of recombination with *nor* (%)					
	Total	*nor*	*nor*$^+$	*leu*	*lys*	*w*	*pdx*	*afl-1*	*arg*
(1-N)/2	117	81	36	0	64	50	54	–	–
(1-N)/3	103	42	61	0	22	31	–	–	–
(1-N)/4	115	10	105	0	12	70	–	0	–
(1-N)/5	83	58	25	–	40	31	–	–	31
Total	418	191	227						

Table 2. *Determination of gene order for nor, afl-1, and leu on linkage group VII*

Culture	No.	Genotype
Heterozygous diploid (1-N)/4	1	+ *nor* +/*afl-1* + *leu*
Recombinant diploids:		
Type A	8	+ *nor* +/+ *nor* +
Type B	9	+ *nor* +/+ *nor leu*
Haploid segregants from:		
Type A	118	+ *nor* +
Type B	50	+ *nor* +
	47	+ *nor leu*

Mitotic recombination provides a means for determining the sequence of genes on a chromosome (Pontecorvo & Kafer, 1958). Since heterozygous gene loci distal to both the centromere and the point of crossing-over can become homozygous, it was concluded that *afl-1* and *nor* were distal to *leu* on the same chromosome arm. Recombination occurred between *leu* and the centromere in eight diploids and distal to *leu* in nine diploids. Since crossing-over was not detected between *afl-1* and *nor* it was not certain which one was closest to *leu*. However, unless *afl-1* and *nor* are closely linked, the results of this study, based on the selection of *nor*/*nor* homozygotes, support the sequence *afl-1 nor leu* centromere.

Nevertheless, selection for *nor*/*nor* diploids may be misleading because of the relationship of *afl-1* and *nor* in the same biosynthetic pathway. Bennett *et al.* (1971) & Bennett (1981) demonstrated the concomitant production of aflatoxin and norsolorinic acid. If *afl-1* is epistatic to *nor*, the only detectable diploids homozygous for *nor* would be those not carrying the dominant *afl-1* allele. This could also account for the apparent lack of *afl-1 nor* haploid recombinants; *nor* could not be expressed in the presence of *afl-1*.

These results indicate that the gene controlling norsolorinic acid accumulation can be useful in mapping studies because the orange-red pigment is easily identified. Mapping aflatoxin genes, however, may present special difficulties.

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