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Six new loci controlling resistance to *p*-fluorophenylalanine in *Aspergillus nidulans*

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SUMMARY

Twelve FPA-resistant mutants were selected on medium containing *p*-fluorophenylalanine and ethionine. Dominance tests in heterozygous diploids showed that 8 out of 12 are dominant and 4 recessive to their wild-type alleles. One mutant, *fpa*60, showed a partial requirement for tyrosine and was found to be allelic to an *fpaA* mutant described previously. A tyrosine non-requirer, *fpa*65, was also assigned to this locus. The other 10 mutants did not show any growth requirement and were simultaneously resistant to ethionine and 3-amino-L-tyrosine. Of the 8 dominant mutants, 3 were allelic to the permease-mutants at the locus *fpaD*. Dominant mutants showed higher degrees of resistance than recessive ones. Six new loci, identified after preliminary genetic analysis, were located on 3 linkage groups: 3 on linkage group VI, and one each on linkage groups I, V, and VIII. The recombinant *fpaD*11;*fpaK*69 was found to be sensitive to FPA.

1. INTRODUCTION

A variety of structural analogues of some essential metabolites have been extensively and successfully used as metabolic probes (for reviews see Richmond, 1962, 1965; Umbarger, 1971). One of the important categories of such analogues comprises those of essential amino acids. The most striking effect of an amino acid analogue on biological systems is the inhibition of growth to various degrees. These inhibitory effects are brought about either by their incorporation into proteins (Cohen & Munier, 1959; Cowie *et al.* 1959; Horowitz *et al.* 1970; Brooks *et al.* 1972) or by their acting as 'false' feed-back inhibitors or repressors (Moyed, 1960; Previc & Binkley, 1964; Ezekiel, 1965).

A large number of mutants resistant to amino acid analogues have been reported in a variety of micro-organisms, but the mechanisms of resistance are known for only a few. Since such resistance mechanisms can be directly correlated with the various effects of analogues on general protein synthesis, and regulation of the biosynthetic pathway of their respective natural metabolites, genetic and biochemical studies on analogue-resistant mutants are an effective probe for understanding these.

One of the most exploited amino acid analogue is *p*-fluorophenylalanine (FPA), an analogue of phenylalanine. Barring a few reports for eukaryotes, detailed bio-

chemical and genetical studies on FPA-resistant mutants, as with other analogue-resistant mutants, are mainly confined to bacterial systems. FPA-resistant mutants of *Escherichia coli* (Cohen & Adelberg, 1958; Fangman & Neidhardt, 1964; Im & Pittard, 1971; Im, Davidson & Pittard, 1971), *Salmonella typhimurium* (Ames, 1964; Gollub & Sprinson, 1969), *Pseudomonas aeruginosa* (Dunn & Holloway, 1971), *Neurospora crassa* (Stadler, 1966; Jacobson & Metzenberg, 1967; Kinsey & Stadler, 1969; Wolfinbarger & DeBusk, 1971; Brooks *et al.* 1972), *Aspergillus nidulans* (Morpurgo, 1962; Warr & Roper, 1965; Sinha, 1967, 1969, 1970*a*, 1972), *Coprinus lagopus* (Barker & Lewis, 1970), and *Schizophyllum commune* (Hannan, 1972) have been reported.

FPA is very toxic to wild-type strains of *A. nidulans* (Sinha, 1967; Verma & Sinha, 1973), indicating its effects on general metabolism. This makes it a favourable system for a detailed biochemical and genetical experimentation. Previous genetic studies on FPA-resistant mutants of *A. nidulans* (Sinha, 1967) described four loci, namely *fpaA*, *fpaB*, *fpaD* and *fpaE*. However, taking into consideration the various effects and the consequent mechanisms by which resistance against FPA can be attained, a fresh search was made for other types of FPA-resistant mutants. The present study identifies six new loci, mutations at which confer resistance to *p*-fluorophenylalanine in *A. nidulans*.

2. MATERIALS AND METHODS

The general techniques and terminology employed were those described by Roper (1952), Pontecorvo *et al.* (1953), Pontecorvo & Käfer (1958), Sinha (1967), Clutterbuck (1970), and Verma & Sinha (1973).

Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was done according to the method standardized for *A. nidulans* by Chattoo & Sinha (1974). Production and replica-plating of microcolonies was done as described by Mackintosh & Pritchard (1963).

The use of 'master strain E' (MSE) for assigning genes to their respective linkage groups and for the test of translocation has been reported by McCully & Forbes (1965).

All the chemicals were of analytical grade. The strains used were made available to us from the Glasgow Stock. Other relevant strains were selected during the course of the work.

3. RESULTS

(i) *Probable number of FPA-resistant loci*

To determine the probable number of loci, the method described by Balassa (1969) for the quantitative survey of sporulation mutants of *Bacillus subtilis*, and subsequently adopted by Martinelli & Clutterbuck (1971) for conidiation mutants of *A. nidulans*, was used. The number was calculated by comparing the frequency of FPA-resistant mutants with the frequency of auxotrophic mutants. Dorn

(1967) has listed 61 auxotrophic loci. For the present studies this number was taken to be 70.

Conidia of a *riboA*1 *biA*1 strain were treated with NTG (Chattoo & Sinha, 1974) and the colonies obtained on CM from viable conidia, were replicated on suitable media by the method of Mackintosh & Pritchard (1963). The colonies were classified as auxotrophs and/or FPA-resistants by replicating them on MM + biotin + riboflavin and CM + FPA (0·0007 M), respectively. The poorly replicating or non-replicating colonies were not taken into consideration. This gave an approximate number of 28 loci, mutations at any one of which can result into resistance to FPA (Table 1).

Table 1. *Auxotrophs and FPA-resistant mutants obtained after NTG treatment of* riboA1 biA1 *conidia*

Expt no.	No. of colonies examined	Survival (%)	No. of auxotrophs obtained	No. of FPA resistants obtained	Calculated no. of loci for FPA resistance
I	3311	15	66	25	27
II	3648	9	75	32	30

(ii) *Selection of new FPA-resistant mutants*

Twelve FPA-resistant mutants were selected as fast-growing and conidiating sectors about a week after point inoculation of conidia from the clonal culture of a FPA-sensitive strain on CM supplemented with FPA (0·005 M) and ethionine (0·0025 M). Two amino acid analogues were used because most of the mutants selected in the presence of FPA alone are allelic to *fpaA* (Sinha, 1967) while mutants at the *fpaD* locus are resistant to FPA, ethionine and 1,3-aminotyrosine + phenylanthranilic acid (Sinha, 1969). G. T. Jones (personal communication) has found that ethionine-resistant mutants of *A. nidulans* are simultaneously resistant to FPA. The mutants were purified by single colony isolations, re-checked for their resistance to FPA and given isolation numbers 58–69 and the locus symbol *fpa*.

(iii) *Nutritional requirements and cross-resistance of new isolates*

Since some of the previously described mutants have requirements for tyrosine (TYR) or tryptophan (TRYP) (Sinha, 1967), the newly selected isolates were also tested for these requirements, so as to get a preliminary idea about their probable nature. Resistance of the newly selected mutants to DL-ethionine and 1,3-aminotyrosine was also tested. All the isolates except *fpa*60 showed no growth requirement and were found to be simultaneously resistant to DL-ethionine (0·0014 M) and 1,3-aminotyrosine (0·0007 M). The isolate *fpa*60 showed a requirement for TYR and inhibition by aminotyrosine, suggesting thereby that it could be allelic to mutants at the locus *fpaA*.

(iv) *Tests of dominance*

To characterize the mutants further, their dominance in heterozygous diploids was determined. Diploids with the following genotype were synthesized: *suA*1 *adE*20 *yA*2 *adE*20; *wA*3; *galA*1; *pyroA*4; *facA*303; *sB*3; *nicB*8; *riboB*2/*riboA*1 *biA*1; *fpaX*, where *X* = isolation numbers 58–69. A comparison of the growth rates of 12 heterozygous diploids on MM and MM + FPA (0·0007 M) showed that 7 were dominant, 1 semidominant and 4 recessive (Table 2).

Table 2. *Degrees of resistance and dominance testing of different FPA-resistant mutants*

Isolate	Colony diameters of haploids after 48 h on FPA (mm)		Growth of heterozygote (*fpaX*/+) on MM + 1400 mg/l FPA as compared with no FPA (%)	Dominant or recessive
	10 mg/l	100 mg/l		
*fpaA*7	24·5	18·33	17	Recessive
*fpaD*11	22·5	10·0	95	Dominant
*fpa*58	23·3	9·0	92	Dominant
*fpa*59	24·5	7·0	92	Dominant
*fpa*60	22·5	4·5	22	Recessive
*fpa*61	22·0	2·0	14	Recessive
*fpa*62	22·0	7·0	65	Semi-dominant
*fpa*63	23·3	9·0	93	Dominant
*fpa*64	25·5	18·33	95	Dominant
*fpa*65	25·0	19·0	27	Recessive
*fpa*66	24·5	14·0	100	Dominant
*fpa*67	23·0	13·0	98	Dominant
*fpa*68	24·0	6·0	21	Recessive
*fpa*69	25·0	15·0	100	Dominant

(v) *Degrees of resistance*

The mutants, when tested for their resistance to different concentrations of FPA, showed appreciably higher degrees of resistance than the wild type. Dominant mutants showed higher degrees of resistance than the recessive mutants (Table 2).

(vi) *Genetic characterization*

(*a*) *Linkage to riboA*1. Since by far the most common type of FPA-resistant mutants have been assigned to the locus *fpaA*, which is tightly linked (0·05 % recombination) to *riboA* (Sinha, 1967), the segregation of newly selected mutants with respect to *riboA* was analysed. Results of crosses of the type MSE × *riboA*1 *biA*1; *fpaX* (where *X* = isolation number), indicate that isolates *fpa*60 and *fpa*65 are closely linked to *riboA*, and therefore could be allelic to *fpaA*. All other isolates are either loosely linked or unlinked to *riboA*.

(*b*) *Complementation tests*. Recessive isolates *fpa*60, *fpa*61, *fpa*65 and *fpa*68 and mutants at previously identified loci *fpaA* and *fpaB* were tested for complementation in all possible combinations. Diploids synthesized between suitable strains

were tested for their growth on MM and MM + FPA (0·0007 M). Results (Table 3) indicate that *fpa*60 and *fpa*65 are allelic to *tyrA*, whereas isolates *fpa*68 and *fpa*61 represent mutations of hitherto unknown types. The isolates *fpa*61 and *fpa*68 were therefore given locus symbols *F* and *G*, respectively.

(c) *Crosses between dominant mutants.* Complementation tests cannot determine allelism between dominant mutants, which can only be inferred from their linkage relationships. The dominant mutants were therefore crossed with a mutant at the only dominant locus known so far, *fpaD*. Out of the eight mutants tested, *fpa*58, *fpa*59 and *fpa*63 were allelic to the mutant *fpaD*11. Isolates *fpa*62, *fpa*64 and *fpa*66 showed no linkage to *fpaD*11 and must represent mutations at new dominant

Table 3. *Complementation tests of recessive FPA-resistant loci*

Strains	*fpa*60	*fpa*61	*fpa*65	*fpa*68	*tyrA*8	*fpaB*37
*fpaB*37	+	+	+	+	+	−
*tyrA*8	−	+	−	+	−	
*fpa*68	+	+	+	−		
*fpa*65	−	+	−			
*fpa*61	+	−				
*fpa*60	−					

+, Complementing. −, Non-complementing.

locus/loci. The dominant mutants *fpa*62, *fpa*64 and *fpa*66 were crossed among themselves to find out whether they represent mutations at the same or different loci. Recovery of about 25 % FPA-sensitive colonies in all the crosses indicate that these are mutations at three different loci. The isolates *fpa*62, *fpa*64 and *fpa*66 were given the locus symbols *H*, *I* and *J* respectively. However, the segregation of FPA-resistance and FPA-sensitivity in crosses *fpa*67 × *fpaD*11 and *fpa*69 × *fpaD*11 was 121 : 81 and 88 : 110 respectively. Since these mutants behaved identically with respect to *fpaD*11, they were crossed together to find out their mutual relationship. Non-recovery of a single FPA-sensitive segregant out of 208 viable ascospores from a single hybrid perithecium analysed suggested their allelic nature. The isolates *fpa*67 and *fpa*69 probably represent mutations at the same locus, which was given the symbol *K*. In a normal case, involving two non-allelic FPA-resistant loci, only 25 % or less of the progeny should be sensitive to FPA. Recovery of about 50 % FPA-sensitive progeny in crosses involving *fpaD* and *fpaK* suggested that these loci are interacting and that the double mutant is sensitive to FPA. To test this, a few of the FPA-sensitive progeny from the cross *fpaD*11 × *fpaK*69 were outcrossed to a wild-type strain; recovery of about 50 % colonies which were resistant to FPA from one of three such crosses confirmed that *fpaD*11 *fpaK*69 double mutants are sensitive to FPA.

(d) *Formal genetics.* All the loci were assigned to linkage groups by mitotic haploidization of heterozygous diploids between the FPA-resistant strains and MSE. Loci *fpaF*, *fpaH* and *fpaJ* are in linkage group VI, whereas *fpaG*, *fpaI* and *fpaK* are in linkage groups V, I and VIII respectively. With the help of suitable

Table 4. *Genetic analysis of different* fpa *loci*

Serial no.	Strains involved	Loci considered	Genotypes of progeny: + +	+ −	− +	− −	Recombination (%)
1	*riboA*1 *biA*1; *fpaF*61 × MSE	*fpaF* × *sB*	21 (R)	Selected against	94 (P)	Selected against	18·3 ± 1·66
2	*riboA*1 *yA*2 *biA*1; *fpaF*61 × *lysA*1	*fpaF* × *lysA*	28 (R)		115 (P)		19·6 ± 1·59
3	*fpaG*68 × MSE	*fpaG* × *facA*	33 (R)		138 (P)		19·3 ± 1·44
4	*yA*2; *fpaG*68 *facA*303 *riboD*5 × *biA*1	*fpaG* × *riboD*	87 (P)	25 (R)	40 (R)	40 (P)	33·9 ± 2·41
		fpaG × *facA*	99 (P)	13 (R)	29 (R)	51 (P)	21·9 ± 1·54
5	*biA*1; *fpaH*62 × MSE	*fpaH* × *sB*	45 (R)	50 (P)	87 (P)	18 (R)	31·5 ± 2·19
6	*riboA*1 *yA*2; *fpaH*62 × *lysA*1	*fpaH* × *lysA*	11 (R)	Selected against	138 (P)	Selected against	7·4 ± 0·56
7	*riboA*1 *fpaI*64 *biA*1 × *galD*5 *adG*14 *pabaA*1 *yA*2	*fpaI* × *adG*	36 (R)	86 (P)	105 (P)	13 (R)	20·4 ± 1·28
		fpaI × *riboA*	113 (P)	9 (R)	19 (R)	99 (P)	11·7 ± 0·72
		adG × *riboA*	49 (R)	92 (P)	83 (P)	16 (R)	27·1 ± 1·71
8	*riboA*1 *biA*1; *fpaJ*66 × MSE	*fpaJ* × *sB*	17 (R)	Selected against	145 (P)	Selected against	10·5 ± 0·78
9	*riboA*1 *yA*2; *fpaJ*66 × *lysA*1	*fpaJ* × *lysA*	48 (R)		65 (P)		42·5 ± 3·95
10	*riboA*1 *biA*1; *fpaK*69 × *pabaA*1; *facB*101 *riboB*2 *palB*7 *chaA*	*fpaK* × *facB*	41 (R)		66 (P)		38·3 ± 3·65
		fpaK × *chaA*	39 (P)		17 (R)		30·4 ± 3·99
11	*riboA*1 *biA*1; *fpaK*69 × *pabaA*1; *wA*3; *ornB*7 *facB*101 *riboB*2	*fpaK* × *facB*	54 (R)		76 (P)		41·5 ± 3·59
		fpaK × *ornB*	73 (R)		99 (P)		42·4 ± 3·19

P, Parentals. R, Recombinants.

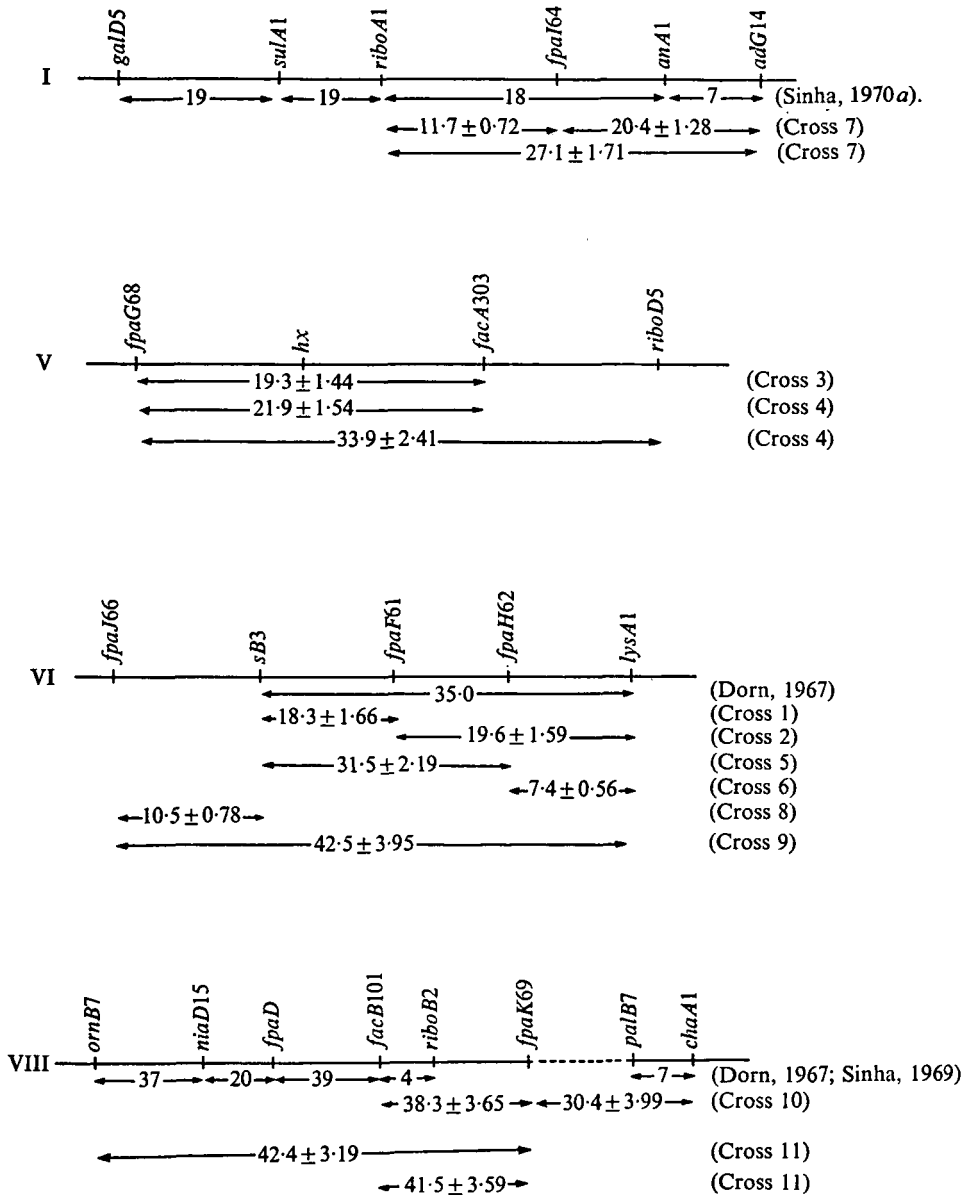

Fig. 1. Relevant parts of linkage groups I, V, VI, and VIII showing the locations of loci *fpaF*, *fpaG*, *fpaH*, *fpaI*, *fpaJ*, and *fpaK* with respect to previously known loci.

crosses these loci were mapped meiotically (Table 4, Fig. 1). No *ornB*7 *fpaK*69 recombinants were recovered in the last cross, perhaps because *fpaK* mutants are deficient in ornithine transport and also because ornithine requiring colonies have poor viability. *fpaD*11 *argB*2 recombinants too are very rare (Sinha, 1969). Isolate *fpaK*69 was found to be loosely linked to *chaA*1 and *facB*101. Its location was

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therefore confirmed by analysing the products of mitotic crossing over. A heterozygous diploid of the genetic make up

$$\left(\frac{riboA1}{+}\ \frac{+}{pabaA1}\ \frac{biA1}{+};\ \frac{+}{facB101}\ \frac{+}{riboB2}\ \frac{fpaK69}{+}\ \frac{+}{palB7}\ \frac{+}{chaA1}\right)$$

was allowed to grow on CM and conidia from two chartreuse-coloured heads were isolated and purified by single colony isolations. Both of them turned out to be *facB*101$^+$ *riboB*2$^+$ *fpaK*69 *chaA*1, confirming the location of *fpaK* distal to *riboB* and proximal to *chaA*.

4. DISCUSSION

With the identification of six new loci controlling resistance to *p*-fluorophenylalanine in *A. nidulans*, the total number of such loci identified so far becomes 10. The estimated number of 28 loci, mutations at any one of which can result into resistance to FPA, is perhaps an overestimate because the procedure employed selects all the FPA-resistant mutants but only a fraction of the auxotrophs. Of these ten loci, mutations at five (*fpaD*, *fpaH*, *fpaI*, *fpaJ* and *fpaK*) are dominant over their wild-type alleles. Dominant mutants for FPA-resistance could be preferentially selected by using two or more amino acid analogues in the medium. Mutants at the previously known dominant locus (*fpaD*) are deficient for a permease system which transports phenylalanine and other related amino acids (Sinha, 1969).

Wild-type strains of *A. nidulans* synthesize an active permease which concentrates FPA in the mycelium, and some of the FPA-resistant mutants are deficient or defective in this permease (Sinha, 1970*b*). In a heterozygous diploid, therefore, the amount of active permease will be less as compared to a FPA-sensitive homozygous diploid. Thus, the former will be comparatively more resistant to the analogue, i.e. the permease mutants will be dominant over the wild-type. FPA-resistant mutants which are permease-deficient are cross-resistant to a variety of analogues, perhaps because all these analogues are taken up by the same system.

FPA is not destroyed either in the mutants or in the wild type. It could well be that dominance in some of the FPA-resistant mutants results from the mutants being constitutive for a barrier between the conversion pool and the activation site. Bussey & Umbarger (1970) have put forward arguments for the existence of such a barrier in yeast.

Identification of five dominant loci for FPA-resistance further indicates that the aromatic amino acid permease in *A. nidulans* could be a polymeric protein. The fact that the mutants *fpaD* and *fpaK* individually are resistant to FPA but the recombinant *fpaD fpaK* is sensitive to this analogue supports this contention. Perhaps *fpaD* is defective for one protein and *fpaK* for another, but when the two individually defective proteins are brought together, the activity of enzyme is restored. Alternatively, both *fpaD* and *fpaK* are mutants in regulator genes. The structural gene for permease synthesis, however, is yet to be characterized. The second alternative seems more plausible, because Sinha (1970*b*) has presented evidences to suggest that *fpaD* is a mutant in a regulator gene.

The ten loci are distributed on five out of eight linkage groups, i.e. on linkage groups I, II, V, VI and VIII. There is no indication of clustering of similar loci. The dominant mutants have been found to be more resistant to FPA than recessive ones and may have a more effective resistance mechanism.

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