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Isolation and characterization of a new class of amino-acid-analogue-resistant mutants in *Aspergillus nidulans* using reduced carbon flow

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SUMMARY

Four recessive amino-acid-analogue-resistant mutants were isolated on a medium containing acetate as the sole carbon source and the amino acid analogues p-fluorophenylalanine and ethionine. None of the mutants showed any growth requirement. Analysis of growth on media containing an amino acid as the sole nitrogen source indicated that two mutants out of the four possess normal systems for utilization of acidic, neutral, basic and aromatic amino acids. The mutants *fpa*70 and *fpa*71 showed reduced growth on tryptophan as the sole source of nitrogen. Three new loci, identified after preliminary genetic analysis, were located on three linkage groups: one each on linkage groups I, VI and VIII.

1. INTRODUCTION

A number of structural analogues of essential metabolites have been successfully used for unravelling the intricacies of various metabolic processes (Richmond, 1962, 1966; Umbarger, 1971). Of these, the analogues of amino acids have been the most frequently utilized in procaryotes as well as in eucaryotes.

Amino-acid-analogue-resistant mutants have been reported in fungi, e.g. *Aspergillus nidulans* (Morpurgo, 1962; Warr & Roper, 1965; Sinha, 1967*a*, *b*, 1969, 1970, 1972; Srivastava & Sinha, 1975; Kinghorn & Pateman, 1975), *Neurospora crassa* (Stadler, 1966; Jacobson & Metzenberg, 1967; Brooks *et al.* 1972), *Saccharomyces cerevisiae* (Surdin *et al.* 1965), *Coprinus lagopus* (Barker & Lewis, 1970) and *Schizophyllum commune* (Hannan, 1972) and have yielded valuable information regarding the synthesis and regulation of various metabolites in these eucaryotes.

Auxanographic tests with a number of amino acid analogues (β-2 thienylalanine, 5-methyltryptophan, norvaline, homoserine, canavanine, ethionine and FPA) showed that *A. nidulans* is resistant to most of the analogues except p-fluorophenylalanine (FPA) and ethionine (Sinha, 1967*b*). FPA is the most potent inhibitor of growth in this fungus while the effect of ethionine is much less pronounced (Verma & Sinha, 1973).

A thorough understanding of the mode of action of FPA and the mechanism of resistance to this analogue in eucaryotes can be possible only if a large number of

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FPA-resistant mutants are isolated and characterized, genetically as well as biochemically. *A. nidulans* offers a simple and highly suitable eucaryotic system for such investigations.

Three different selection procedures, namely the selection of mutants in the presence of a single analogue, in the presence of two analogues, and in the presence of glutamate as sole nitrogen source, have resulted in the identification of 12 FPA-resistant loci in *A. nidulans*, each procedure facilitating the isolation and identification of new loci. Singh, Srivastava & Sinha (1977) observed that some of these mutants lose their resistance to FPA on a poor source of carbon or nitrogen. The synthesis of aromatic amino acids is limited during growth on a poor source of carbon (Calhoun & Jensen, 1972), which in turn leads to increased sensitivity of the organism to the analogue. Therefore a poor source of carbon in combination with the aromatic amino acid analogue(s) can be utilized as a selection system for the identification of hitherto unknown loci, mutations at which can lead to analogue resistance. We report the isolation and characterization of FPA-resistant mutants in *A. nidulans* at three hitherto unknown loci, using acetate as the only source of carbon in the presence of amino acid analogues.

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*a*), Clutterbuck (1974), Srivastava & Sinha (1975), Singh *et al.* (1977) and Singh & Sinha (1976, 1979).

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

All the chemicals were of analytical grade. DL-p-Fluorophenylalanine and DL-ethionine were obtained from Sigma Chemical Co., U.S.A. The strains used were originally obtained from the Glasgow Stock.

The acetate medium used was the same as the minimal medium except that 1 % glucose was replaced by 1 % sodium acetate.

3. RESULTS

(i) *Selection of FPA-resistant mutants*

Conidia of a FPA-sensitive strain (*riboA*1, *biA*1) were point-inoculated on an acetate medium containing FPA (700 μM), ethionine (3 mM) and the required growth factors. FPA-resistant mutants were selected as fast-growing and conidiating sectors after about a week of incubation. To avoid the isolation of clonal sectors, only one sector was picked up from each inoculum. Two amino acid analogues were used because most of the mutants selected in the presence of FPA alone are allelic to *fpaA* (Sinha, 1967*a*). The mutants were purified by single colony isolations and re-checked for their resistance to FPA. After final screening,

four FPA-resistant mutants were selected and given isolation numbers 70–73 and the locus symbol *fpa*. All the four newly isolated mutants were found to be simultaneously resistant to FPA and to ethionine and none showed growth requirement for either tyrosine or tryptophan.

(ii) *Degrees of resistance*

The degree of resistance of each mutant was tested to have an idea as to how efficient a particular metabolic block was in bringing about resistance to FPA. For this, each mutant was grown in the presence of eight different concentrations of the analogue in liquid shake cultures at 37 °C in a gyrotory shaker at 150 rev/min. Mycelia were filtered on pre-dried and weighed filter papers. Dry weight was measured after drying the filter papers and mycelia at 90 °C for 24 h. All the four isolates showed much higher degrees of resistance as compared to the wild type (Fig. 1). A concentration of 10 mg/l of FPA considerably reduced the growth of the sensitive strain, whereas the resistant mutants were practically unaffected. These became sensitive only to a FPA concentration higher than 1000 mg/l.

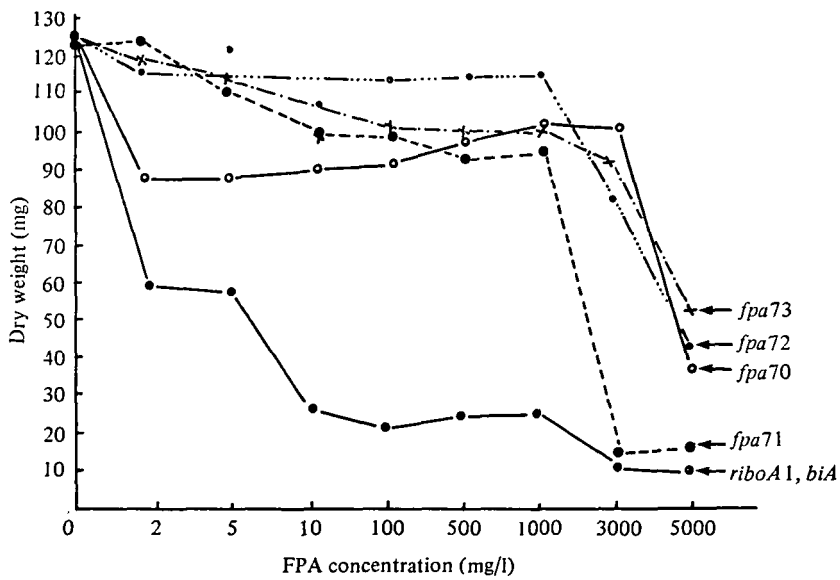

Fig. 1. Growth in 48 h in terms of dry weight of various strains of *Aspergillus nidulans* in the presence of different concentrations of p-fluorophenylalanine. Average of four replicates.

(iii) *Utilization of amino acids as sole nitrogen source*

Development of resistance to an amino acid analogue in *A. nidulans* often leads to impaired utilization of the corresponding natural amino acid when it is provided as the sole source of nitrogen, and this feature has been used as one of the criteria for biochemical characterization of amino acid analogue resistant mutants (Kinghorn & Pateman, 1975). Therefore, the growth of *fpa*70, *fpa*71, *fpa*72 and

Table 1. *Percentage growth of FPA-resistant mutants on different amino acids used as sole source of nitrogen (% dry weight* as compared to that of* riboA1, biA1) *after 48 h of growth in liquid shake cultures*

(Figures in parentheses represent the normalized values against growth on nitrate.)

	Amino acid used								
Mutant	Phe	Tyr	Tryp	Val	Arg	Meth	Hist	Sodium glutamate	Sodium nitrate
*fpaD*11	47·7 ± 4·9 (47·6)	36·8 ± 2·5 (36·7)	35·3 ± 6·4 (35·4)	148·0 ± 13·8 (147·8)	113·0 ± 10·9 (112·8)	53·1 ± 0·5 (53·0)	31·9 ± 2·2 (31·8)	81·0 ± 7·5 (80·9)	100·1 ± 4·0
*fpa*70	42·0 ± 2·7 (60·8)	94·5 ± 6·8 (134·0)	31·2 ± 1·5 (45·2)	89·5 ± 12·6 (129·7)	65·9 ± 4·1 (95·5)	104·4 ± 4·9 (151·3)	83·6 ± 0·7 (121·7)	84·8 ± 6·9 (122·8)	69·0 ± 4·1
*fpa*71	85·0 ± 2·7 (79·9)	92·5 ± 10·0 (87·0)	50·0 ± 1·9 (47·0)	137·6 ± 5·3 (129·4)	107·9 ± 5·5 (101·5)	113·7 ± 2·6 (106·9)	121·2 ± 5·0 (114·0)	131·3 ± 3·0 (123·5)	106·3 ± 4·0
*fpa*72	94·6 ± 2·3 (92·7)	96·8 ± 5·5 (94·9)	77·7 ± 7·9 (76·1)	99·0 ± 15·2 (97·0)	60·8 ± 1·9 (59·7)	68·9 ± 9·7 (67·5)	53·9 ± 8·8 (52·8)	105·1 ± 1·6 (103·8)	102·0 ± 2·7
*fpa*73	78·1 ± 2·2 (84·3)	98·9 ± 3·5 (106·4)	77·0 ± 7·9 (83·1)	88·5 ± 12·7 (95·5)	60·9 ± 8·7 (65·7)	123·6 ± 6·2 (132·8)	154·1 ± 11·1 (166·4)	78·9 ± 1·0 (85·2)	92·6 ± 4·9

* Average of four readings.

*fpa*73 was measured using different amino acids belonging to aromatic, neutral, basic and acidic categories. About 1×10^8 conidia of each strain were separately inoculated in 50 ml of nitrateless media containing the required growth factors and the amino acid to be tried, in 250 ml flasks. Incubation was carried out at 37 °C in a gyratory shaker at 150 rev/min. The mycelia were filtered on pre-dried and weighed Whatman No. 1 filter papers. The dry weight was taken after keeping the filter papers with the mycelia at 90 °C for 24 h. The strain *riboA*1, *biA*1 (normal uptake) and *riboA*1, *biA*1, *fpaD*11 (uptake defective) were kept as controls. Percentage dry weight of each mutant with reference to that of *riboA*1, *biA*1 was calculated for each nitrogen source (Table 1). Less than 50% growth of a mutant was taken as non-utilization of the amino acid concerned (Kinghorn & Pateman, 1975). For a better comparison, the extent of growth of the mutants was normalized by calculating percentage growth on different amino acids as compared to that on sodium nitrate (Table 1).

The isolates *fpa*70 and *fpa*71 showed less than 50% growth on tryptophan, indicating poor utilization of tryptophan as the sole source of nitrogen by these mutants. Mutants *fpaM*72 and *fpaN*73 could utilize all types of amino acids for their optimal growth. It seems that the four newly isolated mutants possess normal uptake systems for phenylalanine and methionine, the natural counterparts of the amino acid analogues (FPA and ethionine) used in the present studies.

(iv) *Genetic characterization*

(*a*) *Dominance tests.* The mutants were further characterized by determining their dominance in heterozygous diploids. Diploids of the following genotypes were synthesized: *suA*1, *adE*20, *yA*2, *adE*20; *galA*1; *pyroA*4; *facA*303; *sB*3; *nicB*8; *riboB*2/*riboA*1, *biA*1; *fpa*X, where X = isolation numbers 70–73. A comparison of the growth rates of heterozygous diploids on minimal medium (MM) and MM + FPA (700 μM) showed that all the newly isolated mutants were recessive to their wild-type alleles. Diploids *fpaA*7/+ (recessive) and *fpaD*11/+ (dominant) served as controls.

(*b*) *Complementation analysis.* Strains containing the recessive alleles *fpa*70, *fpa*71, *fpa*72, *fpa*73 and the previously known recessive alleles *fpaA*7, *fpaB*37, *fpaF*61 and *fpaG*68 were tested for complementation in all possible combinations. Heterozygous diploids were synthesized between suitable strains and were tested for growth by point-inoculating on MM and MM + FPA (700 μM). Colony diameters were measured after 48 h of growth at 37 °C. A complementing diploid showed colony diameters between 19·0 and 25·0 mm, while a non-complementing one did not grow more than 2·5 mm in diameter. Results (Table 2) indicate that isolates *fpa*70 and *fpa*71 are allelic to each other but non-allelic to the rest of the mutants. Hence, these two isolates represent mutations at a new FPA-resistant locus, henceforth assigned the locus symbol *L*. The isolates *fpa*72 and *fpa*73 represent mutations at two different and hitherto unknown loci and were given the locus symbols *M* and *N*, respectively.

Table 2. *Complementation analysis of recessive FPA-resistant loci*

Strains	*fpaA*7	*fpaB*37	*fpaE*48	*fpaF*61	*fpaG*68	*fpa*70	*fpa*71	*fpa*72	*fpa*73
*fpaA*7	−	.	.	.	.	.	.	.	.
*fpaB*37	+	−	.	.	.	.	.	.	.
*fpaE*48	+	+	−	.	.	.	.	.	.
*fpaF*61	+	+	+	−	.	.	.	.	.
*fpaG*68	+	+	+	+	−	.	.	.	.
*fpa*70	+	+	+	+	+	−	.	.	.
*fpa*71	+	+	+	+	+	−	−	.	.
*fpa*72	+	+	+	+	+	+	+	−	.
*fpa*73	+	+	+	+	+	+	+	+	−

+, Complementing. −, Non-complementing.

(*c*) *Formal genetics*. All the newly identified loci were assigned to linkage groups by mitotic haploidization of heterozygous diploids synthesized between the FPA-resistant strains and MSE, using chloral hydrate as the haploidizing agent (Singh & Sinha, 1976, 1979). Loci *fpaL*, *fpaM* and *fpaN* are on linkage groups VI, I and VIII, respectively. The assignment of these loci to their respective linkage groups was further confirmed using FPA as the haploidizing agent. Meiotic mapping of these newly identified loci was carried out with the help of suitable crosses (Table 3, Fig. 2). The locus *fpaL* showed free recombination with *methB*3 (Table 3). The paucity of suitable markers and multiply marked strains for linkage group VI is a hindrance in mapping new markers. As no colour marker has yet been identified on linkage group VI, mitotic mapping of the locus *fpaL* could not be carried out.

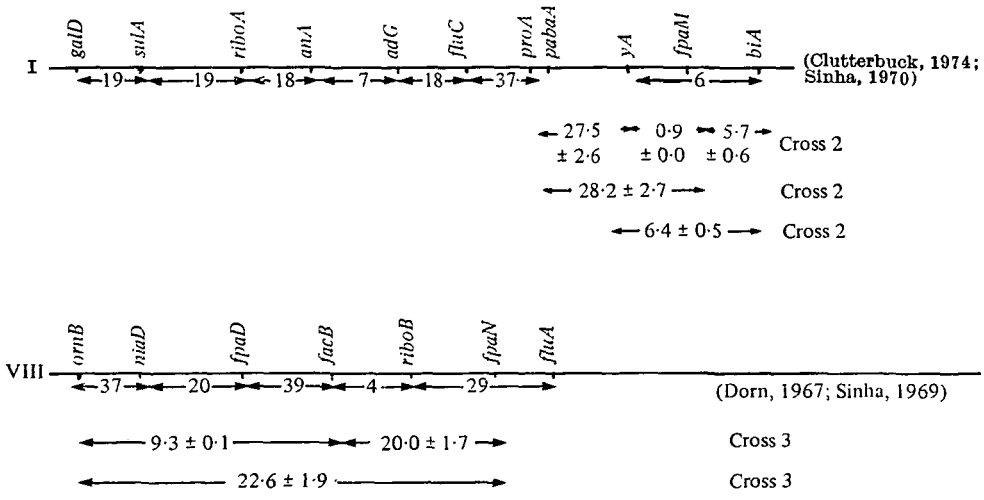

Fig. 2. Relevant parts of linkage groups I and VIII showing the locations of loci *fpaM* and *fpaN* with respect to previously known loci.

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Table 3. *Genetic analysis of different* fpa *loci*

Serial no.	Strains involved	Loci considered	Genotypes of progeny* ++	+−	−+	−−	Recombination (%)
1	*riboA*1, *biA*1; *fpaL*71 × *yA*2; *pyroA*4; *methB*3	*fpaL* × *methB*	99 (R)	S.A.	50 (P)	S.A.	60·4 ± 4·9
2	*riboA*1, *biA*1, *fpaM*72 × *galD*5, *adG*14, *pabaA*1, *yA*2	*fpaM* × *biA*	97 (P)	6 (R)	1 (R)	0 (P)	5·7 ± 0·5
		fpaM × *yA*	S.A.	106 (P)	S.A.	1 (R)	0·9 ± 0·0
		fpaM × *pabaA*	28 (R)	78 (P)	0 (P)	1 (R)	28·2 ± 2·7
		biA × *yA*	S.A.	101 (P)	S.A.	6 (R)	6·4 ± 0·6
		pabaA × *adG*	23 (P)	5 (R)	53 (R)	26 (P)	48·6 ± 4·6
		pabaA × *yA*	S.A.	28 (R)	S.A.	79 (P)	27·3 ± 2·6
3	*riboA*1, *biA*1; *fpaN*73 × *pabaA*1; *wA*3; *ornB*7, *facB*101, *riboB*2, *galC*7	*fpaN* × *ornB*	26 (R)	103 (P)	S.A.	S.A.	22·6 ± 1·9
		fpaN × *facB*	28 (R)	101 (P)	S.A.	S.A.	20·0 ± 1·7
		fpaN × *wA*	53 (R)	76 (P)	S.A.	S.A.	40·1 ± 3·5
		ornB × *facB*	53 (P)	11 (R)	9 (R)	109 (P)	9·3 ± 0·1

* S.A., Selected against. P, Parentals. R, Recombinants.

4. DISCUSSION

With the addition of three new loci controlling resistance to p-fluorophenylalanine in *A. nidulans*, the total number of such loci identified so far becomes 15 (Table 4). Mutations at seven loci are dominant over their wild-type alleles whereas eight are recessive. The predominance of dominant loci noted in earlier studies (Sinha, 1969; Srivastava & Sinha, 1975) was most probably due to the selection process involving more than one amino acid analogue in the medium containing glucose as the sole carbon source.

The present selection procedure using acetate instead of glucose as the sole source of carbon seems to be quite efficient in identifying new FPA-resistant loci, as out of the four FPA-resistant isolates studied, three new loci could be identified.

A comparison of the degree of resistance of various FPA-resistant mutants to FPA on acetate had shown that except *fpaD*11, *fpaK*69 and *fpaA*7, the previously isolated mutants became sensitive to FPA (Singh *et al.* 1977). The newly isolated mutants maintain high degrees of resistance to FPA on acetate, indicating their dissimilarity to those mutants which lose their resistance under similar conditions. Mutants *fpaL*71, *fpaM*72 and *fpaN*73, unlike *fpaA*7 and *fpaE*, are prototrophic for tyrosine or tryptophan requirement. The newly isolated mutants also differ from *fpaD*11 and *fpaK*69 as the former are recessive, while the latter are dominant. These observations indicate that the mutants *fpaL*71, *fpaM*72, and *fpaN*73 are in fact different from the previously isolated mutants.

The high degree of FPA resistance in *fpaL*71, *fpaM*72 and *fpaN*73, which are all recessive, is contrary to the earlier belief that dominant mutants, in general, are more resistant to FPA than the recessive ones (Srivastava & Sinha, 1975; Singh *et al.* 1977). It seems that there is no correlation between the degree of resistance

and dominance of a particular mutant. Amino acid utilization studies showed that *fpaL*70 and *fpaL*71 were unable to utilize tryptophan as the sole source of nitrogen. The similar nature of these two mutants was further supported by genetic complementation where these isolates were found to be allelic to each other. On the other hand, mutants *fpaM*72 and *fpaN*73 could grow normally on all the amino acids tried, indicating that they possess normal systems for the utilization of these amino acids.

The fifteen FPA-resistant loci are distributed on five out of eight linkage groups (Table 4). There is no clustering among any of the loci isolated so far. As the total number of FPA-resistant loci has been estimated to be 28 (Srivastava & Sinha, 1975), more than 50 % of these have already been identified in *A. nidulans*.

Table 4. *FPA-resistant loci in* Aspergillus nidulans

S. no.	Locus symbol	Selected on	Phenotype	Dominant/ recessive	Linkage group	Reference
1	fpaA	CM+FPA	FPA resistant	Recessive	I	Morpurgo, 1962; Sinha, 1967 (*a*)
2	fpaB	CM+FPA	FPA resistant	Recessive	I	Sinha, 1970
3	fpaD	CM+FPA	FPA resistant	Dominant	VIII	Sinha, 1969
4	fpaE	CM+FPA	FPA resistant	Recessive	II	Sinha, 1967 (*a*)
5	fpaF	CM+FPA +ethionine	FPA and ethionine resistant	Recessive	VI	Srivastava & Sinha, 1975
6	fpaG	CM+FPA +ethionine	FPA and ethionine resistant	Recessive	V	Srivastava & Sinha, 1975
7	fpaH	CM+FPA +ethionine	FPA and ethionine resistant	Dominant	VI	Srivastava & Sinha, 1975
8	fpaI	CM+FPA +ethionine	FPA and ethionine resistant	Dominant	I	Srivastava & Sinha, 1975
9	fpaJ	CM+FPA +ethionine	FPA and ethionine resistant	Dominant	VI	Srivastava & Sinha, 1975
10	fpaK	CM+FPA +ethionine	FPA and ethionine resistant	Dominant	VIII	Srivastava & Sinha, 1975
11	aauC	Glutamate	Glutamate non-utilizer	Dominant	II	Kinghorn & Pateman, 1975
12	aauD	Glutamate	Glutamate non-utilizer	Dominant	VIII	Kinghorn & Pateman, 1975
13	fpaL	Acetate+FPA +ethionine	FPA and ethionine resistant	Recessive	VI	Present study
14	fpaM	Acetate+FPA +ethionine	FPA and ethionine resistant	Recessive	I	Present study
15	fpaN	Acetate+FPA +ethionine	FPA and ethionine resistant	Recessive	VIII	Present study

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