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Genetic Control of Amino Acid Transport in *Aspergillus nidulans*: Evidence for Polymeric Amino Acid Permease

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Abstract. On a medium containing either acetate as the sole source of carbon or arginine as the sole source of nitrogen and the two amino acid analogs, *p*-fluorophenylalanine (FPA) and ethionine, eight FPA-resistant mutants were selected. Dominance tests in heterozygous diploids showed that 3 out of 8 are recessive, 1 semidominant, and 4 dominant to their wild-type alleles. Mutants were characterized by the nature of amino acid transport detected on the basis of amino acid utilization patterns. Six new loci identified after genetic analysis were located on two linkage groups: three each on linkage groups I and II. Recombinants between pairs of loci *fpa*D and *fpa*Q, and *fpa*K and *fpa*P, were found to be sensitive to FPA. The pattern of segregation of resistant markers and amino acid utilization were considered to characterize the specificity of transport mutants.

Structural analogs of some essential metabolites have been successfully used as metabolic probes [14, 26, 27]. These analogs are taken into the cell by the same mechanism and route as their natural counterparts [20]. Selection of mutants resistant to inhibitory amino acid analogs has thus been widely used as a means of obtaining mutants deficient in specific as well as general permeases [5, 6, 19, 21–24, 28]. Genetic characterization of FPA-resistant mutants isolated in *Aspergillus nidulans* have led to the identification of 16 loci, scattered over five out of eight linkage groups [25]. Mutations at only four out of 16 loci have been reported to affect the uptake of aromatic amino acids [7, 9, 20, 21]. On the basis of reports that biosynthesis of aromatic amino acids becomes limited on a poor source of carbon [3], and that aromatic amino acid permease becomes derepressed under conditions of reduced carbon and/or nitrogen flow [18], leading to increased sensitivity of analog-resistant transport mutants to FPA, a method for preferential selection of amino acid transport mutants was developed. The present article deals with the isolation, genetic characterization, and growth pattern of six new FPA-resistant mutants of *A. nidulans*, of which three are concerned with the transport of amino acids.

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Materials and Methods

The auxotrophic strains of *Aspergillus nidulans* used in the present investigation were obtained from Glasgow Stock through the courtesy of A. J. Clutterbuck (Department of Genetics, Glasgow University, Glasgow, Scotland). Genetic and biochemical characteristics of FPA-resistant strains used for characterization of newly isolated mutants have been described elsewhere [25]. Locus symbol and phenotype of genetic markers used in this study have been described by Clutterbuck [4]. Cultures were maintained on complete medium (CM) slants and were routinely purified and subcultured.

Minimal medium (MM) was a Czapek-Dox medium supplemented with 1% (wt/vol) glucose (pH 6.5). CM was a complex medium (pH 6.2) containing 1 g/liter yeast extract, 2 g/liter peptone, 1.5 g/liter casamino acid, and vitamin mixture (riboflavin, biotin, thiamine–HCl, pyridoxin–HCl, nicotinamide, and para-aminobenzoic acid). Acetate medium and arginine medium used for the isolation of mutants were essentially the same as MM, except that the glucose and sodium nitrate of MM were replaced by sodium acetate (1% wt/vol) and arginine (12.5 m*M*), respectively. Any of the media described above was prepared as liquid medium by excluding agar agar (1.5% wt/vol). DL-para-Fluorophenylalanine (FPA) and DL-ethionine (Sigma) were used at final concentrations of 0.7 m*M* and 3 m*M*, respectively. Two amino acid analogs were used simultaneously to avoid the selection of partial tyrosine-requirer mutants [21].

Amino acids used either for screening of amino acid transport mutants or for studying their growth patterns were filter sterilized and added to nitrateless MM at a final concentration of 10 m*M* [7]. The pH of the media was adjusted to 6.5–6.7. Thir-

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Table 1. Preliminary screening and dominance tests of different FPA-resistant mutants

Isolation numbers	Growth on amino acids[a]				Growth of heterozygote (fpa-x/+) on MM + FPA(%) MM (100%)	Dominant or recessive
	Aromatic	Acidic	Neutral	Basic		
fpa-74	+	−	−	−	21	Recessive
fpa-75	+	−	−	−	21	Recessive
fpa-76	+	+	+	+	96	Dominant
fpa-77	+	+	+	+	23	Recessive
fpa-78	+	+	+	+	92	Dominant
fpa-79	−	−	−	−	54	Semidominant
fpa-80	−	−	−	+	100	Dominant
fpa-81	+	+	+	±	96	Dominant
*fpa*D11	−	±	±	±	100	Dominant
*fpa*M72	+	+	+	+	25	Recessive

[a]+, growth; ±, 50% growth; and −, no growth.

teen amino acids used in the present investigation were grouped as follows: aromatic (phenylalanine, PHE; tyrosine, TYR; tryptophan, TRY), acidic (aspartic acid, ASP; glutamic acid, GLU), neutral (alanine, ALA; serine, SER; leucine, LEU; valine, VAL), and basic (glutamine, GLN; asparagine, ASN; ornithine, ORN; arginine, ARG).

All the chemicals were of analytical grade and obtained from commercial sources (E. Merk, Loba Chemie, and Difco Laboratories).

Liquid cultures were raised in 250-ml flasks containing 50 ml of medium. Flasks were shaken incubated at 37°C at 150 rpm on a gyratory shaker. Conidial suspension was prepared in normal saline containing 0.01% (vol/vol) Tween-80 as detergent, and the mixture was thoroughly shaken on a vortex mixer to break the chains of conidia. The number of conidia ml^{-1} were counted with the help of a hemocytometer. Usually 0.05–0.1 ml of the desired dilution was spread over the solid medium plate with a sterile glass spreader.

Genetic techniques employed for characterization of mutants were the same as originally described by Pontecorvo et al. [10]. Heterokaryon was established between desired strains, first by inoculating conidia in liquid CM and then by transferring the resulting mycelial mat on MM. For selection of heterozygous diploids, a conidial suspension prepared from the "balanced heterokaryon" was mixed with cooled molten MM (40°C) and poured in sterile plates. Colonies appearing on MM plates were analyzed for diploidy on the basis of conidial size, the size of diploid conidia being double the size of either of those of the parent strains.

Mitotic assignment of mutants to their respective linkage groups was done by analyzing the haploid segregants obtained after haploidization of heterozygous diploids synthesized between each of the mutant strains and Master strain "G" (MSG = *gal*A1; *pyro*A4; *fac*A303; *s*B3; *nic*B8; *ribo*B2) [8], with chloral hydrate (0.02 *M*) as the haploidizing agent [16]. During the process of mitotic haploidization, markers of different linkage groups show free recombination, whereas those of the same linkage group show no recombination. Meiotic mapping of an assigned locus was carried out by analyzing progeny obtained from ascospores of a single hybrid cleistothecia. Colonies, arranged on master plates, were replicated on different sets of media with the help of a multipin replicator to determine their genotype. In some cases, the ascospores were plated on selective medium so as to allow the growth of colonies of only desired genotypes for further analysis.

Results

Isolation of FPA-resistant amino acid transport mutants. Conidia of the wild-type (FPA-sensitive) strain were point-inoculated separately on acetate and arginine media supplemented with required growth factors, FPA, and ethionine. After 7 days of incubation, only one of the many fast-growing and well-conidiating sectors from each inoculum was picked up to avoid the isolation of clonal sectors. Mutants thus isolated were purified by single-colony isolation and again tested for their resistance to FPA and ethionine. Altogether 86 isolates, 71 from the sodium acetate medium and 15 from arginine medium, were selected.

A preliminary screening for the amino acid transport mutants was carried out on the basis of their growth pattern on 13 different amino acids supplied exogenously as sole sources of nitrogen. Tests were made on solid medium plates as well as in liquid cultures. On the basis of the observations, isolates were classified under four broad categories. Finally, eight mutants representing the four classes were given the isolation numbers *fpa*-74 to *fpa*-81 (Table 1). Except for *fpa*-81, which was selected on an arginine medium, the other seven were from media containing acetate as the sole source of carbon.

Tests of dominance. Mutants were characterized for their dominance or recessivity in heterozygous diploids by comparison of the growth of diploids of

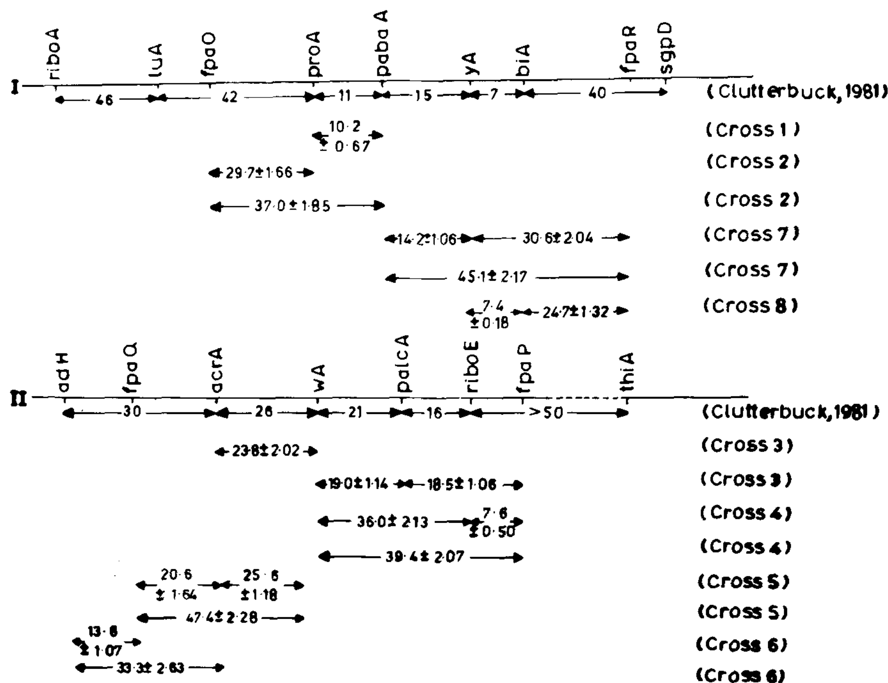

Fig. 1. Relevant parts of the genetic map of linkage groups I and II of *Aspergillus nidulans*, showing location of new *fpa* loci.

genetic constitution *fpa*-x/+ (where x = isolation number 74–81) on MM and MM + FPA (0.0007 *M*). As shown in Table 1, three mutants (*fpa*-74, *fpa*-75, *fpa*-77) were recessive, one (*fpa*-79) was semi-dominant, and four (*fpa*-76, *fpa*-78, *fpa*-80, *fpa*-81) were dominant. Heterozygous diploids *fpa*M72/+ and *fpa*D11/+ served as recessive and dominant controls, respectively.

Degrees of resistance. Since resistance to an antimetabolite may develop owing to a block in the metabolic pathway, either at the level of utilization or uptake or biosynthesis of the natural metabolite, the degrees of resistance of mutants were tested under liquid shake culture condition in the presence of seven different concentrations (5, 10, 100, 500, 1000, 1500, and 2000 mg/liter). Growth in terms of dry weights indicated that dominant mutants possessed a higher degrees of resistance than recessive ones and could be arranged in the order *fpa*-80 > *fpa*-79 > *fpa*-76 > *fpa*-74 = *fpa*-78 > *fpa*-81 > *fpa*-77 > *fpa*-75.

Complementation analysis. Recessive isolates and previously known recessive mutants were tested for complementation in all possible paired combinations; *fpa*-74 and *fpa*-75 were found allelic to each other but nonallelic to the rest of the mutants and represented mutation at a hitherto unknown locus, which was assigned the symbol *fpa*O. The isolate *fpa*-77 represented a mutation at another unknown locus, to which the symbol *fpa*P was assigned.

Crosses between dominant mutants. Allelism between dominant mutants was tested by crossing them in paired combinations, the result of which showed that the semidominant mutant *fpa*-79 and dominant mutant *fpa*-80 were allelic because not a single *fpa*-sensitive recombinant could be obtained out of 210 segregants analyzed. These two mutants were assigned to a single locus *fpa*Q. Other dominant isolates *fpa*-76, *fpa*-78, and *fpa*-81 did not show any linkage among themselves and, therefore, led to the identification of three new loci, which were assigned the symbols *fpa*R, *fpa*S, and *fpa*T, respectively.

Formal genetics. Loci *fpa*O, *fpa*R, and *fpa*T were mitotically assigned to linkage group I, and *fpa*P, *fpa*Q, and *fpa*S to linkage group II by haploidization of heterozygous diploids synthesized between each of the mutant strains and MSG on media containing chloral hydrate. Meiotic mapping of only four loci (*fpa*O, *fpa*P, *fpa*Q, and *fpa*R) was done with the help of suitable crosses (Fig. 1). It is evident from the data of different crosses (Table 2) that there was some interaction between the locus *fpa*O and leucine requirement of the auxotroph. Only three

Table 2. Genetic analysis of different *fpa* loci of *Aspergillus nidulans*

Sl no.	Strains involved in cross	Loci considered	Genotypes of progeny[a] ++	+−	−+	−−	Recombination (%)
1.	*proA1, pabaA1, yA1, fpaO74* × *MSG*	*proA* --- *pabaA*	S.A.	S.A.	16(R)	140(P)	10.2 ± 0.67
2.	*proA1, pabaA1, yA1, fpaO74* × *luA1, biA1; phenA3*	*fpaO* --- *proA*	222(P)	94(R)	S.A.	S.A.	29.7 ± 1.66
		fpaO --- *pabaA*	199(P)	117(R)	S.A.	S.A.	37.0 ± 1.85
		luA --- *fpaO*	269(R)	67(P)	47(P)	03(R)	>50
3.	*proA1, pabaA1, yA1, fpaP77* × *biA1; acrA1, wA3, palcA1*	*acrA* --- *wA*	99(P)	31(R)	S.A.	S.A.	23.8 ± 2.02
		wA --- *palcA*	98(P)	23(R)	S.A.	S.A.	19.0 ± 1.14
		fpaP --- *palcA*	26(R)	114(P)	S.A.	S.A.	18.5 ± 1.06
4.	*yA1; fpaP77* × *biA1; wA3, riboE6*	*fpaP* --- *riboE*	09(R)	110(P)	S.A.	S.A.	7.6 ± 0.50
		fpaP --- *wA*	47(R)	72(P)	S.A.	S.A.	39.4 ± 2.07
		wA --- *riboE*	55(P)	31(R)	S.A.	S.A.	36.0 ± 2.13
5.	*proA1, pabaA1, yA1; fpaQ79* × *biA1; acrA1, wA3, thiA4, cn* × *E16, adD3*	*fpaQ* --- *acrA*	S.A.	S.A.	77(P)	20(R)	20.6 ± 1.64
		acrA --- *wA*	S.A.	S.A.	22(R)	64(P)	25.6 ± 1.18
		fpaQ --- *wA*	S.A.	S.A.	51(P)	46(R)	47.4 ± 2.28
6.	*pabaA1, yA1; fpaQ79* × *biA1; adH23, acrA1, wA3, nicB8*	*fpaQ* --- *adH*	S.A.	S.A.	81(P)	13(R)	13.8 ± 1.07
		adH --- *acrA*	68(P)	34(R)	S.A.	S.A.	33.3 ± 2.63
7.	*proA1, pabaA1, yA1, fpaR76* × *MSG*	*pabaA* --- *yA*	114(P)	19(R)	S.A.	S.A.	14.2 ± 1.06
		yA --- *fpaR*	86(P)	38(R)	S.A.	S.A.	30.6 ± 2.04
		pabaA --- *fpaR*	68(P)	56(R)	S.A.	S.A.	45.1 ± 2.17
8.	*proA1, yA1, fpaR76* × *galD5, anA1, luA1, biA*1	*fpaR* --- *biA*	S.A.	S.A.	79(P)	26(R)	24.7 ± 1.32
		yA --- *biA*	S.A.	S.A.	99(P)	08(R)	7.4 ± 0.18

[a] P, parentals; R, recombinants; and S.A., selected against.

Table 3. Crosses showing genetic interaction between loci *fpa*D & *fpa*Q and *fpa*K & *fpa*P

Sl no.	Strains involved in the cross[a]	Pairs of loci considered	Number of progeny	Segregation of markers: FPA resistant	FPA sensitive	Yellow	Green
1.	*riboA1, biA1; fpaD11* × *proA1, pabaA1, yA1; fpaQ79*	*fpaD11-fpaQ79*	355	186	169	184	171
2.	*riboA1, biA1; fpaD11* × *proA1, pabaA1, yA1; fpaQ80*	*fpaD11-fpaQ80*	198	97	101	98	100
3.	*riboA1, biA1; fpaK69* × *proA1, pabaA1, yA1; fpaP77*	*fpaK69-fpaP77*	271	130	141	139	132
4.	*riboA1, biA1* × *pabaA1, yA1; fpa*⁺ *(fpaD11; fpaQ79)*	—	201	132	69	95	106
5.	*pabaA1, yA1* × *biA1; fpa*⁺ *(fpaD11; fpaQ80)*	—	238	126	112	118	120
6.	*riboA1, biA1* × *proA1, yA1; fpa*⁺ *(fpaK69; fpaP77)*	—	209	73	136	100	109

[a] Markers in parentheses represent FPA-sensitive recombinants.

small and poorly growing *lu*A1, *fpa*074 recombinants were obtained out of 386 colonies analyzed, even after 72 h of incubation. Loci *fpa*S and *fpa*T could not be mapped meiotically because of nonrecovery of hybrid perithecia.

Genetic interaction between transport mutants. Interaction studies of six newly identified loci with two previously known aromatic amino acid transport defective/deficient loci *fpa*D and *fpa*K were carried out to have an idea of the nature of aromatic permease(s) in *Aspergillus nidulans*. Analyses of various crosses (Table 3) reveal that the locus *fpa*Q was interacting with the locus *fpa*D, and the locus *fpa*P was interacting with another permease-defective locus *fpa*K by yielding about 50% FPA-sensitive progeny. Such interaction could have arisen due to sensitivity of the double mutant classes (i.e., *fpa*D11; *fpa*Q79, *fpa*D11; *fpa*Q80 and *fpa*K69; *fpa*P77) to FPA. To test the validity of assumption,

Table 4. Growth of FPA-sensitive recombinants and FPA-resistant segregants on 13 amino acids as sole sources of nitrogen[a]

Mutants	Amino acids used as nitrogen source												
	PHE	TYR	TRY	ASP	GLU	SER	LEU	VAL	ALA	ASN	GLN	ORN	ARG
fpaD11; fpaQ79	+	+	+	±	±	±	+	+	+	±	±	±	±
fpaD11; fpaQ80	+	+	+	±	±	±	+	+	+	+	+	+	+
fpaK69; fpaP77	+	+	+	+	+	+	+	+	+	+	+	+	+
fpaD11	−	−	−	±	±	±	±	±	±	±	±	±	±
fpaQ79	−	−	−	−	−	−	−	−	−	−	−	−	−
fpaQ80	−	−	−	−	−	−	−	−	−	+	+	+	+
fpaK69	−	−	±	±	±	+	+	+	+	+	+	+	+
fpaP77	+	+	+	+	+	+	+	+	+	+	+	+	+
Wild type	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] +, 100% growth; ± 50% growth; and −, no growth.

Table 5. FPA-resistant amino acid transport defective/deficient loci in *Aspergillus nidulans*

Sl no.	Locus	Isolation number	Linkage group	Dominant/ recessive	Defective/deficient for uptake of amino acids	References
1.	*fpa*D	11	VIII	Dominant	Aromatic	[19, 20]
2.	*fpa*K	69	VIII	Dominant		[21]
3.	*fpa*P	77	II	Recessive		(Present study)
4.	*aau*C	1	II	Dominant	Aromatic, acidic, neutral	[7]
5.	*aau*D	1	VIII	Dominant		[7]
6.	*fpa*Q	80	II	Dominant		(Present study)
		79	II	Semidominant	Aromatic, acidic, neutral, basic	(Present study)
7.	*fpa*O	74	I	Recessive	Acidic, neutral, basic	[24]

three FPA-sensitive progeny from the cross *fpa*D11 × *fpa*Q79, three from *fpa*D11 × *fpa*Q80, and five from *fpa*K69 × *fpa*P77 were randomly selected from the original master plate and outcrossed to a wild-type strain. Recovery of FPA-resistant colonies from some of these crosses (crosses 4, 5, and 6 in Table 3) confirmed that the double mutant types had become FPA sensitive.

Growth pattern of double mutants and segregants on different amino acids as sole nitrogen sources. In order to characterize for the specificity of transport mutants, the growth patterns of the sensitive double mutants, the resulting segregants, original mutants and wild type on different amino acids as sole source of nitrogen were tested. The double mutants could utilize all the amino acids in a manner similar to that by the wild-type strain. Among the original strains, *fpa*D11 and *fpa*K69 could utilize all the amino acids (although the growth was quite poor), but not the aromatic ones; *fpa*Q79 was unable to utilize any of the amino acids, whereas *fpa*Q80 could utilize only basic amino acids (Table 4). Out of 104 resistant colonies replicated on different amino acids, 21 showed a phenotype similar to *fpa*D11, 18 similar to *fpa*Q79, 10 similar to *fpa*Q80, 39 similar to *fpa*K69, and 16 similar to *fpa*P77.

Discussion

Identification of six new loci extends the list of FPA-resistant loci in *Aspergillus nidulans* to 22. The present selection methods with reduced carbon or nitrogen flow appear to be highly specific for the identification of amino acid transport-defective loci. This is evident from the fact that out of eight FPA-resistant isolates analyzed, six new loci could be identified, and 50% of these appeared to be concerned with transport systems of amino acids.

Mutations at 7 of the 22 hitherto known loci appear to affect the specific as well as general permeases of amino acids (Table 5). Scattering of uptake-defective loci on linkage groups I, II, and VIII suggests that an 'operon organization' for per-

meases, as in the case of bacteria, is not possible in *A. nidulans* and that the genetic regulation of amino acid transport is a more intricate process, like those in higher eukaryotes. Furthermore, predominance of dominant permease mutants in this fungus is contrary to those reported in bacterial [1] and other fungal systems [29]. One plausible hypothesis for this could be that the heterozygous diploid containing the wild-type and mutant alleles synthesizes suboptimal levels (50% or more) of active permease, which is able to transport only subinhibitory concentrations of FPA. As a result, the diploids are not inhibited by the concentration of the analog normally used, and the mutations appear to be dominant. In a situation in which a mutation affects the permease partially, the level of enzyme synthesized in diploids could be more than 50%, the consequence of which would be semidominance of the mutant concerned. An alternative hypothesis is that the dominant mutants may be produced at loci whose products act in polymeric complexes, in which one defective polypeptide may be able to abolish the activity of the whole complex.

Growth pattern of *fpa*O mutants on different amino acids as sole nitrogen source and interaction of this locus with the leucine requirement of the auxotroph in luA1, *fpa*O74 recombinants suggest that a mutation at the locus *fpa*O affects the uptake of acidic, neutral, and basic amino acids. Similar interaction between amino acid auxotrophs on the one hand and uptake-defective locus *fpa*D on the other has been reported [20]. By analogy, *fpa*O mutants appear to be similar to pm-g mutants of *Neurospora crassa* [12] defective in Pall's system II.

Mutant *fpa*Q79 does not utilize aromatic, acidic, neutral, and basic amino acids and seems to be a "general permease" mutant. On the other hand, *fpa*Q80 is able to utilize only basic amino acids and appears to be deficient in the transport of aromatic, acidic, and neutral amino acids. The growth pattern of *fpa*Q80 is comparable to the two previously known uptake mutants, *aau*C1 and *aau*D1 [7]. It seems that, in spite of being mutations in the same gene, the ultimate expression of the mutation is different in *fpa*Q79 and *fpa*Q80. There are two possibilities for such pleiotropy of a mutation. One of these is that the DNA sequence needed for coding the genetic message for aromatic, acidic, neutral, and basic amino acid permeases is overlapping. The locus *fpa*Q may code for two polypeptides by initiating or terminating the transcription at two different sites in the same reading frame. Alternatively, *fpa*Q locus may produce a transcription regulator protein that has to act on a number of different operators, or an activity regulator that interacts with a number of different uptake complexes. In either case, different mutants may synthesize products that have lost their specificity for some interactions but retain affinities for others [2].

Genetic interaction studies between different transport-defective mutants and phenotypes of segregants obtained from the crosses between FPA-sensitive double mutants and wild type on different amino acids as sole sources of nitrogen provide evidence for the specificity of the four aromatic amino acid transport mutants. The question arises why certain pairs of FPA-resistant loci would interact among themselves by yielding FPA-sensitive recombinants. Because of similar patterns of interaction, it has been suggested that different loci code for various structural proteins that are shared by different amino acid permeases [7]. The present study provides strong evidence based upon genetic analyses that aromatic permease, at least in *A. nidulans,* is a high molecular weight polymeric protein, and the loci *fpa*D, *fpa*K, *aau*C, *aau*D, *fpa*P, and *fpa*Q code for different but intimately associated polypeptides. The product of these genes is needed for the normal transport function, a mutation in any one of which leads to FPA resistance. In combination (recombinants), two defective polypeptides complement and lead to the formation of an active permease as a result of which the double mutant types become sensitive to FPA. Alternatively, there may be common carriers or overlapping control switches as reported in *Neurospora crassa* [5, 15], *Rhizobium leguminosarum* [11], *Asticcacaulis biprosthecum* [23], and in higher plants [13]. It is yet to be established whether one or several carriers mediate transport of aromatic amino acids in *A. nidulans*. Detailed biochemical analyses are in progress to characterize genetically identified permease mutants by study of the amino acid-binding protein and uptake pattern of radiolabeled amino acids.

Conclusions

1) Mutants defective in amino acid transport could be preferentially selected under conditions of reduced carbon or nitrogen flow and in the presence of amino acid analogs.
2) Mutation at the locus *fpa*O leads to an uptake defect for acidic, neutral, and basic amino acids.
3) On the basis of pattern of segregation and amino acid utilization, two new loci *fpa*P and *fpa*Q

were identified as defective in the transport of aromatic and other amino acids.

4) Aromatic amino acid permease in *Aspergillus nidulans* is possibly a polymeric protein.

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