Mutations which Affect Amino Acid Transport in *Aspergillus nidulans*

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SUMMARY

Mutants deficient in amino acid transport (*aau*), and unable to utilize L-glutamate as a sole carbon and nitrogen source, have been isolated. There are four unlinked genes involved: *aauA*, *aauB*, *aauC* and *aauD*. The transport levels of certain amino acids, and the growth characteristics on certain nitrogen and carbon sources and toxic amino acid analogues, indicate that: *aauA1* has defective transport of acidic amino acids; *aauB1* has defective transport of acidic and neutral amino acids; *aauC1* and *aauD1* have defective transport of acid, neutral and aromatic amino acids. *aauA1* and *aauB1* are recessive for all three characteristics in the heterozygous diploid; *aauC1* and *aauD1* are dominant.

INTRODUCTION

In the fungi, a number of specific transport systems for amino acids and related substances have been described. These include ammonium (Pateman, Kinghorn, Dunn & Forbes, 1973; Pateman, Dunn, Kinghorn & Forbes, 1974) and urea (Dunn & Pateman, 1972) in *Aspergillus nidulans*, arginine, lysine, methionine and histidine in *Saccharomyces cerevisiae* (Grenson, 1966; Grenson, Mousset, Wiame & Béchet, 1966; Gits & Grenson, 1967; Crabeel & Grenson, 1970), and ammonium, methionine, cysteine, proline, lysine and arginine, as well as distinct acidic and basic systems, in *Penicillium chrysogenum* (Benko, Wood & Segel, 1967; Hackette, Skye, Burton & Segel, 1970; Skye & Segel, 1970; Hunter & Segel, 1971). Moreover, the isolation of mutants defective in the transport of specific amino acids is strong evidence for the existence of specific uptake systems. However, general amino acid transport systems have also been described in the fungi. The presence of a 'general' amino acid system has been reported for *Botrytis fabae* (Jones, 1963), *Arthrobotrys conoides* (Gupta & Pramer, 1970), *S. cerevisiae* (Grenson, Hou & Crabeel, 1970; Grenson & Hou, 1972), *Neurospora crassa* (Pall, 1969), and *P. chrysogenum* (Benko, Wood & Segel, 1969). Mutants have been isolated which have simultaneously lost the ability to transport a number of amino acids, e.g. *gap* and *apf* in yeast (Surdin *et al.* 1965; Grenson *et al.* 1970; Grenson & Hennaut, 1971), *un-t*, *nap* and *mtr* in *N. crassa* (Kappy & Metzenberg, 1965; Stadler, 1966; Lester, 1966; Jacobson & Metzenberg, 1968) and *fpaD* in *A. nidulans* (Sinha, 1969).

We describe the isolation and properties of new classes of mutants mapping at four loci in *A. nidulans* which are defective in the transport of a number of amino acids. A preliminary report of this work was given at a meeting of the Genetical Society of Great Britain (Kinghorn & Pateman, 1972).

METHODS

Chemicals. Analytical grade chemicals were used whenever possible: ^{14}C -labelled L-amino acids, ^{14}C -methylammonium and thio ^{14}C -urea were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

Strains. A biotin- and putrescine-requiring strain, *bir puA2* (Glasgow No. 0171), was used as the prototroph with respect to nitrogen metabolism.

Media. Medium used for genetic analysis was essentially that described by Pontecorvo *et al.* (1953). Nitrogen-less minimal medium (–N medium) described by Cove (1966), and carbon- and nitrogen-less minimal medium (–CN medium) were used. Most nitrogen and carbon sources used in media were kept as sterile stock solutions at 1 M. Others such as urea, L-asparagine and L-glutamine, which are unstable with ammonium as one of the products, were made up immediately before use.

Growth tests of mutants were carried out in Petri dishes containing 20 ml –N or –CN medium (solid) plus the nitrogen or carbon source under test. These were carefully point-inoculated with fresh conidia and incubated at 37 °C for approximately 30 to 36 h. Growth, in terms of colony size and density, was recorded as ++, + or –.

Selection of transport mutants. The putrescine technique of Herman & Clutterbuck (1966) was used to isolate mutants unable to utilize L-glutamate as a sole carbon and nitrogen source. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treated conidia (Adelberg, Mandel & Chen, 1965) of *bir puA2* were point-inoculated on to –CN medium plus limiting putrescine (9.7×10^{-7} M) plus 100 mM L-glutamate. After 3 to 4 days' incubation at 37 °C, sectors showing spidery growth were isolated and purified.

Genetic analysis. The genetic techniques were those described by Pontecorvo *et al.* (1953) and McCully & Forbes (1965), with the exception that acridine yellow (0.06 mg/ml) was used as the haploidization agent instead of *p*-fluorophenylalanine.

Growth of mycelium for uptake assays. The uptake of ^{14}C -labelled amino acids was determined during exponential growth phase in an attempt to study transport in young cells under as standard and reproducible conditions as possible. Mycelium was grown in culture shaken at 200 rev./min (rotary motion describing a 1 in circle) in a controlled environment incubator shaker (Pycrotherm; New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) for approximately 18 to 20 h at 25 °C, and harvested by filtering on nylon cloth (Cove, 1966). When mycelium was given some special treatment, it was transferred after harvesting to the preheated treatment medium for the appropriate time and then reharvested. The mycelium was pressed dry with paper towels and weighed. Yields were usually 5 to 8 g/l medium. Cells were used immediately for uptake assays.

Amino acid transport assay. The method is basically that described in previous communications (Kinghorn & Pateman, 1973, 1974*a*). The growth and treatment media are described in the text. The final concentration (μM) and specific activity (mCi/mmol, in parentheses) of each amino acid used were as follows: L-alanine, 100 (169); L-arginine, 100 (42); L-glutamate, 100 (231); L-glutamine, 200 (42); L-phenylalanine, 200 (4); L-serine, 100 (162); methylammonium, 500 (54); thiourea, 200 (18.3). The final approximate specific activities of each amino acid were as follows (μCi): L-alanine, 6.0; L-arginine, 1.5; L-glutamate, 0.5; L-glutamine, 3.5; L-phenylalanine, 2.0; L-serine, 9; methylammonium, 2.5; thiourea, 2.5. The amino acids were uniformly labelled.

RESULTS

Isolation of mutants

A large number of mutants unable to grow on L-glutamate as a sole carbon and nitrogen source were isolated (Kinghorn & Pateman, 1973, 1974*b*). Six of these mutants possessed low levels of L-glutamate transport.

Fig. 1. Linkage relationships of *aauB* and *aauC* to established markers, given as the mean percentage recombination $\pm$ S.E.M. An explanation of symbols and a complete linkage map are given by Clutterbuck (1974). The data in (b) do not exclude the possibility that *aauB* is located to the left of *wetA*.

Genetic analysis

Mitotic and meiotic genetic analysis, as discussed below, showed that four gene loci were involved. These loci were designated *aauA* (amino acid uptake), *aauB*, *aauC* and *aauD*.

aauA locus. Haploidization of the diploid between *bir*; *puA2*; *aauA1* and master strain D by the method of McCully & Forbes (1965) yielded segregants which showed free assortment between *aauA1* and all chromosome markers except *nicB*. Analysis of a single mixed perithecium from the cross: *bir*; *puA2*; *aauA1* by *yA2*; *palD nicB malA wetA* failed to locate *aauA1*, since *aauA1* showed approximately 50 % recombination with *palD*, *nicB*, *malA* and *wetA* (available markers in linkage group VII) amongst 161 progeny tested. Therefore the position of *aauA1* in linkage group VII is not known.

aauB locus. Haploidization analysis showed that a further two mutants were located in linkage group VII. The mutations were allelic, or closely linked, since no wild-type recombinant progeny were isolated in a total of 500 progeny scored. The locus was designated *aauB* and the mutations *aauB1* and *aauB2*. Crosses between *bir*; *puA2*; *aauB1* and *yA1*; *pyroA4*; *aauA1* gave approximately 50 % recombination for the two genes (280 progeny tested). Crosses between *bir*; *puA2*; *aauB1* or *bir*; *puA2*; *aauB2* and *yA2*; *palD nicB malA wetA* located the *aauB* locus near *wetA* (Fig. 1).

aauC locus. Haploidization analysis of the diploids between *bir*; *puA2*; *aauC1* or *bir*; *puA2*; *aauC2* and the tester strain D was carried out with complete medium plus acridine yellow (0.06 mg/ml). Acridine yellow was used instead of *p*-fluorophenylalanine since the *aauC1* and *aauC2* mutations are dominant and resistant to *p*-fluorophenylalanine. Both mutant characteristics assorted freely with all chromosome markers except *acrA*, and consequently were assigned to linkage group II. The results of crosses between *bir*; *puA2*; *aauC1* and *yA2*; *pyroA4*; *aauC2* showed they were allelic or closely linked; no wild-type recombinants were isolated in 450 progeny. The results of crosses between *bir*; *puA2*; *aauC1* or *bir*; *puA2*; *aauC2* and *acrA wA thiA abA cnxE ygA* located the *aauC* locus in the *cnxE*–*ygA* interval in linkage group II (Fig. 1).

aauD locus. As before, acridine yellow was used as the haploidization agent. *aauD1* assorted freely with all chromosome markers except *riboB* and was therefore assigned to

Fig. 2. L-Glutamate transport by wild-type and *aau* mutants. The cells were grown on —N medium + 10 mM-urea for 18 h. ■, Wild type; △, *aauAI*; ○, *aauBI*; ◇, *aauCI*; ▲, *aauDI*.

linkage group VIII. A cross between *aauDI* and *fpaD43*, which is also an amino acid transport mutant and maps in this linkage group (Sinha, 1969), showed that *aauDI* and *fpaD43* are not allelic but loosely linked (approximately 20 % recombination in 410 progeny tested).

Transport of amino acid by wild-type and *aau* mutants

The following amino acids and inorganic nitrogen compounds were used to determine the transport characteristics of *aau* mutants: L-glutamate (acidic), L-phenylalanine (aromatic), L-alanine and L-serine (neutral), L-arginine and L-glutamine (basic), thiourea and methylammonium (inorganic). From the data in Fig. 2, it can be seen that all classes of uptake mutants had low uptake of L-glutamate (< 10 % of the wild type). The heterozygous diploids *aauAI/aauA*⁺ and *aauBI/aauB*⁺ appeared to have wild-type transport while *aauCI/aauC*⁺ and *aauDI/aauD*⁺ showed the mutant level, indicating that *aauAI* and *aauBI* mutants are recessive to their wild-type alleles while *aauCI* and *aauDI* are dominant (Fig. 3). The mutants *aauBI*, *aauCI* and *aauDI*, but not *aauAI*, had impaired transport of the neutral amino acids L-serine (Fig. 4) and L-alanine (Fig. 5), while only *aauCI* and *aauDI* had impaired uptake of the aromatic representative L-phenylalanine (Fig. 6). All genotypes had normal uptake of the basic amino acids L-arginine and L-glutamine, and also normal uptake of thiourea and methylammonium.

In summary, *aauAI* shows low uptake of only acidic amino acids and is recessive. *aauBI* shows low uptake of acidic and neutral amino acids and is recessive. *aauCI* and *aauDI* have low uptake of acidic, neutral and aromatic amino acids, and in contrast to *aauAI* and *aauBI*, are dominant.

Growth responses of *aau* mutants

Table 1 shows the growth characteristics of the four classes of uptake mutants on a range of amino acids provided as the sole nitrogen source. The *aauAI* mutant grows as wild type on inorganic nitrogen sources (e.g. nitrate, ammonium or urea), neutral amino acids (e.g. L-serine, L-alanine or L-valine), aromatic amino acids (e.g. L-phenylalanine, L-tryptophan or L-tyrosine) and basic amino acids such as L-arginine, L-ornithine, L-asparagine or L-glutamine, but grows poorly on the acidic amino acids, L-glutamate and L-aspartate. The heterozygous

Fig. 3

Fig. 4

Fig. 3. L-Glutamate transport by heterozygous diploids of wild-type and *aau* mutants. The cells were grown on $-N$ medium + 10 mM-urea for 18 h.

■, $\frac{\text{Wild type}}{\text{wild type}}$; Δ , $\frac{aauAr}{aauA^+}$; $\circ$, $\frac{aauBr}{aauB^+}$; $\diamond$, $\frac{aauCr}{aauC^+}$; $\blacktriangle$, $\frac{aauDr}{aauD^+}$.

Fig. 4. Transport of L-serine by wild-type and *aau* mutants. The cells were grown on $-N$ medium + 10 mM-urea for 18 h. ■, Wild type; Δ , *aauAr*; $\circ$, *aauBr*; $\diamond$, *aauCr*; $\blacktriangle$, *aauDr*.

Fig. 5

Fig. 6

Fig. 5. Transport of L-alanine by wild-type and *aau* mutants. The cells were grown on $-N$ medium + 10 mM-urea for 18 h. ■, Wild type; Δ , *aauAr*; $\circ$, *aauBr*; $\diamond$, *aauCr*; $\blacktriangle$, *aauDr*.

Fig. 6. Transport of L-phenylalanine by wild-type and *aau* mutants. The cells were grown on $-N$ medium + 10 mM-urea for 18 h. ■, Wild type; Δ , *aauAr*; $\circ$, *aauBr*; $\diamond$, *aauCr*; $\blacktriangle$, *aauDr*.

Table 1. Growth responses of *aau* mutants on certain nitrogen sources

Nitrogen source*	Wild type	<i>aauAI</i>		<i>aauBI</i>		<i>aauCI</i>		<i>aauDI</i>	
		<i>aauAI</i>	<i>aauA</i> ⁺	<i>aauBI</i>	<i>aauB</i> ⁺	<i>aauCI</i>	<i>aauC</i> ⁺	<i>aauDI</i>	<i>aauD</i> ⁺
Urea	++	++	++	++	++	++	++	++	++
Ammonium	++	++	++	++	++	++	++	++	++
Nitrate	++	++	++	++	++	++	++	++	++
L-Aspartate	++	+	++	+	++	+	+	+	+
L-Glutamate	++	+	++	+	++	+	+	+	+
L-Alanine	++	++	++	+	++	+	+	+	+
L-Serine	++	++	++	+	++	+	+	+	+
L-Valine	++	++	++	+	++	+	+	+	+
L-Phenylalanine	++	++	++	++	++	+	+	+	+
L-Tryptophan	++	++	++	++	++	+	+	+	+
L-Tyrosine	++	++	++	++	++	+	+	+	+
L-Glutamine	++	++	++	++	++	++	++	++	++
L-Asparagine	++	++	++	++	++	++	++	++	++
L-Arginine	++	++	++	++	++	++	++	++	++
L-Ornithine	++	++	++	++	++	++	++	++	++

++, Wild-type growth (this varies with the nitrogen source); +, about 50 % of wild-type growth.

* Added at 10 mM final concentration to solid -N medium.

Table 2. Growth responses of *aau* mutants on certain carbon sources

Carbon source*	Wild type	<i>aauAI</i>		<i>aauBI</i>		<i>aauCI</i>		<i>aauDI</i>	
		<i>aauAI</i>	<i>aauA</i> ⁺	<i>aauBI</i>	<i>aauB</i> ⁺	<i>aauCI</i>	<i>aauC</i> ⁺	<i>aauDI</i>	<i>aauD</i> ⁺
Galactose 1 %	++	++	++	++	++	++	++	++	++
Maltose 1 %	++	++	++	++	++	++	++	++	++
Sedoheptulose 1 %	++	++	++	++	++	++	++	++	++
Acetate 1 %	++	++	++	++	++	++	++	++	++
Glycerol 1 %	++	++	++	++	++	++	++	++	++
L-Glutamate 100 mM	++	-	++	-	++	-	-	-	-
L-Aspartate 100 mM	++	-	++	-	++	-	-	-	-
L-Alanine 100 mM	++	++	++	-	++	-	-	-	-

++, Wild-type growth (this varies with the carbon source); -, poor growth.

* Basal medium = solid -N medium + 10 mM-ammonium.

diploid *aauAI/aauA*⁺ grows as wild type on L-glutamate and L-aspartate, indicating that the *aauAI* mutation is recessive. Mutation in the *aauB* gene results in poor growth on acidic and neutral amino acids and is recessive for growth characteristics. The *aauCI* and *aauDI* mutants are rather similar to each other in that they both grow poorly on acidic, neutral and aromatic, but as wild type on basic amino acids. They are dominant in the heterozygous diploid.

All classes of mutants appear to have wild-type growth on certain carbon sources tested (Table 2). However, the utilization of L-glutamate, L-aspartate and L-alanine as sole carbon sources by the mutants was relatively poorer than their utilization of these amino acids as sole nitrogen sources. This is not surprising since larger quantities of the amino acid would be necessary to provide a sufficient energy source. The mutants *aauAI* and *aauBI* appear recessive for growth on L-glutamate as a carbon and nitrogen source, while *aauCI* and *aauDI* are dominant (Fig. 7).

Fig. 7. Growth of a homozygous wild-type diploid and diploids heterozygous for *aau* mutants with L-glutamate as sole carbon and nitrogen source. — CN medium (solid) plus 100 mM-L-glutamate for 48 h. +, Wild-type allele.

*Resistance of aau mutants to certain toxic analogues of amino acids
and inorganic nitrogen*

An attempt was made to correlate the transport deficiencies of the various *aau* mutants with resistance to certain toxic analogues. A number of amino acid analogues reported to be inhibitory to bacteria and yeast did not inhibit wild-type *A. nidulans*. These included analogues of L-glutamate (D-glutamate), L-aspartate (D-aspartate), L-arginine (canavanine), L-proline (azetidine-2-carboxylic acid), L-lysine (thiosine), L-methionine (ethionine) and glutamine (glutamyl-hydrazine). However, three analogues were found to inhibit the growth of *A. nidulans*: aspartate hydroxamate, glutamate hydroxamate and D-serine. Auxanographic tests of a wild-type strain showed that the inhibitions of growth by the three analogues were reversed by L-asparagine, L-glutamine and L-serine, respectively. In addition, the *aau* mutants were tested for resistance to *p*-fluorophenylalanine, 3-amino tyrosine HCl (Sinha, 1969), methylammonium (Arst & Cove, 1969) and thiourea (Dunn & Pateman, 1972), which are toxic analogues of L-phenylalanine, L-tyrosine, ammonium and urea, respectively. The mutants *meaA8*, *uruA1*, SER-9, *fpaD43*, *ahyA1* and *ghyA1*, each known to be resistant to at least one of the analogues, were tested as controls and to determine if *aau* mutants shared any resistance characteristics with various known resistant mutants (Table 3). *aauA1* is sensitive to all seven toxic analogues; *aauB1* is resistant to D-serine only; *aauC1* and *aauD1* are resistant to *p*-fluorophenylalanine, D-serine and 3-amino tyrosine. *aauB1* is recessive, while *aauC1* and *aauD1* are dominant for resistance. *uruA1*, *meaA8*, SER-9, *ahyA1* and

Table 3. Resistance of *aau* mutants to toxic analogues of certain nitrogen sources

Metabolite	Toxic analogue of metabolite	-N medium (solid) + 10 mM	Control strains										<i>aau</i> strains					
			Wild type	<i>meaA8</i>	<i>uruA1</i>	SER-9	<i>spaD43</i>	<i>ahyA1</i>	<i>ghyA1</i>	<i>aauA1</i>	<i>aauA1</i>	<i>aauA1</i>	<i>aauA1</i>	<i>aauB1</i>	<i>aauB1</i>	<i>aauC1</i>	<i>aauC1</i>	<i>aauD1</i>
L-Phenyl- alanine	<i>p</i> -Fluorophenyl- alanine (0.025 %)	Nitrate	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+
L-Serine	D-Serine (5 mM)	Nitrate	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+
L-Asparagine	L-Aspartate hydroxamate (0.32 mM)	Nitrate	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
L-Glutamine	L-Glutamate hydroxamate (5 mM)	Nitrate	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
L-Tyrosine	3-Amino tyrosine HCl (10 mM)	Nitrate	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+
Ammonium	Methylammonium (200 mM)	Arginine	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	Thiourea (5 mM)	Nitrate	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Control strains:

- uruA1* Resistant to thiourea only (Dunn & Pateman, 1972)
meaA8 Resistant to methylammonium only (Arst & Cove, 1969)
spaD43 Resistant to *p*-fluorophenylalanine and 3-amino tyrosine HCl only (Sinha, 1969)
 SER-9 Resistant to D-serine only (Kinghorn, unpublished work)
ahyA1 Resistant to aspartate hydroxamate only (Kinghorn, unpublished work)
ghyA1 Resistant to glutamate hydroxamate only (Kinghorn, unpublished work)
 +, Appreciable growth (relatively normal colony size); -, extremely poor growth.

aauA mutants have no common resistance characteristics. *fpaD43* shares some resistance characteristics with *aauC1* and *aauD1*, i.e. dominance and resistance to *p*-fluorophenyl-alanine and 3-amino tyrosine (Sinha, 1969). However, *fpaD43* differs in that it is not resistant to D-serine.

DISCUSSION

It is clear that mutation in at least four loci, *aauA*, *aauB*, *aauC* and *aauD*, in *A. nidulans* results in impaired transport of certain amino acids. These are in addition to *fpaD* described by Sinha (1969). There are three main lines of evidence to support this: the transport levels of amino acids in the mutants; the phenotypes of the mutants on certain nitrogen or carbon sources; and their cross-resistance patterns to toxic analogues of certain nitrogen metabolites. Moreover, since the growth rates of the *aau* mutants are similar to that of the wild type when grown on minimal medium, it is concluded that the physiological defect is limited to the transport function.

The existence of *aauA1*, in which only L-glutamate and L-aspartate transport is impaired, suggests the presence of a specific system or component for L-glutamate and L-aspartate transport rather similar to that in *N. crassa* (Pall, 1969) and *P. chrysogenum* (Hunter & Segel, 1971).

Mutation in the *aauB* gene reduces the uptake of acidic and neutral amino acids and is rather similar to the *nap* and *un-t* mutations in *N. crassa*. Several explanations can be advanced to explain such mutations. The most likely is that *aauB1* results in an altered functional component (e.g. an energy-generating system) which is shared by both the acidic and neutral amino-acid transport systems. The fact that the *aauB1* mutation is recessive tends to support this.

Mutation at the *aauC* and *aauD* loci results in altered transport levels of acidic, neutral and aromatic amino acids. In contrast to *aauA1* and *aauB1*, but similar to *fpaD43*, both *aauC1* and *aauD1* are dominant in the heterozygous diploid.

Sinha (1969) discussed the possibility that *fpaD* plays a regulatory role in amino acid transport. However, the isolation of similar dominant mutations at another two loci rather discounts this hypothesis. It seems unlikely that all three loci are involved in regulation. An alternative explanation is that the loci code for structural proteins which are intimately associated with and shared by the acidic, neutral and aromatic amino acid transport systems. The proteins determined by these three loci (and perhaps others presently unknown) are essential for normal amino acid transport function. An extension of this hypothesis provides a plausible explanation for the dominance or semi-dominance of these mutations. The heterozygous diploid contains normal and mutant alleles which specify both normal and abnormal transport proteins. An actual transport site might contain all normal, all abnormal, or some combination of molecules of the transport protein. If all transport sites contained sufficient abnormal components to reduce transport, the mutation would appear dominant.

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