Tryptophan Transport in *Neurospora crassa* by Various Types of *mtr* Revertants

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SUMMARY

Reversions in a strain of *Neurospora crassa* carrying the *mtr*-26 allele may result from at least two types of mutations: (i) intralocus mutations which may or may not occur at the primary mutational site; (ii) unlinked suppressor mutations which may or may not be allele specific. The uptake of ^{14}C -labelled tryptophan in *mtr*⁺, *mtr* and *mtr* (revertant) strains has been studied. Revertant strains have been isolated in which the rate of tryptophan transport is one half of that in *mtr*⁺ cells. These revertants include those resulting from intragenic suppression of the *mtr* phenotype as well as those resulting from mutation at unlinked suppressor loci. Possible explanations for this reduced rate of transport are discussed. This paper also reports the occurrence of an unlinked allele specific suppressor in *Neurospora* which may suppress a frameshift mutation.

INTRODUCTION

Previous studies (Stadler, 1966, 1967) have indicated that *mtr* mutants of *Neurospora crassa* (resistant to 4-methyltryptophan (4MT) and *para*-fluorophenylalanine) are defective in the uptake of tryptophan. In rapidly growing mycelia tryptophan transport is mediated by an energy requiring permease system (Transport System I). This tryptophan transport is subject to competitive inhibition in the presence of excess concentrations of phenylalanine and other aromatic amino acids (Stadler, 1967; Pall, 1969). Unlinked suppressor mutations located on linkage group I (*su*^{*mtr*}) have been isolated and these appear to restore normal levels of tryptophan transport in strains carrying both the suppressor and an *mtr* allele. These suppressors appear to be allele non-specific and it is postulated that this mutation alters the structural gene for another permease system thereby increasing its substrate range so that it can now take up the aromatic amino acids (Stadler, 1967).

Some intralocus revertants of allele *mtr*-26 were found to segregate *mtr*⁻ colonies with a low frequency (about 1×10^{-5}) when crossed to wild (*mtr*⁺) strains (Brink, Kariya & Stadler, 1969). These 4MT-resistant colonies were not produced by spontaneous mutation, but arose as a result of recombinational events between the primary mutational lesion and a secondary lesion within the locus. These two lesions together resulted in reversion of the *mtr* phenotype.

This paper reports a further type of revertant which results from forward mutation at an unlinked locus. This suppressor is apparently allele specific, since of those *mtr* alleles tested, only *mtr*-26 was suppressed. Some properties of tryptophan transport in *mtr*⁺, *mtr*⁻ and *mtr* revertant strains are described.

Table 1. *Expected growth responses of various wild, mutant and revertant strains*

Strain	Media			
	HPS	HP ₄ MTS	HPFPAS	H ₁ PArg ₆ S
<i>hist; pdx mtr</i> ⁺	+	—	—	+
<i>hist; pdx mtr-26</i>	+	+	+	—
<i>hist; pdx mtr-10</i>	+	+	+	—
<i>hist; pdx mtr-26 (Rev)</i>	+	—	—	+

METHODS

Strains. Two basic strains of *Neurospora crassa* have been used: *hist-2; pdx-1 (hpA)* and *hist-2; col-4 (hca)*. Mutant *mtr-26* was induced in the *hpA* strain by u.v. irradiation (Brink *et al.* 1969). Revertants of this mutant have been induced by ICR-170* (R1364, R1373), DES* (R2435) and NA* (R5035, R5037, and R5042). Methods used for the ICR-170 and DES treatments have been described previously (Brink *et al.* 1969), whilst the nitrous acid treatments utilized the procedure outlined by Seale (1968).

Media. The minimal sorbose medium of Lester & Gross (1959) served as the basic medium. It was supplemented as follows: (i) HPS medium = histidine (50 mg/l) and pyridoxine (5 mg/l); (ii) HP₄MTS or HPFPAS = histidine, pyridoxine and either 4MT (90 mg/l) or FPA (10 mg/l); (iii) H₁PArg₆S = histidine (10 mg/l), pyridoxine, and arginine (60 mg/l). The various strains described in these experiments show typical growth responses on each of these media (Table 1). For corresponding liquid media, 'S' is omitted.

Liquid growth tests. Liquid growth tests were sometimes used to measure the growth of various strains on different media. Erlenmeyer flasks containing 20 ml of liquid minimal medium with appropriate supplements were inoculated with about 10⁵ conidia. The mycelia were grown in still culture at 32 °C for about 30 h, harvested, washed, dried and weighed.

Classification of revertants. Phenotypic revertants of allele *mtr-26* were confirmed by growth tests and further analysed by crossing to *hist-2; col-4 mtr-10*. Ascospores from the cross were heat shocked and plated on to HPS, HP₄MTS, HPFPAS and H₁PArg₆S medium. Three types of result are expected depending on the type of phenotypic revertant (see Table 2). This classification takes advantage of the fact that the *mtr* and *col-4* loci are tightly linked.

Genetic mapping. For those revertants resulting from a forward mutation at an unlinked suppressor locus, initial mapping of this mutant was carried out by crossing the revertant strain to 'alcoy' (*T (I; II) al-1; T (IV; V) cot; T (III; VI) ylo-2*). To obtain a more accurate chromosomal location for the mutant, the revertant was then crossed to another strain carrying several markers on a particular linkage group.

Intragenic recombination events in those revertants resulting from a second mutation within the *mtr* locus were studied by crossing them to *mtr*⁺ strains and selecting for the rare 4MT-resistant recombinants which sometimes occurred. These intragenic recombinants were then backcrossed to a *hist-2; pdx-1 mtr-26* strain and selection made for the rare recombination events which would yield 4MT-sensitive colonies.

Uptake experiments. Flasks (2-litre) containing 200 ml of liquid minimal medium (Vogel's) supplemented with histidine (5 mg/100 ml) and pyridoxine (0.5 mg/100 ml) were inoculated with conidia. The flasks were shaken overnight on a Paton Shaking Incubator for about 20 h at 25 °C. Mycelia was harvested in a Buchner funnel and washed three times with glass-

* ICR-170 = 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)amino-propylamine] acridine dihydrochloride; DES = diethylsulphate; NA = nitrous acid.

Table 2. *Types of progeny expected when various hist⁻; pdx mtr-26 col-4⁺ (Rev) strains are crossed to hist-2; col-4, mtr-10*

The letter *x* refers to the expected proportions of the *col* and *col*⁺ progeny which will grow on the various media.

Type of revertant	Media					
	HPS		HP ₄ MTS		H ₁ PArg ₆ S	
	<i>col</i>	<i>col</i> ⁺	<i>col</i>	<i>col</i> ⁺	<i>col</i>	<i>col</i> ⁺
Locus	<i>x</i>	<i>x</i>	<i>x</i>	—	—	<i>x</i>
Unlinked specific suppressor	<i>x</i>	<i>x</i>	<i>x</i>	$\frac{1}{2}x$	—	$\frac{1}{2}x$
Unlinked non-specific suppressor	<i>x</i>	<i>x</i>	$\frac{1}{2}x$	$\frac{1}{2}x$	$\frac{1}{2}x$	$\frac{1}{2}x$

distilled water. Surplus water was removed from the resultant pad by pressing between thicknesses of blotting paper. Approximately 1.5 g of this pad was fragmented and the mycelia dispersed in 15 ml of glass-distilled water. This was achieved by placing a magnetic flea in the water containing the pad and vortexing for about 30 s. This mycelial suspension was then added to the uptake flask containing 35 ml of 0.1 mM-DL-[¹⁴C]tryptophan and 0.04 ml L-[¹⁴C]tryptophan (supplied by the Radiochemical Centre, Amersham, Buckinghamshire, specific activity 277 μ Ci/mg, total radioactivity in sample 0.5 μ Ci). Samples (5 ml) were removed from the uptake solution at time zero and at 2 min intervals. The samples were filtered and washed with 100 ml ice-cold glass-distilled water (4 °C) and the resultant pads placed in 2 ml of ice-cold water in a standard centrifuge tube.

Extraction and counting. The pads were extracted by placing the centrifuge tubes in a boiling water bath for 10 min. The tubes were then centrifuged to sediment the mycelial pellet. Samples (0.5 ml) of the supernatant were added to 4.5 ml of scintillation fluid (Bray, 1960) and counted on a Nuclear-Chicago scintillation counter. The mycelial pellets were dried and weighed.

RESULTS

Growth tests. Revertant strains grown on H₁PArg₆S medium. However, some of the revertant strains studied differed from *mtr*⁺ in that they retained partial resistance to 4MT. Consequently, in liquid growth tests these strains grew on HP₄MT as well as H₁PArg₆ (see Table 3). This growth on HP₄MT was most pronounced in revertants R5035, R5037 and R5042, whilst R1364, R1373 and R2435 were similar to *hpA* in their growth response.

Classification of revertants. Classification makes use of the fact that the *mtr* locus is tightly linked to *col-4* on linkage group IV. Previous studies had indicated that R1364, R1373 and R2435 were intralocus revertants (Brink *et al.* 1969). The other three revertants (R5035, R5037 and R5042), which are all non-colonial, were crossed to *hist-2; col-4 mtr-10*. The results of the crosses are shown in Table 4. These three reversions are apparently associated with mutation at an unlinked suppressor locus since non-colonial colonies grew on HP₄MTS medium (see Table 2). The suppressor appears to be allele specific, since no colonial colonies grew on H₁PArg₆S medium. Similar results were obtained when these three revertants were crossed to five other *mtr* alleles. Thus the suppressor mutation appears to be specific for allele *mtr-26*.

The locus revertants result from a 'second site' mutation within the *mtr* locus, since 4MT resistant colonies occur with low frequency when they are crossed to *mtr*⁺ strains (Table 5).

Table 3. *Dry weight (mg) of mycelial pads obtained from 30 h liquid growth tests of various revertant strains*

Strain	HP4MT	H ₁ PArg ₆
<i>mtr</i> ⁺	3	56
<i>mtr-26</i>	61	4
<i>mtr-26</i> (R1364)	2	52
<i>mtr-26</i> (R1373)	1	26
<i>mtr-26</i> (R2435)	0	22
<i>mtr-26</i> (R5035)	13	36
<i>mtr-26</i> (R5037)	11	35
<i>mtr-26</i> (R5042)	19	30

Table 4. *Crosses of nitrous acid induced revertants (hist-2; pdx mtr-26 col-4⁺ (Rev)) to hist-2; col-4, mtr-10 to determine the genetic nature of the revertant*

Figures refer to the observed numbers of colonial and non-colonial progeny types obtained when ascospores were plated on various media.

Revertant	Media					
	HPS		HP4MTS		H ₁ PArg ₆ S	
	<i>col</i>	<i>col</i> ⁺	<i>col</i>	<i>col</i> ⁺	<i>col</i>	<i>col</i> ⁺
R5035	27	35	34	10	0	58
R5037	51	56	63	21	0	79
R5042	98	90	62	26	0	66

This Table also shows that the 'second site' mutation must also have the *mtr* phenotype since some of the 4MT-resistant colonies isolated with low frequency recombine with *mtr-26* when they are backcrossed to this allele. On the other hand, self crosses of *mtr-26* have not yielded any 4MT-sensitive colonies.

Mapping of unlinked suppressor mutations. Strains R5035, R5037 and R5042 were each crossed to 'alcoy' linkage tester. This indicated that the suppressor mutation in R5035, R5037 and R5042 was located on either linkage group III or VI. Furthermore, crosses with an *a*; *ad-8*, *lys-5*, *ylo-1*, *pan-2* and *try-2* strain showed that the suppressor was located on the right arm of linkage group VI.

[¹⁴C]Tryptophan uptake. The rates of [¹⁴C]tryptophan uptake in wild-type (*mtr*⁺) and mutant (*mtr-26*) strains are shown in Fig. 1. The rate is negligible in *mtr-26*, whilst in *mtr*⁺ there is a steep increase in the first 6 min after which the rate of uptake is reduced. In *mtr*⁺ strains following the addition of 1 mM-DL-phenylalanine there is a slower rate of tryptophan transport, but a similar concentration of DL-arginine added to the uptake medium did not affect the rate.

Rates of tryptophan transport in revertants arising as a result of intragenic suppression within the *mtr* locus are shown in Fig. 2. In R1364 the rate is similar to that of *mtr*⁺, but in R1373 and R2435 it is only about half that of the wild-type strain. This difference is statistically significant at the 5% level. When these revertant strains were crossed to wild-type strains, some colonies resistant to 4MT were obtained and these, like *mtr-26*, had negligible uptake rates.

Fig. 3 shows the rates of tryptophan transport in two of the strains (R5035 and R5037) which had the *mtr* phenotype suppressed by a forward mutation at another locus. The rate was reduced to about 50% of the wild-type value.

Table 5. *Genetic analysis of the locus revertants*

Revertant	Self cross		Cross to <i>hca</i>		Liquid growth tests of 4MT-resistant colonies			Cross of 4MT-resistant colonies to <i>hcm26a</i>		Flanking markers		
	4MT-resistant colonies	Total spores ($\times 10^3$)	4MT-resistant colonies	Total spores ($\times 10^3$)	HP4MT	Dry wt (mg)	H ₁ PArg ₆	4MT-sensitive colonies	Total spores ($\times 10^3$)	<i>p</i> +	+ <i>co</i>	<i>pco</i> + +
R1364	0	1545	2 (all <i>col</i> ⁺)	210	{ 24 Not tested	{ 0 Not tested	{ 0 Not tested	44	304			Not tested
								34	234			Not tested
R1373	0	1019	4 (all <i>col</i> ⁺)	693	{ 55 37 37 53 63	{ 0 0 0 0 0			Not tested			Not tested
R2435	0	1564	7 (4 <i>col</i> ⁺)	887	{ 29 21 30 26	{ 3 0 0 2		28	242	9	10	1 8

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Uptake of ^{14}C -labelled tryptophan by growing mycelia of strain *hpA* and mutant strain *hist-2; pdx-1, mtr-26 (hpmtr-26A)* of *Neurospora*. Uptake was in distilled water which contained 10^{-4} M- ^{14}C tryptophan only or in addition, 10^{-3} M-arginine or 10^{-3} M-phenylalanine. Uptake is expressed as counts/mg/min. ●, *hpA*; ■, *hpmtr-26A*; ◇, *hpA* with arginine in the medium; ○, *hpA* with phenylalanine in the medium.

Fig. 2. Uptake of ^{14}C -labelled tryptophan by growing mycelia of strain *hpA* (●) and various revertant strains resulting from intragenic suppression (▼, R1364; ▲, R1373; ◆, R2435). In addition the rate of uptake in 4MT-resistant strains derived from these revertants is also shown (▽, R1373-1; ◇, R2435-1; △, R2435-2).

Fig. 3. The effect of either arginine or phenylalanine on the rate of [^{14}C]tryptophan uptake in growing mycelia of strain *hpA* and revertant strains resulting from forward mutation at an unlinked suppressor locus. Only tryptophan in uptake medium: —●—, *hpA*; —▲—, R5037; —◆—, R5035. Tryptophan and arginine in uptake medium: —◇—, *hpA*; —□—, R5037; —▼—, R5035. Tryptophan and phenylalanine in uptake medium: --○--, *hpA*; --△--, R5037; --▽--, R5035.

In addition, 10^{-3} M concentrations of DL-phenylalanine resulted in a markedly reduced rate of uptake in these strains compared with that found in wild-type cells. On the other hand, uptake of tryptophan was also affected by excess amounts of arginine (12×10^{-4} M) in contrast to the results observed in *mtr*⁺ cells. This concentration of arginine resulted in a threefold reduction in the rate of tryptophan transport.

DISCUSSION

The results of this and other studies (Stadler, 1967; Brink *et al.* 1969) indicate that the *mtr* phenotype can be suppressed by at least four types of mutations: (i) reversion of the *mtr* allele; (ii) forward mutation at a second site within the *mtr* locus; (iii) forward mutation at the *sumtr* locus on linkage group I—these suppressors are not allele specific; (iv) forward mutation at one other locus at least (on linkage group VI)—these suppressors appear to be allele specific for *mtr-26* of those *mtr* alleles tested.

It was previously suggested (Brink *et al.* 1969) that *mtr-26* may be a frameshift mutation. This conclusion was largely based on the fact that locus revertants of this allele were readily produced by ICR-170, a mutagen which has been reported to mainly induce and revert

frameshift mutations in *Neurospora* (Malling, 1967; Brusick, 1969). Support for this proposal comes from the present study, in which the second site has been genetically isolated. These mutants are 4MT-resistant and also have reduced rates of tryptophan uptake. Also, the rates of tryptophan transport by various locus revertants are not identical. This may be related to the variable length of 'nonsense' between the primary and secondary lesions of the different mutants. However, the alternative possibility that both mutations (*mtr-26* and the intragenic suppressor) are base substitutional changes which compensate the effects of each other and thus at least partially restore enzyme activity (Helsinki & Yanofsky, 1963) cannot be entirely discarded.

If *mtr-26* is a frameshift mutation, the isolation of allele specific unlinked suppressors is of interest since normally this type of lesion is not affected by mutation at an unlinked suppressor locus. However, it has been reported (Riyasaty & Atkins, 1968; Riddle & Roth, 1970) that unlinked suppressors of frameshift mutations occur in *Salmonella*. The suppressors isolated in this study may be similar to those reported by these authors, particularly as none of them appear to map near any of the reported super-suppressors in *Neurospora* (Seale, 1968; Case & Giles, 1968*a, b*). In each of the three strains studied, the suppression of the *mtr* phenotype was not complete in that partial resistance to 4MT was retained. The rate of tryptophan transport appears to be only half that of *mtr*⁺ cells. This observation was made in seven separate experiments and undoubtedly represents a true reduction and not merely an experimental artifact. As observed in wild-type cells, phenylalanine (10^{-3} M) almost completely blocks tryptophan transport; however, excess arginine, which has no effect in *mtr*⁺ strains, also reduces the amount of tryptophan uptake in these two strains. If these suppressor mutations were either general membrane mutants or alternatively forward mutations in a structural gene of another permease system, thereby expanding its substrate range to include tryptophan in addition to arginine (Pall, 1969; Wolfinbarger & Debusk, 1971), then the suppressor should not be allele specific (i.e. only suppressing *mtr-26*). On the other hand, it may be a missense suppressor which partially restores tryptophan uptake, but at the same time enables some arginine to be transported via the *mtr* system. This would explain both the specificity of the suppressor as well as the sensitivity of the system to competitive arginine inhibition.

The frequency of 4MT-resistant recombinants obtained from revertant $\times$ wild crosses, is different to that of sensitives obtained when these resistant colonies are backcrossed to *mtr-26*. In each case, the frequency of recombination between the same two mutant sites is being measured, thus the different frequencies may be indicative of different gene conversion frequencies from 'wild' to 'mutant' and 'mutant' to 'wild'. Self crosses of *mtr-26* have not produced any sensitive colonies, and the frequencies of recombinants are considerably greater than the spontaneous mutation frequencies reported by Brink *et al.* (1969).

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