SUPPRESSORS OF AMINO ACID UPTAKE MUTANTS OF NEUROSPORA[1]

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DETAILED analyses of mutation and recombination in a single gene would be facilitated by a system which permitted the effective selection of mutants from a preponderantly wild-type population and of rare nonmutants from a population of mutants. There have been many genetic studies of genes controlling nutritional requirements, employing selection to detect rare nonmutants. However, in such cases, selection of mutants has not been achieved, and the less efficient "enrichment" methods have been employed. Mutants have been selected in genes controlling resistance to growth inhibitors, but here the selection of nonmutants has been the problem. There have been very few cases in fungi in which selection in both directions has been accomplished (REISSIG 1963; APIRION 1965; CALVORI and MORPURGO 1966).

The growth of wild-type Neurospora is inhibited when minimal medium is supplemented with either of the amino acid analogs 4-methyltryptophan (4MT) or para-fluorophenylalanine (FPA). Mutants resistant to these analogs were selected and were shown to result from the loss of a normal uptake system for tryptophan, phenylalanine and several other amino acids (LESTER 1966; STADLER 1966). Mutants at this locus (called *mtr*) can be efficiently selected on medium supplemented with the inhibitors. It seemed that this system might also be suitable for the selection of nonmutants, if the strains involved were auxotrophs requiring one of those amino acids of which the uptake was controlled by the *mtr* gene. A medium supplemented with a very low level of the required amino acid would then become selective for the recovery (by either mutation or recombination) of the normal uptake system.

This report describes the selection of revertants of *mtr* in strains requiring tryptophan. The revertants have been analysed genetically and some properties of their recovered uptake systems have been observed. A preliminary report of this work appeared earlier (STADLER 1965).

MATERIALS AND METHODS

The same methods were employed as in the previous study of *mtr* mutants (STADLER 1966). Tests for resistance were performed on plates of minimal sorbose medium supplemented with 110 mg DL-4MT or 10 mg DL-FPA per liter. Uptake measurements were performed on mycelial pads grown 48 hours in still culture. They were incubated for 10 minutes at 25°C with agitation in solutions of tritium-labelled L-amino acids at a concentration of 10^{-4}M. The pads were washed

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and extracted in hot water for counting by liquid scintillation. The pads were dried and weighed, and the concentrations are reported here in terms of uptake per unit mass of mycelium. (The rates of tryptophan uptake reported here are about twofold lower than in the previous study. Those high rates have been traced to a particular preparation of tritiated tryptophan, but the reason is not understood. However, the *relative* rates for different strains and the conclusions drawn from them are the same as in the earlier study.)

RESULTS

Revertants were selected in strains of the genetic constitution *tryp-1 mtr* by plating in medium supplemented with very low levels of tryptophan. In order to determine the most satisfactory concentration, the *tryp-1 mtr* strain was compared to *tryp-1 mtr*$^+$ for growth on sorbose medium with various levels of tryptophan. On plates containing 0.5 mg of L-tryptophan per liter, individual conidia of *tryp-1 mtr*$^+$ could form colonies, while conidia of *tryp-1 mtr* did not grow. This low tryptophan (LT) medium was used in the selection of revertants.

Conidia of three *tryp-1 mtr* stocks (*mtr* 17, 18 and 21) were suspended in water and treated with doses of ultraviolet light sufficient to kill 50 to 80% of the cells. These suspensions were mixed with LT sorbose medium kept liquid at 45°C and immediately poured in plates. Each plate contained approximately 10^5 cells. The plates were incubated at 25° for a period of 4 to 6 days and then examined for revertants. There was rather heavy background growth on these plates and on the control plates with untreated conidia; although isolated cells of this strain cannot grow on LT, these concentrated preparations achieved considerable growth. Even so, there were many points of especially dense growth on the plates of treated conidia, and these were much less frequent on the untreated controls.

Samples were cut from the centers of ten of these dense growth points from each of the three strains, and these were grown in slants of minimal medium plus tryptophan. When the slant cultures had matured, conidial suspensions were prepared and duplicate samples were plated on minimal sorbose medium and on the same medium supplemented either with LT or with a high level of tryptophan (HT: 100 times the concentration of LT). About half the cultures tested grew on minimal as well as on LT and HT; these were revertants to *tryp*$^+$ and were discarded. The remaining strains were uptake revertants and still required tryptophan. For each of these, counts were made of colonies produced on LT and HT by duplicate inocula. If the LT count was significantly below the HT count, a single robust colony was reisolated from LT, grown and retested. A maximum of three successive reisolations was sufficient to purify all the revertants. Thirteen revertants were carried through vegetative purification, but four of them failed to give viable revertant progeny in crosses and were discarded. The remaining nine (three from each *tryp mtr* strain) were the subject of further study.

The revertants were tested for resistance to 4MT and FPA. These tests could not be performed on medium supplemented with tryptophan, because it blocks the inhibition by the analogs, and even the sensitive strains can grow. However, the *tryp-1* mutant is capable of growth on medium supplemented with indole (10 mg/liter) instead of tryptophan, and indole does not block the inhibition. The

TABLE 1

Growth tests on sorbose plates of the nine revertants and their mutational ancestors

Strain	Minimal	High tryptophan	Low tryptophan	Indole	Indole + 4MT	Indole + FPA
tryp-1	0	+	+	+	0	0
tryp-1 mtr	0	+	0	+	+	+
revertants	0	+	+	+	+	0

4MT: 4-methyltryptophan. FPA: para-fluorophenylalanine.
Concentrations used per liter: 50 mg (HT) on 0.5 mg (LT) L-tryptophan; 10 mg indole; 110 mg DL-4MT; 10 mg DL-FPA.

nine revertants were compared to the *tryp mtr* stocks from which they were derived for growth on minimal sorbose plates supplemented with indole plus 4MT or FPA (Table 1). All the revertants were sensitive to FPA, but they appeared to retain the parental resistance to 4MT. In order to make a quantitative measurement of the growth inhibition, mycelial pads were weighed after growth in liquid medium supplemented with the inhibitors (Table 2). (These tests were made on revertant strains which did not require tryptophan, which were recovered from crosses of the original revertants to *mtr* $tryp^+$ strains). The results indicate that the revertants have become fully sensitive to FPA while remaining partially resistant to 4MT. We have no explanation for this differential effect on the two kinds of inhibition.

Genetic analysis of revertants. Each of the nine revertants was crossed to the *mtr* allele from which it was derived. The analysis of random spores from one of these crosses is shown in Table 3. Eleven dissected asci from this cross all showed 4:4 segregation for resistance to FPA. The following conclusions may be drawn from these results: (1) sensitivity to FPA is determined by a single-gene differ-

TABLE 2

Inhibition by 4MT and FPA in liquid growth tests

Strain	Minimal	4MT	FPA
mtr^+:			
col-4	46	10(22%)	7(15%)
mtr:			
*mtr*17 *col-4*	44	46(104%)	33(75%)
*mtr*18 *col-4*	47	50(106%)	40(85%)
*mtr*21 *col-4*	50	46(92%)	32(64%)
Revertants:			
*mtr*17 *col-4 R*17-2	40	34(85%)	10(25%)
*mtr*18 *col-4 R*18-1	43	33(77%)	5(12%)
*mtr*18 *col-4 R*18-3	36	31(86%)	6(17%)
*mtr*21 *col-4 R*21-1	48	30(63%)	7(15%)
*mtr*21 *col-4 R*21-3	33	20(61%)	3(9%)

Cultures were grown 72 hours at 25°C in 20ml liquid minimal medium without shaking. Inhibitor concentrations were 20mg 4MT/liter and 1mg FPA/liter. Results are given as dry weight in milligrams. Percent of control (no inhibitor) growth is given in parenthesis.

TABLE 3

Analysis of the progeny from random spores of the cross tryp-1 mtr*17* col-4 R*17-3* × *mtr*17 *by growth tests on sorbose plates*

Number of spores	Growth habit	Growth test on: Minimal	Low tryptophan	Indole	Indole + FPA	Genotype
6	col	+	+	+	+	*tryp*+ *mtr col su*+
9	col	+	+	+	0	*tryp*+ *mtr col su*
5	col	0	0	+	+	*tryp mtr col su*+
4	col	0	+	+	0	*tryp mtr col su*
9	col+	+	+	+	+	*tryp*+ *mtr col*+ *su*+
7	col+	+	+	+	0	*tryp*+ *mtr col*+ *su*
7	col+	0	0	+	+	*tryp mtr col*+ *su*+
5	col+	0	+	+	0	*tryp mtr col*+ *su*

ence between the two parents of this cross; (2) the same gene which permits growth of mutants which require tryptophan on LT determines sensitivity to FPA; (3) this gene is not closely linked to *col-4; mtr* is tightly linked to *col-4* (STADLER 1966); therefore the revertant carries a suppressor of *mtr* which is not closely linked to the *mtr* locus; it is not closely linked to *tryp-1* either. This interpretation was confirmed by crossing one of the putative *mtr col su* progeny to wild type. Approximately half of the *col* progeny of this cross showed the unsuppressed *mtr* phenotype.

Corresponding crosses of the other eight revertants gave the same general result, indicating an unlinked suppressor mutation in every case. Several of these crosses gave a distinct majority of FPA-resistant progeny, but the proportion of resistant isolates was very similar in the *col* and the *col*+ classes. Such a result indicates an unlinked suppressor mutation with a lower viability than its nonsuppressing allele. The suppressor mutants will be designated by the symbol su^{mtr}, but in the following account it will be shortened to *su* for convenience. The individual suppressor mutations will be designated by numbers which include the number of the *mtr* allele with which each was isolated. Thus *su*18-2 is the second suppressor of *mtr*18.

In order to learn whether the suppressors were specific to the alleles with which they were recovered, crosses of suppressed *mtr* strains to other unsuppressed alleles were analysed. The closely linked *col* marker was used to distinguish the two *mtr* alleles in the progeny. An example of this type of cross is shown in Table 4. The result shows that *su*18-3 is capable of suppressing *mtr*17 as well as *mtr*18. This same type of test for cross-suppression has been made in 11 different combinations involving five of the suppressors (listed in Table 4) and the result has been positive in every case. It is therefore concluded that these suppressors do not show allele specificity in their action.

The location of the suppressor mutants with respect to each other was investigated by crosses of the type shown in Table 5. Analysis of 60 random ascospores from the cross between two different suppressed *mtr* strains revealed that every

TABLE 4

Genetic test for specificity of action of the suppressors.

Number of spores	Growth habit	Resistance to FPA
*mtr*18 *col-4 su*18-3 × *mtr*17 (random spores):		
11	col	0
12	col	+
19	col+	0
14	col+	+
Combinations tested (all gave suppression):		
su	*mtr*	
17–1	15, 16, 17, 18, 21	
17–2	15, 17	
17–3	15, 16, 17, 21	
18–2	6, 15, 18	
18–3	17, 18	

spore showed the suppressed *mtr* phenotype on the inhibitor tests. This indicates that the *su* genes in the two parents were closely linked to each other. Eight other crosses of this type were examined (Table 5). In order to detect su^+ (nonsuppressed) recombinants with a higher resolution, the ascospores were germinated on plates of sorbose medium supplemented with FPA. From 400 to 800 ascospores were germinated from each cross, and *no* FPA-resistant colonies appeared. This result suggests that all of the *su* mutations which were involved in these crosses may be in the same gene.

TABLE 5

Analysis of linkage relations between the suppressors.

Number of spores	Growth habit	Resistance to FPA	Resistance to 4MT
*mtr*17 *su*17-1 × *mtr*18 *col-4 su*18-3 (random spores):			
31	col+	0	+
29	col	0	+
Controls:			
wild type	col+	0	0
mtr18	col+	+	+

Crosses analyzed by germination of 400 to 800 spores on minimal sorbose plus FPA:
*mtr*17 *su*17-1 × *mtr*17 *col-4 su*17-2
× *mtr*17 *col-4 su*17-3
*mtr*21 *col-4 su*21-1 × *mtr*17 *su*17-1
× *mtr*17 *su*17-2
× *mtr*17 *su*17-3
*mtr*6 *su*18-2 × *mtr*17 *su*17-1
× *mtr*17 *su*17-2
× *mtr*17 *su*17-3
Result: *all* spores sensitive.

In several of the crosses segregating for one or the other of the suppressors, it was noted that *su* showed linkage to the mating type locus (which is in the left arm of linkage group I). This was further demonstrated by the analysis of 11 ordered asci from the cross *mtr*17 *su*17-1 *a* × *mtr*17 *A*. There were seven parental ditypes for *su* and mating type, and there were four tetratypes. In every one of the parental ditypes, both loci showed 1st division segregation patterns. The four tetratypes included three with 2nd division segregation for mating type and one with 2nd division segregation for *su*. These results show that the suppressor locus is near the centromere of linkage group I. (The fact that the single ascus with 2nd division segregation for *su* had 1st division segregation for mating type suggests that *su* is in the right arm.)

The location of *su* was further specified by the cross *hist-2 mtr*32 *A* × *mtr*17 *su*17-2 *a*. The *hist-2* locus is in the right arm of linkage group I, very near the centromere. The analysis of 23 dissected asci gave no recombination between *hist* and *su* and the centromere (all parental ditypes with 1st division segregation at both loci). The $hist^{+}$ su^{+} recombinants were selected from random spores of this cross by plating on minimal sorbose medium plus FPA. Among approximately 750 germinated spores there were six selected growers. There were isolated to confirm their classification and to determine their mating types. All six proved to be $hist^{+}$ su^{+} *a*. This reveals that *su* is to the right of *hist-2* and closely linked to it.

Uptake properties of suppressed mtr *strains*. Rates of uptake of phenylalanine and tryptophan by the suppressed mutants are shown in Table 6. Uptake of these amino acids is greatly retarded by the *mtr* mutation. It may be seen that the suppressor mutation has restored the uptake to near-normal rates. In like manner the suppressed mutants have been shown to have recovered efficient uptake of those other amino acids of which the uptake was strongly retarded in the *mtr* strains (tryrosine, leucine, isoleucine, valine, methionine and cysteine.)

The suppressed mutants approximate wild type in their simple uptake rates. However, a basic difference from wild type was revealed in studies of the blocking of tryptophan uptake by other amino acids. Blocking by a large variety of

TABLE 6

Uptake properties of suppressed mtr *strains*

Strain	Phenylalanine	Tryptophan	Tryptophan blocked by arginine	Tryptophan blocked by lysine
wild type	19.8	19.0	18.2	18.8
*mtr*16	4.1	1.4	0.2	0.3
*mtr*18	2.0	0.3	..	..
*mtr*16 *su*17-3	14.2	17.9	2.4	2.3
*mtr*18 *su*18-1	22.5	13.3	2.2	..
*mtr*18 *su*18-3	29.2	15.4	1.2	..

Pads were in 10^{-4}M L-phenylalanine-H^3 or L-tryptophan-H^3 for 10 minutes at 25°C before extraction. The concentration of the unlabelled blocking amino acids was 2 × 10^{-4}M. Concentrations are expressed as moles per liter × 10^4, assuming a pad volume equal to the volume of water weighing five times the dry weight of the pad.

other amino acids is very pronounced. Especially notable is the effective blocking of tryptophan uptake by arginine and lysine (Table 6); these amino acids are completely ineffective in blocking this uptake by wild type.

Further uptake studies have shown that arginine itself is taken up at similar rates by all the strains (wild type, *mtr* mutants, suppressed mutants). Tryptophan is not an effective inhibitor of arginine uptake in any of these strains. The behavior of lysine is much like that of arginine in these experiments.

DISCUSSION

The genetic and physiological studies of the revertants from *mtr* mutants seem most simply explained by the following interpretation: recovered uptake of aromatic amino acids (and their analogs) has resulted from mutation in a different gene controlling a different uptake system. This second system does not normally carry the aromatic amino acids; it has a high affinity for arginine and lysine, and it may be the normal vehicle for the uptake of these materials. The mutation has modified this uptake system so as to expand its substrate range, but it still has special affinity for arginine and lysine.

The suppressor mutations may have revealed the structural gene for a normal permease for arginine and lysine. If so, we might hope to obtain another class of mutants at the same locus by selecting for resistance to inhibitory analogs of these amino acids. We have no information on this point as yet. Attempts to isolate mutants (from wild type 74A) which are resistant to canavanine have been unsuccessful.

The hypothesis that two uptake systems are involved has been substantiated by studies of the uptake of histidine. It appears that histidine is taken up in wild type by both systems. MATHIESON and CATCHESIDE (1955) demonstrated that histidine mutants were unable to grow on histidine-supplemented medium if excess amounts of both tryptophan and arginine were also present. We have confirmed this observation and have found that uptake of histidine is *partially* blocked by either of these amino acids. The uptake of histidine by *mtr* strains is cut to about half the wild-type rate, and it can be *completely* blocked by arginine. Growth of *hist mtr* double mutants on medium supplemented with histidine is prevented if an excess of arginine is also present. This serves as a selective medium for the recovery of the mtr^+ phenotype. Studies of mutation and recombination at the *mtr* locus is strains requiring histidine will be the subject of a later report.

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SUMMARY

Mutations at the *mtr* locus (methyltryptophan resistance) result in greatly retarded uptake of aromatic amino acids. An *mtr* strain with a requirement for tryptophan cannot grow on a medium supplemented with very low levels of tryptophan. This system has permitted the selection of nine genetic revertants which have recovered efficient uptake of the aromatic amino acids. All nine appear

alike in that they result from suppressor mutations at another locus, unlinked to *mtr*. The suppressors appear to be nonspecific: they suppress all *mtr* alleles with which they have been combined. A comparison of uptake properties of the suppressed *mtr* strains with those of wild type has suggested that the suppressor mutations have altered a second uptake system.

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