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Forward and back mutation in the *pyr3* region of Neurospora. I. Mutations from arginine dependence to prototrophy*

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1. INTRODUCTION

An important limitation of most screening systems available for mutation studies is that they allow selection of the mutations in one direction only, e.g. from nutritional deficiency to prototrophy, from inactive to active allele. The recent work of Freese (1959) on bacteriophage has strikingly vindicated the importance of working with systems which permit the selection of mutants in both directions (forward and back) at a single locus. As pointed out by Westergaard (1960), it is desirable that systems of this kind be established in organisms of different degrees of complexity. Moreover, it is desirable to be able to screen not only for the extreme phenotypes, but also for the widest possible range of intermediate mutant phenotypes.

With such considerations in mind, we have been studying the behaviour of a system for screening forward and back mutations at the *pyr3* (*pyr*) locus of Neurospora (Reissig, 1958, 1959). Selection for forward mutations at the *pyr* locus is possible since *pyr* suppresses a certain arginine mutant, *arg2* (*arg*); i.e. the double mutant *arg pyr* is pyrimidine-dependent, but arginine-independent. On plating uninucleate conidia of the *arg* strain on a medium supplemented with pyrimidine, but lacking arginine, two phenotypically distinct classes of mutants are obtained: pyrimidine-dependent mutants of the *arg pyr* constitution (I in Fig. 1), and prototrophs. The back-mutation pattern of the *pyr* mutants can be studied by the usual back-mutation technique (II in Fig. 1).

The present paper describes the prototrophs obtained after treating a macroconidial *arg* strain with u.v., and plating on a medium free of arginine or pyrimidines. As will be shown, such reversions can result from any of the following events (Fig. 1): back mutation at *arg* (III); forward mutation from pyr^+ to *pyr* (I), followed by heterokaryon formation; or forward mutation from pyr^+ to $pyr^{su\text{-}arg}$ (IV).

2. MATERIALS AND METHODS

Strains

The arginine-dependent strain carries the *arg2* (*arg*) allele, isolated as 33442 by Beadle & Tatum (1945) after u.v. treatment. As *arg* can utilize citrulline as well as

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arginine for growth, this allele has been occasionally referred to as *cit* (Mitchell & Mitchell, 1952; Reissig, 1958, 1959). Other mutant alleles used are: *pyr*, obtained by spontaneous mutation (Reissig, 1958) and allelic to the *pyr3* gene isolated as 37301 (Mitchell & Mitchell, 1956; Reissig, unpublished); *colonial-4* (*co*), isolated as 70007 (Mitchell & Mitchell, 1954); and *cot*, isolated as C102 (Mitchell & Mitchell,

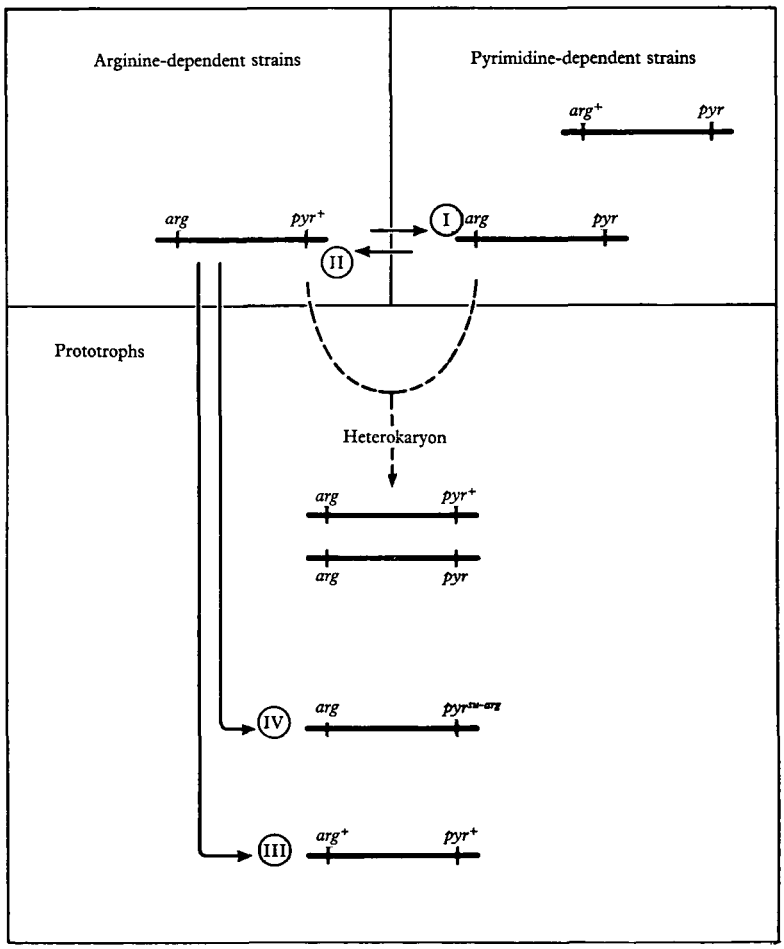

Fig. 1. Mutations at the *pyr* and *arg* locus which are readily obtained by screening methods reported in this study. The solid arrows indicate mutational steps. The broken arrow indicates the composition of the heterokaryon.

1954). The latter two mutants determine colonial growth: *cot* gives small colonies at 32° C., but spreading growth at 25° C.; while *co* grows colonially at all temperatures. The *cot co* and *cot co*+ strains are easily distinguished from each other by culturing at 32° C., cooling to 25° C., and some hours later examining the halos which form around the small colonies. Intermediate colonial phenotypes, such as those obtained in pseudo-wild types (Mitchell, Pittenger & Mitchell, 1952), can also be identified by this method, but not with certainty.

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All the above markers are linked (Mitchell & Mitchell, 1954). The reported map distances are: centromere–13–*co*–0·4–*arg*–1·1–*pyr*–25–*cot*, on chromosome IV.

Strain ED38-35a was used in all mutation experiments. It is macroconidial, and carries the *arg*, *co* and *cot* markers. The *cot* allele was present in all the strains used.

Culture media and temperature

The M/15 phosphate medium of Strauss (1951) was arbitrarily chosen for the screening of mutants. It was adjusted to pH 4·5, and supplemented with 1 gm./l. of ammonium sulphate. The salt solution was autoclaved separately from the other ingredients. Crosses were performed on the medium of Westergaard & Mitchell (1947). In all other cases, the solid medium of Fries (Beadle & Tatum, 1945) was used.

All cultures were grown at 25° C. In order to obtain colonial growth, all plates were incubated at 32° C., unless otherwise indicated.

Supplements

L-Arginine HCl and L-lysine mono HCl were obtained from Light and Co. The L-canavanine sulphate was prepared from jack beans (*Canavalia ensiformis*) by the method of N. H. Horowitz and M. Fling (personal communication). Hydrolysed RNA was prepared by the following method. A sample of yeast nucleic acid (The British Drug Houses, Ltd.) was divided into two equal portions. These portions were dissolved in N HCl and N NaOH, respectively, heated for 20 minutes at 100° C., mixed, neutralized, and filtered while hot. Hydrolysed RNA was added at a concentration of 0·5 to 1 g. of nucleic acid per litre of medium. Arginine was added at 0·05 to 0·1 g./l. for vegetative cultures, and 0·4 g./l. for crosses.

Production and screening of revertants

Strain ED38-35a was grown for 2–4 weeks in flasks with 50 ml. of arginine-supplemented medium. The conidia were harvested in water, filtered through cotton wool and washed three times in water by centrifugation. A portion of this suspension was irradiated with u.v. light. The control series was handled in a similar fashion, with the exception that the irradiation step was omitted. Two alternative procedures were used for irradiation. In one, the conidial suspension was exposed to u.v. while being magnetically agitated, washed once by centrifugation, diluted as required and plated at concentrations ranging from 5×10^5 to 3×10^6 conidia per plate. In the second procedure, the conidia were first plated and then irradiated within 2 hours of plating. Since the sensitivity of the conidia to u.v. increases significantly in the first few hours after plating (Reissig, 1960), the latter procedure cannot be relied upon for quantitative work.

The u.v. source was either a Philips germicidal tube TUV 15 W, or a Hanovia Germicidal Unit. In both cases most of the output of the source was at 254 mμ. All operations during and after irradiation were performed under low-intensity yellow light.

The plates for the screening of mutants were poured in three successive layers. The bottom layer consisted of 12 ml. of minimal medium. If the irradiation was to be performed on the plates, the conidial suspension was spread on top of this layer. If the conidia had been irradiated in water, they were mixed with 3 ml. of minimal medium kept liquid at 42° C., and poured as a second layer. This second layer was also added on top of the conidia after irradiation on the plates. The *arg* mutant is a leaky one; i.e. it can grow slowly in the absence of arginine. This slow growth can be inhibited by canavanine. Further supplementation with lysine protects revertant clones from partial inhibition by canavanine. Therefore, a third layer (LC) of medium supplemented with 0·05 g./l. of lysine, and 0·15 g./l. of canavanine, was added on top of the previous two layers. Unless otherwise specified, LC was added 9–12 hours after plating (cf. section 3—I).

On the fourth day the plates were cooled to 25° C. for about 7 hours. In this way the revertant colonies spread out, permitting a better differentiation between the fast-growing revertants and background clumps. The plates were scored on the fifth day. Viability was scored in a similar fashion, adding an arginine-supplemented overlayer instead of LC.

Some of the above-specified conditions for the detection of revertants are critical, and appreciable departures from them may affect the residual growth of non-mutant conidia, and the recovery of mutants. Less growth, and in general fewer mutants, are obtained by using older cultures, lower conidial densities, more canavanine, less lysine, or by adding LC at an earlier time.

It is not possible to know whether all types of revertants are recovered; indeed, there could be types which completely escape identification under the conditions employed. We believe, however, that the recovery is essentially complete for the (major) types of revertant being scored. Evidence for this comes from reconstruction experiments with four different revertants, and from the fact that a plateau in the yield of revertants is reached when plating density is increased or when addition of LC is delayed.

The effect of plating density on recovery alerted us to the possibility of falling into the pitfalls described by Grigg (1952) (see also Kølmark & Westergaard, 1952). We tested for plating interactions by two methods (Srb, 1958); plating varying dilutions of treated conidia, and plating mixtures of treated and untreated conidia. The only effect detected was a drop in the recovery of revertants in plates containing less than approximately 5×10^5 conidia. While this effect may lead to an underestimate of the reversion frequency under conditions of low survival, it points reassuringly in the opposite direction from the 'Grigg effect'.

Analysis of crosses

Ascospores were washed by centrifugation, first with sodium hypochlorite solution (*ca.* 0·7% of available chlorine), and then with water, thereby killing all vegetative cells. The suspension was then heat-activated and plated in the usual way.

The detection of infrequent arginine-dependent segregants among a population of arginine-independent colonies was accomplished by the use of replica-plating

(Reissig, 1956). In this method minimal plates are covered with discs of cigarette paper ('pipe-papers' from Rizla Cigarette Papers, Ltd., Wembley, England). The ascospores are spread on top of the paper at a density of *ca.* 100 viable spores per plate, and incubated until the colonies grow into the agar. The paper is then lifted with forceps and transferred to an arginine-supplemented plate. Colonies developing on the second plate only are either slow growers (*ca.* 1% of the population in our crosses) or arginine-dependent. The recovery of arginine-dependent colonies is affected by the extent to which the agar surface is covered with prototrophic colonies. Reconstruction plates were set up with each experiment. In such a plate, an aliquot of the population being assayed and an aliquot of a population of arginine-dependent ascospores were inoculated simultaneously. The concentration of spores in the latter population was determined by plating on arginine media. This value can then be compared with that obtained from the reconstruction plates, the ratio of the two being called 'recovery ratio'. Whenever justified, recovery ratios from simultaneous experiments were pooled for tabulation.

Testing for nutritional requirements (spot-test)

This was done by depositing with a loop the conidial suspensions to be tested on the surface of plates with the discriminating media. The plates were scored after 3 days. The revertant phenotype was scored on plates with and without arginine.

3. RESULTS

I. *Expression of the u.v.-induced revertants*

Delay in the occurrence of a mutation or in its expression is known to take place in several systems. If this delay occurs in a system for selecting drug-resistant mutants, the resistant clones would fail to grow up if the drug is added immediately after the treatment. This is precisely what is observed in the present system: the maximum yield of mutants is realized only if the cells are incubated for about 9 hours after plating and before adding LC. Several mechanisms could be responsible for this behaviour:

(*a*) Lag between the primary effect of u.v. and the occurrence of the mutation (delayed mutation).
(*b*) Lag in the phenotypic expression of the newly induced mutation (delayed phenotypic expression).
(*c*) If the resistant trait is partially recessive, a delay in the establishment of the favourable ratio of mutant and wild-type nuclei in the heterokaryon (selection delay).
(*d*) Physiological factors independent of the time when mutation was induced, e.g. the drug prevents conidial germination in the resistant strains (physiological lag).

Mechanisms (*a*) and (*b*) operate only with newly induced mutants, while (*c*) could, and (*d*) would operate regardless of the time when the mutation was induced. It

is therefore possible to decide between (*a*) and (*b*) on the one hand, and (*c*) and (*d*) on the other, by comparing the behaviour of newly-induced mutants with that of a random sample of established mutants.

A population of *arg* conidia was plated on minimal medium, irradiated, and transferred 8 hours later to a culture flask supplemented with arginine. This culture was allowed to grow and conidiate. The proportion of revertants among the conidia formed in this flask was *ca.* 0·2%. This is higher by two orders of magnitude than is

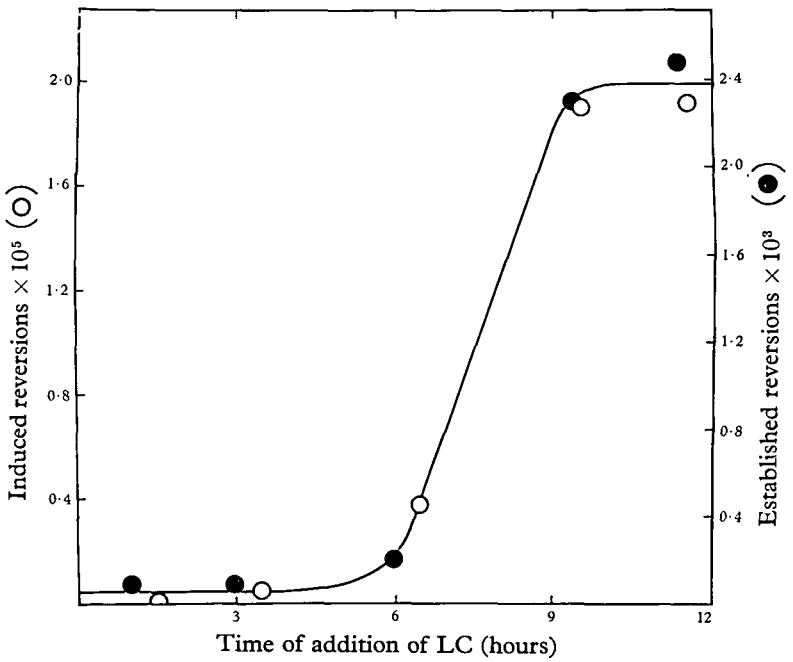

Fig. 2. Lag in the appearance of revertant colonies. The experimental procedure is described in the text. Time zero is the time of plating. Each point represents three to six plates. Closed circles, established reversions; open circles, newly induced reversions.

usually observed when scoring directly after irradiation. The difference was probably due to selection during growth. The high frequency of established mutants encountered permitted further irradiation of the conidial population without causing a significant increase in the number of revertants present. In two parallel series, conidia from the above culture and from an untreated *arg* culture were harvested, irradiated in water, and plated at concentrations of 10^5 and 10^6 per plate, respectively. The former series reflects the behaviour of established mutants, the latter the behaviour of newly-induced mutants. The results (Fig. 2) show that the time-dependence for the appearance of mutants is the same for both series, thus indicating that mechanisms (*a*) and (*b*) are either excluded or of minor importance.

Similar experiments were also performed with microconidial strains. The time of addition of LC again had a pronounced effect on the yield of mutants. In

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this case only mechanism (*d*) could be operating since the microconidia are uninucleate.

The timing of the physiological lag is correlated with conidial germination. In three experiments, the beginning of germination coincided with the time when addition of LC allowed the first clear increase in the yield of mutants. Over 50% of the conidia had germinated when the plateau was reached, supporting the hypothesis that the lag is due to interference of LC with conidial germination.

Granted that the physiological lag is the major cause of the observations depicted in Fig. 2, the evidence does not exclude the occurrence of delayed mutation, delayed phenotypic expression or selection delay as minor components of the observed phenomenon. The following experiment was designed as a more sensitive test for such effects.

Untreated *arg* spores were plated, and 9 or 11 hours later overlayered with LC. Irradiation was performed at varying times after plating and before overlayering. An untreated control series was also run. The results shown in Table 1 indicate

Table 1. *Testing for mutational delay*

Time of irradiation (hrs.)	Revertants per total conidia plated. Time of overlayering		Revertants per survivor $\times 10^5$. Time of overlayering	
	9 hrs.	11 hrs.	9 hrs.	11 hrs.
2	124	138	2·6	2·9
6½	97	95	2·4	2·4
9	115	102	3·5	3·1
	100*		3·0*	
11		117		4·4
		92*		3·4*
Untreated control		1		0·01

Each figure corresponds to eight plates with 8×10^5 conidia per plate.
* See text.

that the revertants were induced by the treatment, and, secondly, that the yield of revertants is independent of the time-lapse between irradiation and overlayering with LC. Even plates treated at 9 hours and overlayered immediately, or those treated and overlayered at 11 hours, show as many mutants as those irradiated earlier. The parallel behaviour of the series overlayered at 9 and 11 hours provides a check on the possible effects of changes in the u.v. sensitivity of the conidia (Reissig, 1960).

Most plates were treated according to the standard procedure: 3 ml. of minimal medium were added on top of the conidia immediately after irradiation. The values indicated with an asterisk are derived from plates in which the LC layer was added before the 3-ml. minimal layer, thus insuring immediate contact between the conidia

and the inhibitor. Slightly lower counts are given by these plates, but the difference is to small to warrant speculation.

The above results provide evidence against the occurrence of delayed mutation, delayed phenotypic expression, or selection delay in the present system.

II. *Dose dependence*

Fig. 3 shows the relation between mutation frequency and dose. The lower curve describes the total number of revertants per treated conidium. There is a net increase in the number of revertants at intermediate doses. The upper curve (mutation

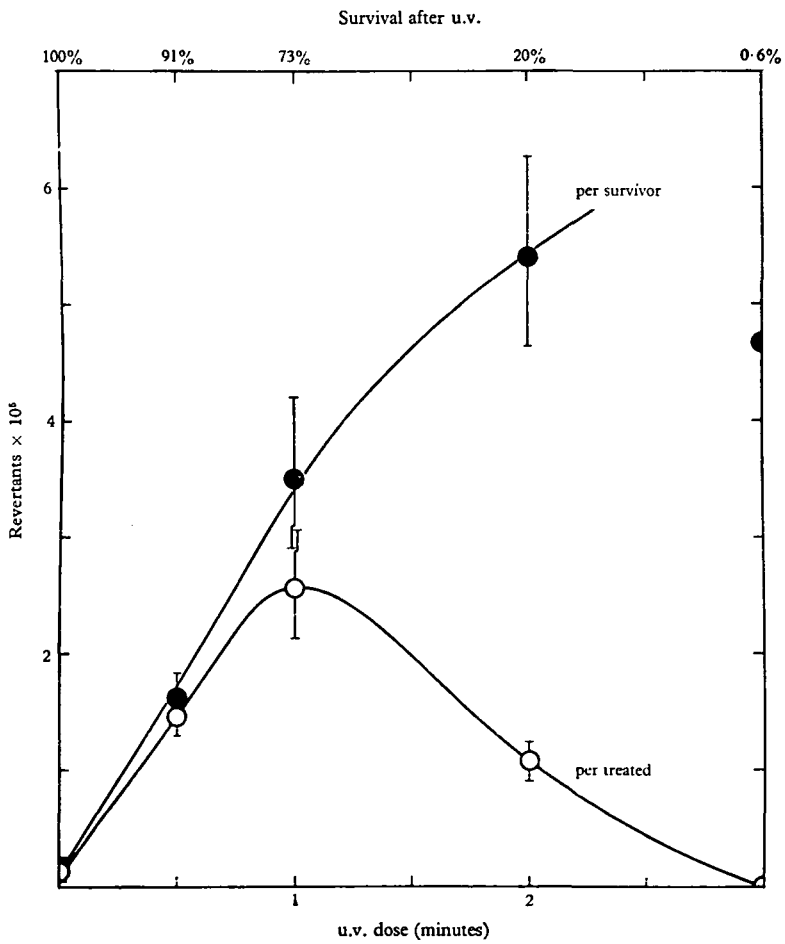

Fig. 3. Relation between reversion frequency and u.v. dose. The irradiation was performed in water. Each point represents six to twelve plates with 10^6 or 2×10^6 total spores per plate. LC was added 11 hours after plating. Confidence limits at the 95% level are indicated; except for the 3-minute points, which are based on only five colonies. The energy of the incident u.v. was $4{\cdot}5 \times 10^6$ ergs/cm^2/min., as determined with the dosimeter of Latarjet. Open circles, reversion frequency per treated conidium; closed circles, reversion frequency per conidium surviving the u.v. treatment. (Treated conidium means conidium viable prior to irradiation.)

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frequency per conidium surviving the u.v. treatment) appears to rise less steeply at the higher than at the lower doses. This is only weakly suggested by the points in Fig. 3. Additional data from the same experiment, which are not included in the figure, provide supporting evidence for this, and an explanation. The experiment included two parallel series; in one series LC was added at 8 hours after plating, in the other (Fig. 3) at 11 hours. The 8-hour counts were lower in every case, indicating that the physiological lag extended beyond this time. The ratios of the 11-hour to the 8-hour mutation frequencies are 1·9:1, 2·7:1, 3·3:1, and 7·2:1, at 0, $\frac{1}{2}$, 1 and 2 minutes of irradiation, respectively (actual figures were 21:11, 235:88, 117:36 and 172:24). The ratio for the 2-minute irradiation series is significantly larger than the others. This indicates that heavy doses of u.v. lengthen the physiological lag, and thus can be expected to cause a decline in the recovery of revertants.

III. *Genetics of the revertants*

(*a*) *Origin and purification of the revertants.* Thirty-three revertants were analysed genetically. One (D1) was of spontaneous origin; the rest were obtained after u.v. irradiation. Calculation from the data in Table 3 (Stevens, 1942) indicates that at most two of the mutants isolated following u.v. treatment may be of spontaneous origin ($P < 0{\cdot}05$). Thus, most or all of the mutants tested were induced by the irradiation, and therefore originated from independent events.

The thirty-three revertants were crossed to an *arg* tester, and the resultant ascospores plated on minimal and on arginine-supplemented medium. Twenty-nine of the crosses yielded prototrophic colonies; one prototroph was isolated from each cross and labelled as indicated in Table 2. In this way, the revertants were isolated as homokaryons, free of unmutated *arg* nuclei. All further work was done with these isolates.

Table 2. *Derivation of the revertants obtained after u.v. treatment*

Experiment	Survival after irradiation (percentage of untreated)	Number of revertants in comparable series		Revertants isolated
		Untreated	Irradiated	
A	78	0	38	A1 to A17
B	29	0	17	B1 to B11
C	10	0	100	C1 to C4

The prototrophs could not have originated by back mutation of the tester, since the majority of the prototrophic segregants from each cross carried the *co* marker, which was present in the treated chromosome IV but not in the tester chromosome.

Of the remaining four crosses, two yielded too few ascospores for testing. The other two (involving B9 and A15) will be discussed in section III (*f*).

(*b*) *Linkage of the reversion site to* co. The presence of the *co* marker at about 0·4 unit from *arg* provides a simple method for testing the hypothesis that reversion is due to back mutation at the *arg* locus. Twenty-five revertants and two control

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arg$^+$ strains, all marked with *co*, were crossed to *co*$^+$ *arg* testers. Three different tester strains were used; the pooled data from crosses to all three testers are presented in Table 3.

Table 3. *Recombination frequencies between* co *and the reversion site*

Crosses: *co reversion* × *co*$^+$ *arg*
or *co arg*$^+$ × *co*$^+$ *arg* (control)

Group	*co* parent	Colonies on minimal (total)	Colonies on minimal (%*co*$^+$)	Colonies on arginine (%*co*$^+$)	% germination
	Control	4026	0·2	47 ± 1	84 ± 1
	Revertants:				
1	A1	1653	0·4	44 ± 4	87 ± 2
	A2	734	0·5	23 ± 3	71 ± 3
	A3	529	0·2	28 ± 3	73 ± 3
	A4	848	0·2	50 ± 3	79 ± 3
	A5	1208	0·2	52 ± 3	90 ± 2
	A6	719	0·0	47 ± 3	61 ± 3
	A7	526	0·4	47 ± 3	57 ± 5
	A8	1135	0·2	54 ± 3	84 ± 3
	A9	504	1·6	31 ± 4	70 ± 3
	A10	409	0·7	45 ± 4	70 ± 3
	A11	620	0·5	64 ± 3	61 ± 3
	B1	753	0·8	46 ± 3	92 ± 2
	B2	463	0·0	52 ± 3	95 ± 2
	B3	1502	0·3	45 ± 2	90 ± 2
	B4	1079	0·9	28 ± 3	82 ± 3
	B5	362	0·8	46 ± 4	77 ± 3
	B6	620	0·3	52 ± 3	83 ± 2
	B7	1053	0·1	50 ± 3	82 ± 3
	C1	46	0·0	33 ± 7	
	C4	14	0·0	43 ± 9	78 ± 4
2	B8	2263	0·7	44 ± 3	80 ± 4
	C2	1167	1·2	23 ± 2	73 ± 3
3	A12	751	4·7	50 ± 4	93 ± 2
	C3	1735	6·3	50 ± 2	66 ± 2
	D1	3163	1·5	48 ± 3	93 ± 2

Standard deviations are indicated for the last two columns.
The classification of the revertants in groups 2 and 3 derives from Table 4. All other strains are classed under group 1.

From the low percentage of *co*$^+$ colonies found on the minimal plates, it can be concluded that the site of reversion is closely linked with *co*; the data, however, are not homogeneous. This is perhaps not surprising since it is known that the genetic background of the strains involved can modify map distances (Stadler, 1956). The revertants used here had all been outcrossed. Alternatively, true differences in the location of the reversion site may be involved, though this point cannot be settled from the data reported so far. Incidentally, comparison of the data from crosses to the different testers showed that the heterogeneity did not derive from the use of several testers.

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No explanation can be offered for the shortage of co^+ colonies on the arginine plates of some of the crosses (Table 3). This shortage affects the tester chromosome and occurs in crosses involving any one of the three testers.

(c) *Mapping the reversions between* co *and* pyr. By the use of three-point crosses it should be possible to eliminate the variability in map distances due to factors located outside the interval covered by the three markers. The markers used were *co*, *arg* and *pyr*. In a control series, ascospores from the cross *co arg*$^+$ *pyr*$^+ \times co^+$ *arg pyr* were plated on arginine-supplemented plates. Less than 3% of the colonies were co^+, i.e. crossovers between co^+ and pyr^+. As expected, spot-tests showed these colonies to be either arginine-dependent (crossovers in the *arg-pyr* region) or prototrophic (crossovers in the *co-arg* region). The results of this cross, and of similar tests involving thirteen different revertants, are shown in Table 4.

Table 4. *Location of the reversion site in relation to* co *and* pyr

Crosses: *co reversion pyr*$^+ \times co^+$ *arg pyr*
or *co arg*$^+$ *pyr*$^+ \times co^+$ *arg pyr* (control)

Group	*co* parent	Colonies on arginine (total)	Colonies on arginine (%co^+)	Colonies on hydr. RNA (%co^+)	% germination	co^+ pyr^+ recombinants tested for arginine dependence: Dependent	co^+ pyr^+ recombinants tested for arginine dependence: Independent	Ratio of distances *co-reversion* / *co-pyr*
	Controls	3449	2·6	50 ± 3	84	74	7	0·09
	Revertants:							
1	A6	1618	1·3	48 ± 3	96	19	0	0·0
	A13	417	6·0	76 ± 5	57	24	0	0·0
	A14	1596	3·6	49 ± 4	90	36	6	0·14
	B1	1802	4·7	*ca.* 50	93	41	4	0·09
	B2	2622	5·3	*ca.* 50	91	50	1	0·02
	B3	1720	2·3	50 ± 3	92	33	5	0·13
	B4	2383	3·2	46 ± 3	94	39	5	0·11
	B5	1268	3·2	51 ± 4	91	31	4	0·11
2	B8	1088	4·6	25 ± 5	92	31	14	0·31
	C2	2036	1·7	32 ± 5		9	22	0·71
3	A12	2055	4·7	52 ± 2	89	0	58	1·0
	C3	2310	5·1	51 ± 4	93	0	66	1·0
	D1	3614	1·3	29 ± 3	79	0	37	1·0

Some standard deviations are indicated. For the germination percentages, the standard deviations range from 1% to 4%.

Because of the suppressor effect of *pyr* on the arginine requirement, it is not necessary to add arginine for the growth of the double mutant. The expectation for co^+ of hydrolysed RNA is slightly lower than 50% since the *arg pyr*$^+$ recombinants cannot grow on this medium. We can offer no explanation for the much larger shortage of co^+ in some of the crosses. This shortage, like the shortage of co^+ observed in some of the crosses in Table 3, affects the tester chromosome.

Testing for the *arg* phenotype in order to classify the recombinants according to genotype (Table 4) would be misleading if pseudo-wild types contributed significantly to the number of presumed recombinants. This possibility was ruled out by

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crossing most of the arginine-independent recombinants to an appropriate tester, and analysing the progeny of such crosses.

The data of Table 4 have been classified in three groups. The revertants giving ratios in agreement with the expectation that the reversion site is allelic with *arg*, have been placed in group 1. Revertants giving ratios significantly different from the controls, but lower than 1·0, were classed in group 2; while those with a ratio of 1·0 were placed in group 3.

The abnormal linkage relations found in groups 2 and 3 could be due to alterations in the actual location of the *arg* locus which resulted from the mutagenic treatment (e.g. a small inversion), or to other factors affecting recombination frequencies differentially in the intervals under consideration. Alternatively, a suppressor locus might be involved. This hypothesis can be submitted to a critical test, since it postulates the persistence of the *arg* allele in the arginine-independent revertants.

(*d*) *The suppressor hypothesis.* According to this hypothesis, a suppressor closely linked or allelic to *pyr* is the cause of reversion in the case of group 3 revertants. In group 2 revertants the suppressor would be located between *arg* and *pyr*. In crosses between a suppressed revertant and a wild-type strain, *arg* would segregate out in half of the recombinants between *arg* and the suppressor. The replica plating method was used to test for the presence of *arg* in such crosses (Table 5).

Column IV of Table 5 indicates the recovery ratio in the reconstruction series of each replica-plating experiment. This ratio is expressed in terms of the actual number of colonies observed. Correction for recovery was made by multiplying the total number of arg^+ colonies by the recovery ratio (column V).

The data of column V show that *arg* is indeed still present in the group 3 revertants, but not in the others. These results can better be discussed by reference to Table 6, in which the data from Tables 3, 4 and 5 are recalculated for the same interval. A small or zero value (depending on the method of calculation) for the *arg-reversion* distance indicates that both sites are allelic, i.e. that back mutation is involved. This is the case for group 1 revertants. A value significantly larger than zero indicates non-allelism, i.e. that a suppressor is involved. This is clearly the case for group 3 mutants, where there is also good agreement for the values obtained by different methods. Conflicting results are obtained for group 2 revertants: the maximum *arg-reversion* distance calculated from Table 5 (see Table 6) is 0·3, but other methods of calculation give significantly larger distances. This discrepancy points to some kind of anomalous linkage relation in the region under consideration for group 2 revertants. Once abnormal linkage relations have to be postulated to explain the discrepancy, the differences between group 1 and group 2 revertants can also be understood. In the absence of positive evidence for the persistence of the *arg* allele in the latter strains, it appears probable that group 2 revertants are the result of back mutation at *arg*.

One might at this stage consider whether contamination, mutation or genotypes mimicking the *arg* phenotype might be responsible for the appearance of arginine-dependent segregants in some of the crosses described above. As a check against mimic genotypes, nine of the arginine-dependent segregants listed in Table 5 (six

Table 5. *Recovery of* arg *from some of the revertants by means of replica plating*

Crosses: revertant (or *arg*+ control) × *arg*+

Group	Strain	*arg*+ segregants: *arg*+	*arg*+ segregants: Recovery ratio	*arg*+ segregants: Corrected *arg*+ III × IV	*arg* segregants	Distance *arg*-*reversion* $\frac{200 \times VI}{V + VI}$
I	II	III	IV	V	VI	VII
1	Controls	282	13/14			
		570	65/3 × 20			
		210	11/16			
	Sum			1023	0	0
	A14	1305	74/200	483	0	0
	C4	387	6/19			
		623	65/3 × 20			
	Sum			797	0	0
2	B8	1516	74/200			
		2860	71/135			
	Sum			2065	0	0
	C2	393	13/14			
		3597	71/135			
	Sum			2257	0	0
3	A12*	*ca.* 200			2	*ca.* 2
	C3-1	814	6/19		6	
	C3-2	667	74/200		5	
	C3-3	704	74/200		8	
	Sum			764	19	4·9
	D1-1	678	6/19		3	
		656	11/16		5	
	D1-2	881	11/16		3	
	Sum			1271	11	1·7

*Crosses involving A12 cannot be easily analysed by means of replica-plating in view of the partial arginine requirement of this strain (cf. section III (*g*)). The tabulated data were obtained by microscopic scanning of a plate and isolation of presumptive arginine-dependent segregants.

derived from D1 and three from C3) were crossed to an *arg* tester. Among more than 200 ascospores plated out from each cross, all were arginine-dependent, confirming that their genetic constitution was *arg*.

There are three lines of evidence supporting the recombinational origin of the *arg* segregants obtained from crosses with group 3 revertants:

(i) Various sexual isolates of C3 and D1 (C3-1, C3-2 and C3-3; D1-1 and D1-2, in Table 5) gave comparable yields of *arg* segregants.

(ii) In the cross C3-1 × wild, where the fourth chromosomes of both strains were differentially marked with *co* and *co*+, all six *arg* segregants carried the marker of C3-1.

(iii) The obvious controls to rule out mutation would have been selfings of the

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Table 6. *Distances between* arg *and the reversion sites calculated by different methods*

Group	Revertant	*arg-reversion* distances		
		From Table 3	From Table 4	From Table 5
1	Pooling data from all	0·1	0	0 (<0·5)
2	B8	0·4	1·2	0 (<0·3)
	C2	0·9	1·0	0 (<0·3)
3	A12	4·4	4·4	*ca.* 2
	C3	6·1	4·9	4·9
	D1	1·2	1·0	1·7

Distances estimated from Table 3 were calculated by subtracting the control *co-arg* distance from the *co-reversion* distances. The same was done for the values from Table 4 after obtaining the *co-reversion* distance from $co\text{-}pyr \times \frac{co\text{-}reversion}{co\text{-}pyr}$. The upper limits, at the 95% level of probability, are indicated for the zero values in the last column.

revertant chromosomes, i.e. crosses of C3-1 × C3-2, D1-1 × D1-2, etc. Such selfings were attempted, but turned out to be sterile or semi-sterile. Alternatively, crosses of the revertants to an *arg pyr* tester can be used as controls. As shown in Fig. 4 (A and B), arginine-dependent segregants are expected in crosses of arg^+ to such a tester, but not in crosses with suppressed mutants if the suppressor is allelic with *pyr*. The results obtained (Table 7) bear out these expectations.

(*e*) *Allelism between* pyr *and the suppressor*. The data in the last column of Table 4, and the agreement between the values in Table 6, support the hypothesis that the suppressor encountered in group 3 revertants is allelic with *pyr*. But the best evidence comes from Table 7. As illustrated in Fig. 4C, if *su* and *pyr* were not allelic, arginine-dependent segregants would be expected in crosses of the suppressed revertants to *arg pyr*. The absence of such segregants among the tested progeny (Table 7) places an upper limit of 0·1 unit on the distance between *pyr* and the suppressor (pooling the data from C3 and D1, for $P < 0{\cdot}05$).

(*f*) *Revertants yielding pyrimidine-dependent segregants*. Ascospores from the cross *co revertant B9* × co^+ *arg* were plated on minimal, on minimal plus arginine, and on minimal plus hydrolysed RNA. Only the last two media supported the growth of colonies. In the RNA plates, thirty of the colonies were *co* and two co^+. One of the latter colonies was subcultured and spot-tested. It had an absolute requirement for either hydrolysed RNA or uridine. On this basis, and with the background of previous studies on this system (Reissig, 1959), it appears that B9 resulted from forward mutation at the *pyr* locus (I in Fig. 1). The growth of the original B9 on minimal, contrasted with its inability to yield prototrophic segregants, is not unexpected from the phenotype of the heterokaryon depicted in Fig. 1. The *pyr* nucleus can complement with the other unmutated nuclei in the macroconidium, but this balance is destroyed when the different genotypes segregate after crossing.

Another revertant (A15) which failed to segregate prototrophic colonies when

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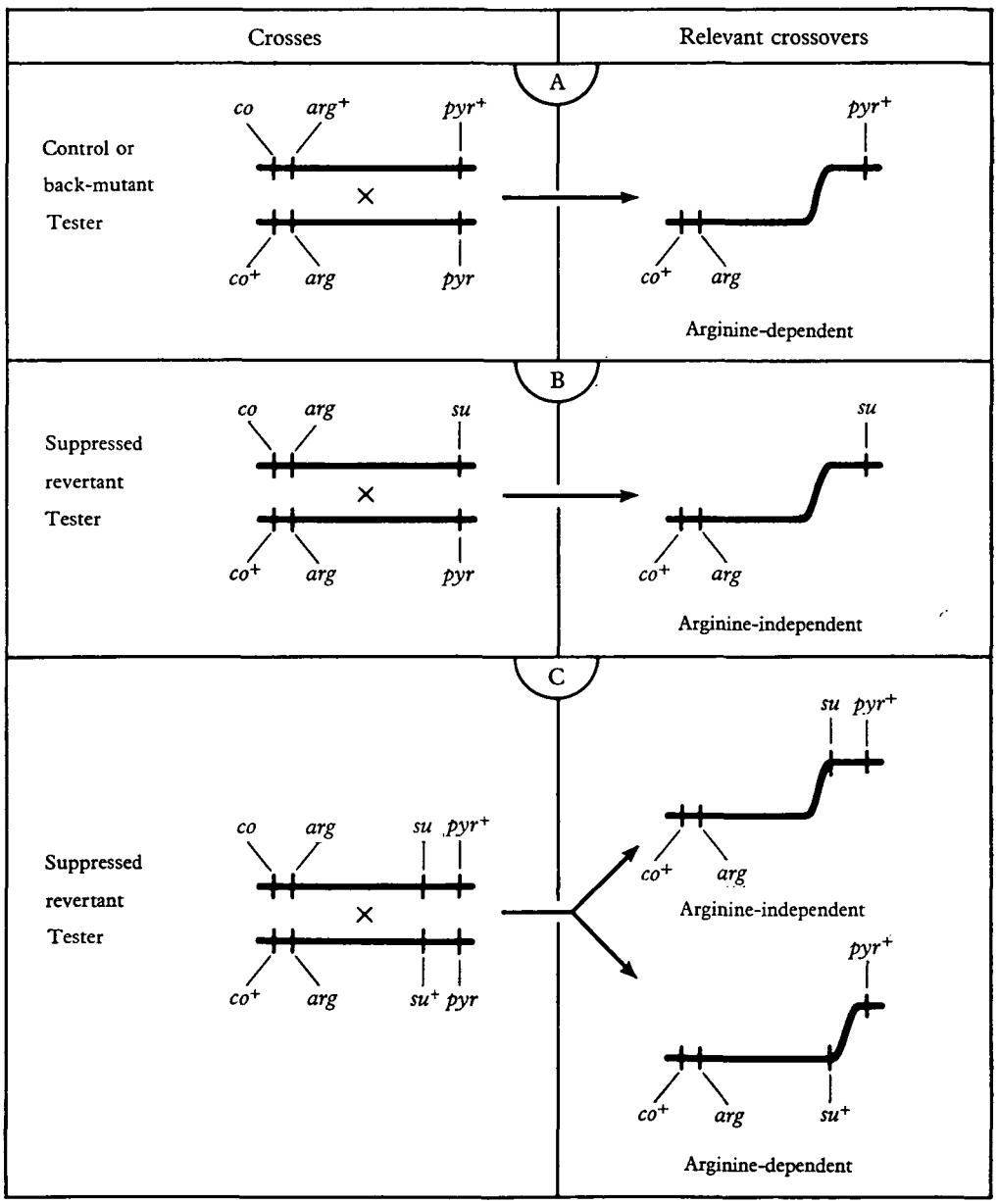

Fig. 4. Some of the segregants expected in crosses between the revertants and an *arg pyr* tester. The expectations vary with the different hypotheses concerning the genotype of the revertants: (A) Reversion is due to back mutation; arginine-dependent segregants are obtained. (B) Reversion is due to a suppressor allelic to *pyr*; no arginine-dependent segregants are obtained. (C) Reversion is due to a suppressor in the *arg-pyr* region; the frequency of arginine-dependent segregants is a function of the distance between *pyr* and the suppressor. The expectations for a suppressor located somewhere else in the genome have not been drawn, but it can readily be seen that the conclusion reached in (C) also holds for other locations of the suppressor. The actual results are shown in Table 7.

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Table 7. *Testing the suppressor hypothesis in crosses to a* co$^+$ arg pyr *tester: recovery of* arg *by means of replica-plating.*

(The crosses are described in Fig. 4)

	arg$^+$ segregants				
Strains	*arg*$^+$	Recovery ratio	Corrected *arg*$^+$ II × III	*arg* segregants	Distance *reversion-pyr* $\frac{200 \times V}{IV + V}$
I	II	III	IV	V	VI
Control	459*	37/47	722	11	
	246†	37/47	194	4	
Sum			916	15‡	3·2
C3-1	1031*	28/81	713		
	1275†	28/81	441		
Sum			1154	0	0
D1-1	1200*	37/47	1889		
	924†	37/47	727		
Sum			2616	0	0

*Replica from minimal to arginine-supplemented plates. To correct for the unscored *pyr* population, the values were multiplied by 2 in column IV.

†Replica from minimal plus hydrolysed RNA plates to same plus arginine.

‡All *co*$^+$.

crossed to *arg*, was lost before its F_1 could be tested on pyrimidine-supplemented medium.

(*g*) *Partial arginine requirements among the revertants.* One might wonder whether reversion restores the capacity to grow at wild-type rate, or whether a partial requirement for arginine remains. Quantitative studies on this point were handicapped by the kind of strains used. Because of their macroconidial character, the revertants had to be purified by outcrossing. Furthermore, the *co* phenotype interfered with the measurement of growth. It may, however, be worth mentioning some preliminary observations.

Only A12 was significantly stimulated by arginine. In crosses of *co revertant A12* × *co*$^+$ *arg*$^+$, the arginine-limited growth habit segregates as if it were closely linked to *co*, indicating that this partial arginine requirement is the property of the *arg-suppressor A12* system, and not an effect of modifying genes. No arginine stimulation was detected when ascospores from crosses of C3 or D1 × *arg*$^+$ were plated, confirming that there is an intrinsic difference between the latter revertants and A12.

Hydrolysed RNA failed to stimulate any of the group 3 revertants.

4. DISCUSSION

The reported observations concerning mutational delay and delayed phenotypic expression (section 3—I) indicate *prima facie* that these two effects either do not occur, or that their occurrence takes minutes instead of hours. However, negative

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evidence of this kind can seldom be conclusive (see, for instance, Lederberg, 1957). It is quite possible that the inhibitor, canavanine in this case, fails to interfere with the pathways leading to mutation and expression, even though it effectively stops the growth of sensitive cells and the germination of all conidia.

Genetic analysis of the revertants showed that those classed in group 3 became arginine-independent as the result of mutation at a suppressor locus (section 3—III (*d*)). No pyrimidine requirement is evident in these mutants (section 3—III (*g*)), and yet the suppressor seems to be allelic to *pyr*. This conclusion is based on the recombination data, which place the suppressor within 0·1 unit of *pyr* (section 3—III (*e*)), and on the functional similarity between *pyr* and the suppressor ($pyr^{su\text{-}arg}$)*. The *pyr* allele is active as a suppressor of *arg*, while the pyr^+ allele is active in the synthesis of pyrimidines. Both these activities seem to be combined in the $pyr^{su\text{-}arg}$ allele, just as they can be combined by making a heterokaryon between *arg pyr* and *arg* pyr^+. This heterokaryon can grow on minimal medium. Differences in the ability of $pyr^{su\text{-}arg}$ alleles to suppress *arg* (section 3—III (*g*)) may reflect differences in the ratios of *pyr* to pyr^+ activities.

The existence of the $pyr^{su\text{-}arg}$ allele has some bearing on the problem of heterosis. The superior growth ability of the *arg pyr*/*arg* pyr^+ heterokaryon as compared with the component homokaryons, provides an example of single-gene heterosis (Reissig, 1958). Is this a quantitative effect, or is the superiority of the heterokaryon due to the presence of alleles making qualitatively different products? Emerson (1952) has given a convincing quantitative explanation of similar cases in Neurospora. Crow (1952) suggested a criterion for distinguishing between the quantitative and the qualitative interpretations: only in the former case could intermediate alleles, capable of replacing the heterotic pair, appear. Thus, the occurrence of $pyr^{su\text{-}arg}$ lends support to the quantitative hypothesis for the pyr^+/*pyr* case. This being so, the heterosis of such pairs as pyr^+/*pyr* could only be of transient importance in evolution. Intermediate alleles like $pyr^{su\text{-}arg}$ would arise and yield a more stable system (Crow, 1952).

Little is known about the biochemical basis of the defects under consideration. Nutritional evidence (Srb & Horowitz, 1944; Srb, 1949) suggests that the *arg* mutant is blocked between ornithine and citrulline. Yet the activity of the enzyme catalysing this step (ornithine transcarbamylase) is at least as high in extracts of *arg* as in wild-type extracts†, suggesting either that the enzyme is inactive under *in vivo* conditions, or that the block affects the synthesis of carbamyl phosphate. The *pyr* alleles studied by Mitchell (1953) respond to orotic acid or to pyrimidines (see also Munkres, Woodward & Suyama, 1958). The same is true for our *pyr* isolate, except that the response to orotic acid is rather poor even in nitrate medium (Reissig & Goldberg, unpublished).

* The full symbol is $pyr3^{su\text{-}arg2}$.

† J. R. S. Fincham (personal communication), R. L. Metzenberg (personal communication) and Reissig (unpublished), working independently of each other. Fincham and Metzenberg also tested other allelic and non-allelic mutants presumed to be blocked at the same step. All had the enzyme.

The biosyntheses of arginine and pyrimidines are known to be related via carbamyl phosphate, which acts as a common precursor (Reichard, 1959). Tracer data (Heinrich, Dewey & Kidder, 1954) supports this scheme for Neurospora. This biochemical relation may underlie the observed interaction between *arg* and *pyr*. On the other hand, there is no reason to suppose that the close proximity between the *arg* and *pyr* genes plays any role in their interaction. Suppressor effects between other unlinked mutant genes affecting arginine and pyrimidine metabolism are widespread in Neurospora (Mitchell & Mitchell, 1952).

The abnormal linkage relations exhibited by group 2 revertants (section 3—III (*d*)) might be due to associated chromosomal rearrangements. De Serres (1958), studying reversions at an adenine locus, also found abnormal linkage relations among them. However, any attempt at a critical evaluation of our data would have to account also for the fact that the revertants in group 2 gave abnormal co^+/co ratios on the RNA-supplemented medium (Table 4).

SUMMARY

A system for the screening of reversions in an arginine-dependent strain of Neurospora has been developed.

Reversion resulted from any one of three events:

(*a*) Back mutation of the original *arg* gene.

(*b*) Forward mutation at the *pyr* locus. This has the pleiotropic effect of suppressing *arg* while determining a pyrimidine requirement. Interaction with the unmutated nuclei of the same conidium permits growth on minimal medium.

(*c*) Forward mutation at the *pyr* locus to alleles of intermediate activity. These alleles suppress *arg*, but do not create a pyrimidine requirement.

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