CAUSES OF THE FORMATION OF AN ABNORMALLY HIGH PROPORTION OF PROTOTROPHS IN SOME INTERALLELIC CROSSES OF *NEUROSPORA CRASSA*

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Abstract

Causes of the formation of a high frequency of prototrophs in interallelic crosses of three tryptophan-1 mutants and one tryptophan-3 mutant have been investigated. The high frequency of prototrophs in the interallelic crosses of the three tryptophan-1 mutants follows from the formation of a large number of pseudo-wild spores. But the formation of an abnormally high proportion of prototrophs in the interallelic crosses of the single tryptophan-3 mutant, A308, cannot be explained on the basis of pseudo-wild formation, action of recombination, accelerating genes, unequal crossing over, reversion of suppressors. It seems that the tryptophan synthetase formed by the damaged tryptophan-3 locus in this mutant can acquire a functional configuration as a result of the action of some other genes which act as modifiers.

The classical researches of Morgan and his school established that chromosomes comprise a linear array of Mendelian factors or genes (Morgan 1926). How are the genes internally organised or what is the fine structure of genes has been the subject of investigation during the recent years (Benzer 1961, Case and Giles 1964, Ahmad *et al.* 1966). The organisation of genes is studied by first obtaining a large number of mutants for a particular gene. One of the mutants for the gene is then combined with a mutant for a closely linked locus or a marker. This double mutant is crossed with the other alleles. The analysis of these triple point interallelic crosses gives an insight into the organisation of the gene.

The most convenient mutants to use are the auxotrophs. An auxotroph is a mutant which fails to grow on a medium (minimal medium) containing the minimum nutrients essential for the growth of the wild type, but will grow if one or more specific substances are added to the medium. A strain which can grow on minimal medium is called a prototroph.

While conducting fine structure studies on loci tryptophan-1 (tryp-1) and tryptophan-3 (tryp-3) of *Neurospora crassa* a number of interallelic crosses

yielded an abnormally high proportion of prototrophs. It was decided to investigate the causes of these abnormal results.

Materials and Methods

Two anthranilic acid mutants (A65 and A106) and six indole mutants (A28, A50, A63, A93, A94 and A121) of locus tryptophan-1 (tryp-1) and three indole mutants (A78, A306 and A308) and four tryptophan mutants (A16, A59, A100 and C83) of locus tryptophan-3 were used in these investigations. The anthranilic acid mutants utilise anthranilic acid, indole or tryptophan; the indole mutants fail to grow on anthranilic acid but grow on indole; while the tryptophan mutants utilise neither anthranilic acid nor indole but grow on tryptophan. All the above mutants except C83 were obtained by irradiating the conidia of Emerson a (5297) with U.V. (Ahmad and Catcheside 1960 and Ahmad and Mozmadar unpublished).

Adenine-2 (STL2) (ad-2) and leucine-1 (33757) (leu-1) were used as markers in studies on tryp-1 mutants, while aromatic-1 (Y7655) (arom-1) was utilised for studying the tryp-3 mutants. We are indebted to Dr. D. D. Perkins for the supply of ad-2 (STL2) and to Dr. W. N. Ogata for sending us arom-1.

Media and methods used in these studies were the same as those reported by Ahmad *et al.* 1966. The double point crosses were plated on V.M. and the growing spores that were observed in these crosses, were isolated on V.M. The triple point crosses were plated and growing spores were isolated on V.M., this time supplemented with the nutrilite required by the mark^{er}.

Experiments and Results

A. Formation of a high proportion of prototrophs in interallelic crosses by three tryptophan-1 mutants

In interallelic crosses with A65, the mutants A28, A50 and A63 gave an abnormally high proportion of tryptophan plus spores as compared with a number of other mutants *e.g.*, A93, A94, A121 and A106 (Table I). To find the cause of this abnormally high proportion of prototrophs, the three mutants, A28, A50 and A63 were crossed with a double mutant A65 ad-2 which was prepared by crossing A65 with the allele STL2 of adenine-2.

About 6,000 to 12,000 spores were counted from each cross and the growing spores were analysed. These data, entered in table 2, showed that the recovery of the abnormally high proportion of growing spores in the interallelic crosses analysed was due to the formation of a large number of pseudo-wilds.

Table 1. *The formation of at least five to ten times more tryp+ spores in interallelic crosses of mutants A28, A50 and A63 as compared to the interallelic crosses of four other mutants*

Cross	Viable spores	Tryp+ spores	Percentage of tryp+ spores
A93 x A65 leu	19,931	13	.0816
A94 x ,,	52,815	14	.0650
A121 x ,,	52,594	15	.0266
A106 x ,,	49,288	37	.0750
A28 x ,,	2,500	12	.4800
A50 x ,,	10,113	83	.8207
A63 leu x A 65	7,938	68	.8566

Table 2. *Formation of pseudo-wilds as the cause of the presence of abnormally high proportion of prototrophs in interallelic crosses of three tryp-*1 *mutants*

Cross	Viable spores	Tryp+ spores	Percentage of tryp+ spores	Type of tryp+ spores		
				Recombinant		Pseudo-wild
				Tryp+ ad-	Tryp+ ad+	
A28 x A65 ad-2	12,051	39	0.32	1	2	36
A50 x ,,	7,660	44	0.58	2	2	40
A63 x ,,	6,941	37	0.55	1	0	36

B. Formation of a high proportion of prototrophs in interallelic crosses by a tryptophan-3 mutant, A308

A cross of the mutant A308 with A78 + arom gave the normal number of prototrophs on twelve hour incubation, but an abnormally high number of prototrophs after twenty-four hour incubation (Table 3). This result was unexpected. The experiment was, therefore, repeated and a similar result was obtained (Table 3). All the ninety-eight growing spores observed in the repeat experiment were isloated. Only fifty-seven of them grew. These fifty-seven single spore cultures were tested for growth on V.M., on V.M. supplemented with nutrients required by aromatic and on V.M. supplemented with tryptophan. The tests showed fifty-three of them to be wild type and four arom. The fifty-three wild type single spore cultures grew more vigorously on V.M. supplemented with tryptophan than on V.M.

Spores from the same cross (A308 X A78 arom) were incubated for the third time for twenty-four hours. A large number of growing spores was observed once again. Ten good growing spores were isloated in V.M. tubes, supplemented with aromatic nutrients and tryptophan. Only nine spores grew. Like the previous fifty-three wild type spores, they gave some growth on V.M. and arom but much better growth on tryptophan supplemented medium. When the parental wild type culture, Ema, was tested on V.M. and tryptophan, its growth was slightly inhibited by tryptophan.

To find out whether the nine wild type spores, W_1—W_9, were true-wild or pseudo-wild, they were crossed with the parental wild type. The crosses of all the nine cultures yielded about fifty per cent mutant spores. Ten mutant spores were isolated from one of the crosses, W-3 x Em. All the ten mutant spores grew on tryptophan supplemented medium but not on V.M. or arom supplemented medium. Hence the prototrophic spore (W-3) was not a true-wild. It could be a pseudo-wild but it was noted that all the ten mutant spores had the phenotype of A308 and none had the phenotype of A78 arom.

Absence in A308 of a gene promoting formation of pseudo-wilds

In order to check if A308 carried a gene which promoted the formation of a large number of pseudo-wilds, it was crossed with the parental wild type and then twenty-five isolates of A308 were obtained. It was expected that if A308 carried such a gene it would segregate in the isolates. The isolates with this gene would form a high proportion of prototrophs when crossed with A78 arom while those lacking it would not.

Table 3. *Recovery of an abnormally high proportion of prototrophs on 24-hour incubation of a triple point interallelic cross*

Expt. No.	Cross	Time of incubation in hrs	Viable spores	Tryp+ spores	Percentage of tryp+ spores
1.	A308 x A 78 arom	12	9352	2	.02
		24	12773	410	3.21
2.	A308 x ,,	12	38840	0	0
		24	2998	98	3.27

Table 4. *Formation of a high proportion of prototrophs in crosses of all the five isolates of A*308 *with A*78 *arom*

Cross	Viable spores	Tryp+ spores	Percentage of tryp+ spores
A308-5 x A78 arom	9,350	68	0.73
A308-9 x ,,	12,701	103	0.81
A308-15 x ,,	402	2	0.498
A308-18 x ,,	606	6	0.909
A308-23 x ,,	3,828	28	0.73

The twenty-five isolates of A308, mentioned above, differed in their capacity to grow on V.M. Thus fifteen of them did not grow on V.M., two showed germination of conidia, five were very slightly leaky, another five were slightly leaky, one was leaky and seven were very leaky. Further studies were conducted on five of these isolates, four of them No. 5, 9, 15 and 23 were completely non-growing on V.M., while the fifth one, No. 18, showed only germination of conidia on V.M. in twenty-four hours. These five isolates were crossed with A78 arom and the number of viable and tryptophan plus spores was counted. The data, as in table 4, did not give any evidence that A308 carried any additional gene for promoting the formation of pseudo-wild spores.

Formation of an abnormally high proportion of prototrophs in crosses of A308 with non-complementing alleles

A78 complements A308, and if the high proportion of prototrophic spores in their cross results from pseudo-wilds, then crosses of A308 with the non-complementing alleles should result in the absence of this abnormality.

An isolate of A308, A308-10A, was crossed with four non-complementing and two complementing alleles (Table 5). When the percentage of prototrophic spores amongst total viable spores was assessed, crosses with non-complementing alleles yielded as high a proportion of prototrophic spores as the crosses with complementing alleles (Table 5). This revealed that the abnormally high proportion of prototrophic spores in these crosses was not due to the formation of a high proportion of pseudo-wilds, but to some other cause.

Further analysis of the cause of the formation of an abnormally high proportion of prototrophs

Whether the high proportion of growing spores in the interallelic crosses of A308 resulted from the formation of pseudo-wilds or from some other cause was investigated further. It was argued that if the former was the cause, crosses of the double mutant A308 arom with A78 should give the same proportion of wild type spores as the reciprocal cross, A308 x A78 arom; while, if the high proportion of prototrophic spores followed from a change in the expression of tryp-3 locus in A308, then tryptophan plus spores formed in the former cross should be arom.

Consequently nineteen A308 arom double mutants were obtained and crossed with A78. Only one cross, A308 arom-3a x A78A, was fertile. When spores from this cross were plated on arom supplemented plates, out of two hundred and eight viable spores counted, sixteen were tryptophan plus. These sixteen spores were isolated in arom tubes. Fifteen of them grew. On testing all the fifteen single spore cultures proved to be arom.

Discussion

The formation of an abnormally high proportion of prototrophs in interallelic crosses has been assigned to five causes: (*a*) the formation of pseudo-wilds due to the recovery of n + 1 spores (Mitchell *et al.* 1952), (*b*) the formation of a normal locus as a result of unequal crossing over (Demerec 1962, Magni and von Borstel 1962, Magni 1962 and Bausum and Wagner 1965), (*c*) the increase in frequency of recombination due to the presence of genes which accelerate recombination (Catcheside *et al.* 1964 and Jha 1967), (*d*) the presence of suppressors and (*e*) the presence of genes which accelerate reversion (Demerec 1963).

The data, presented in the preceding pages, showed that the higher number of prototrophs formed in the interallelic crosses by three mutants, A28, A50 and A63, of locus tryp-1 followed from the formation of pseudo-wilds (Table 2). But the analysis of the cause of the formation of a high proportion of tryptophan plus spores by the tryp-3 mutant, A308, proved difficult.

Most of the prototrophic spores formed in the interallelic crosses of A308 were not pseudo-wild because they were formed with the same frequency in crosses of A308 with non-complementing alleles as with the complementing alleles (Table 5). Further, in the cross of A308 with A78 arom, the tryp + spores were mostly of the wild type, while in the reciprocal cross A308 arom x A78 these spores were arom. Had most of the prototrophs in these crosses been due to pseudo-wild formation, the cross A308 arom x A78 would also have given an excess of wild type spores and not arom.

The majority of the prototrophs formed in the interallelic crosses of A308 showed a slightly delayed growth. Their growth was stimulated by tryptophan. In crosses with the parental wild type they yielded about 50 per cent mutant spores. Hence they are not true wild type spores. They could not be formed therefore by the recombination accelerating genes, or unequal crossing over or by reversion of the tryptophan-3 locus.

No interplay of suppressor loci can be postulated to account for the high frequency of these tryptophan plus spores for two reasons. Firstly both, A308 and A78 have a mutant phenotype, so neither could carry a suppressor which could segregate as a result of the cross when these two mutants are crossed together. Secondly, the crosses of these prototrophs with the parental wild type yielded about fifty per cent mutant spores but the cross of a suppressed mutant with parental wild type always yields a much lower proportion of mutant spores than fifty per cent.

Table 5. Formation of abnormally high proportion of tryp+ spores in crosses of A308 with alleles which complement it and also with alleles which do not complement it

Allele crossed with A308	Complementation of the allele with A308	No. of ascospores in crosses of the allele with A308		Percentage of tryp+ spores
		Viable spores	Tryp+ spores	
A78	+	98	45	46
A306	+	360	175	49
A16	—	113	52	46
A59	—	105	70	60
A100	—	175	100	57
C83	—	96	50	52

If these prototrophs cannot be accounted for by pseudo-wild formation, the presence of recombination promoting genes, unequal crossing over, reversion and suppressor mutations, then how are they really formed? For an explanation of the mechanism of their formation one has first to consider their main features.

Their growth is generally slower than that of the wild type and is stimulated by tryptophan. Phenotypically they are mostly of the wild type in the cross A308 x A78 arom but they are arom in the reciprocal cross A308 arom x A78. Fifty per cent mutant spores are formed in their crosses with the parental wild type. Classification of these mutant spores from the cross of one of them, W3, with the parental wild type revealed that they carried the damaged tryptophan-3 locus of A308 type. The prototrophic spores, W3, therefore, carried the damaged tryptophan-3 locus from A308.

These facts suggest that most of the prototrophs, formed in the interallelic crosses of A308, carry the damaged tryptophan-3 locus of A308. They behave as prototrophs possibly because the tryptophan synthetase formed by this damaged site is capable of becoming functional through the action of other genes present in the same nucleus. This idea of a modified action of tryp-3 locus in A308 in different genetic backgrounds is supported by the fact that thirty-five isolates of

A308, obtained by crossing it with the parental wild type, varied in their phenotype from auxotrophy to various degrees of prototrophy.

When five of the above isolates were crossed with A78 arom, the frequency of prototrophs was reduced to at least one quarter of the frequency of the prototrophs formed by the original mutant, A308, in a similar cross (Tables 3 and 4). On the other hand in the analysis of the reversion rates of four mutants of *Neurospora crassa* Giles (1951) reported that each mutant maintained its definite rate of prototroph production in its progeny.

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