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Cytoplasmic mutations isolated from protoplasts of *Podospora anserina*

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SUMMARY

Sorting out of cytoplasm determinants can be achieved in *Podospora anserina* by the use of protoplasts. In this way four cytoplasmic mutations have been isolated. These mutations affect a precise stage of development of the fungus. In crosses with wild-type strains, the mutants show maternal inheritance when no cytoplasmic contact precedes fertilization. However, when cytoplasmic mixing occurs before fertilization, the cytoplasmic wild-type factor shows dominant and/or suppressive properties over the mutant factor.

1. INTRODUCTION

Interest in cytoplasmic mutations has been stimulated by the development of mitochondrial (Thomas & Wilkie, 1968; Coen *et al.* 1970) and chloroplast genetics (Sager & Ramanis, 1965). In *Neurospora crassa*, although a study of cytoplasmic mutations was started more than 20 years ago, only a small number of mutants have been isolated. Bertrand & Pittenger (1972) have suggested that this is partly due to the difficulty of isolating this type of mutant in a coenocytic organism unless it shows either suppressive and/or dominant properties over the wild-type cytoplasmic state. A possible means of overcoming this can be achieved by using protoplasts. The experiments reported in this paper describe the isolation of four cytoplasmic mutants isolated from protoplasts of *Podospora anserina*. These mutations show no dominant or suppressive properties and are most likely mitochondrial.

2. MATERIALS AND METHODS

(i) *Organism, strains.* The biology, genetics and culture techniques of *Podospora anserina* were first described by Rizet & Engelmann (1949) and reviewed by Esser (1969). *Podospora anserina* only forms microconidia which function as spermatia and do not germinate. All of the strains used are derived from strains *s* and *S* studied by Rizet (1952). The mating-type alleles are designated *mt* + and *mt* − ; *S* and *s* stand for two incompatible alleles of the *s* locus (Rizet, 1952). Mutation *193* belongs to the allelic series of locus *14* and is characterized by the absence of pigment in the mycelium, perithecia and spores (Picard, 1971). The cytoplasmic genotypes are indicated in parentheses: (*s*) and (s^S) stand for the two cytoplasmic

states corresponding to the *s* genotype; (*64*), (*89*), (*119*) and (*561*) designate the four cytoplasmic mutations described here, while (+) stands for the corresponding wild-type cytoplasmic state.

(ii) *Preparation of protoplasts.* The method used was derived from that of Bachmann & Bonner (1959). Cultures were grown in liquid corn-meal medium (Rizet, 1952) enriched with yeast extract (5 g/l.) and gently agitated for 2 days at 27 °C. The mycelia were harvested by filtration on cheese-cloth and incubated for 1 h at 27 °C in an isotonic buffer (200 g/l. saccharose in sodium phosphate buffer: 0·05 M, pH 6). The mycelium was again harvested and incubated for 4 h in the same buffer but containing 5 % of an enzyme preparation (digestive juice from *Helix pomatia*, Industrie biologique de France, 92 Gennevilliers, France). The liquid recovered after incubation and filtration contained approximately 10^6 protoplasts per ml. This suspension was freed from hyphal fragments by filtration on a fritted glass plate (Sovirel, porosity 3). Regeneration of protoplasts towards mycelial form was obtained, after dilution of the suspension, by spreading samples on regeneration medium (corn meal with 200 g/l. saccharose, 1·5 % agar). The proportion of regenerating protoplasts, after 2 days incubation at 27 °C, was usually between 5 and 15 %.

(iii) *Mutagenesis.* In the two experiments summarized in Table 1, 10 ml of the filtered protoplast suspension was irradiated with UV (3000 and 10000 ergs/mm²) in a Petri dish 10 cm in diameter. Immediately after irradiation the suspension was diluted and spread on regeneration medium.

(iv) *Crosses, genetic analysis.* Although *Podospora* asci usually contain 4 binucleate spores, a few asci containing 3 binucleate and 2 smaller uninucleate spores are regularly found and homocaryotic strains can be isolated from them. Crosses between homocaryotic strains of different mating types can be done in two ways: (*a*) by spermatization, i.e. by spreading over a Petri dish culture of one parent (female) a filtered suspension of microconidia harvested from the male parent; (*b*) by confrontation, i.e. by growing the two parental strains in the same Petri dish. Perithecia resulting from the two reciprocal crosses develop along the contact line where numerous anastomoses occur and a narrow heterocaryotic and 'heterocytoplasmic' zone exists. The asci used for genetical analysis were isolated from perithecia.

3. RESULTS

Previous experiments on the $(s)/(s^S)$ system of *Podospora anserina* suggested that protoplasts might be useful for isolating cytoplasmic mutations. Strains (s) and (s^S) differ from each other only by their 'cytoplasmic states' and are distinguished by their vegetative incompatibility reaction with strain *S*. In crosses $(s) \times (s^S)$, the cytoplasmic state is maternally inherited. In spite of the strong infectious power of the (s) state (Rizet, 1952), protoplasts prepared from a strain (s) regularly yielded, after regeneration, about 10 % colonies in the (s^S) state (i.e. colonies which do not contain the cytoplasmic factor responsible for incompatibility with strain *S*) (Belcour, unpublished). These results suggest that

a sorting out of the cytoplasmic determinants can be achieved by protoplast formation. We therefore attempted to isolate mutants among colonies formed by the regeneration of protoplasts, by screening for slow or abnormal growth.

(i) *Isolation of mutant strains.* Table 1 gives the results of two UV mutagenesis experiments on protoplasts. In these experiments the colonies formed by the regeneration of protoplasts were transferred to solid corn-meal medium. After 48 h the strains which had not grown normally were transferred on to fresh Petri dishes of the same medium. The strains which retained their abnormal phenotype on transfer were reciprocally crossed with the wild-type reference strain. From the

Table 1. *Isolation of strains showing abnormal growth by the use of protoplasts*

Expt.	Treatment	Survival (%)	No. of protoplast regenerations transferred (1)	No. of strains showing abnormal growth: After 1st transfer (2)	No. of strains showing abnormal growth: After 2nd transfer (3)	No. of mutant strains: Mendelian inheritance (4)	No. of mutant strains: Non-mendelian inheritance (4)
1	Control	100	690	11	1	0	1
	UV 3000 ergs/mm²	92	1420	162	19	5	1
2	Control	100	470	21	1	0	0
	UV 10000 ergs/mm²	37	820	73	29	9	2

((1) No selection of protoplast regenerations have been made for the first transfer. (2) Phenotype of growth recorded two days after the first transfer. (3) Phenotype of growth recorded 2 days after the second transfer. All of the 50 strains have been crossed with the wild-type-strain by spermatization in the two directions. (4) From analysis of the progeny of crosses with wild-type strain).

progeny of each cross, five asci containing five spores were isolated and the growth of the developing colonies observed. The results of Table 1 show that a few of the selected strains transmitted their character to their progeny. Fourteen of these were classified as nuclear mutants giving monogenic segregations in the crosses; most of them carried UV-induced mutations. Four further strains were classified as cytoplasmic mutants on the basis of two criteria: transmission of the mutant phenotype only when the abnormal strains were used as female and, from these crosses, absence of segregation in the asci. It cannot be ascertained whether the UV treatment actually induced these mutations. The use of protoplasts, however, appears to be successful as such mutations were previously unknown in *Podospora*.

(ii) *Phenotypic properties of the cytoplasmic mutants.* The most conspicuous phenotypic characteristics of the four mutants are the following. (*a*) The germination of spores, formed by crosses in which the female parent is mutant, proceeds normally for the first 8 h and is then much slower. After 48 h, while a wild-type mycelium has reached a diameter of 2·5 cm, the mutant is only a few millimetres in diameter. This period of crisis lasts for 3–4 days, after which growth resumes at

Table 2. *Crosses by confrontation between cytoplasmically wild type and mutant strains*

(In each confrontation two types of perithecia exist derived from the two reciprocal nuclear crosses (♀ nucleus *mt*+ × ♂ nucleus *mt*− and ♀ nucleus *mt*− × ♂ nucleus *mt*+). They can be recognized either by their colour (WP, white perithecia; BP, black perithecia) in crosses involving the alleles *193*/*193*$^+$ or by their position in relation to the barrage line in crosses involving the alleles *S*/*s*. In each perithecium, at least 2 spores have been studied, and generally 3, 4 or 5. Normal or mutant germination phenotypes are recorded after 2 days incubation at 27 °C.)

Mutant	Genotypes of the confronted strains		Type of perithecia	No. of perithecia studied	No. of spores: Mutant-type germination	No. of spores: Wild-type germination	No. of spores: Total
(*64*)	(*64*) *193*$^+$ *mt*+, (+) *193* + *mt*−	1A	WP	10	0	30	30
		1B	BP	30	2	88	90
	(*64*) *193*$^+$ *mt*−, (+) *193 mt*+	2A	WP	6	0	24	24
		2B	BP	8	0	28	28
	(*64*) *193 mt*+, (+) *193*$^+$ *mt*−	3A	WP	9	0	36	36
		3B	BP	5	0	25	25
	(*64*) *s mt*+, (+) *S mt*−	4A	s side	13	46	0	46
		4B	S side	6	0	18	18
(*89*)	(*89*) *193*$^+$ *mt*+, (+) *193 mt*−	5A	WP	5	0	25	25
		5B	BP	24	0	64	64
	(*89*) *s mt*+, (+) *S mt*−	6	s side	12	48	0	48
(*561*)	(*561*) *193*$^+$ *mt*+, (+) *193 mt*−	7A	WP	6	0	30	30
		7B	BP	6	1	23	24
	(*561*) *s mt*+, (+) *S mt*−	8	s side	9	33	0	33
(*119*)	(*119*) *193*$^+$ *mt*+, (+) *193 mt*−	9A	WP	10	0	39	39
		9B	BP	19	20	67	87
	(*119*) *s mt*+, (+) *S mt*−	10	s side	6	24	0	24

a normal rate. (*b*) Although their growth rate is eventually normal, the mutant mycelia have fewer aerial hyphae. This trait is more pronounced during the early stages of growth. (*c*) The mutant strains have a slightly diminished female fertility.

(iii) *Genetic analysis of mutants: crosses initiated by spermatization.* The three phenotypic characteristics listed above are always transmitted together and maternally inherited when crosses are made by spermatization. The mutant strains are stable and in more than a thousand asci no reversion was observed. Furthermore, during five successive generations of isogenization of the mutants, no phenotypic variation was observed.

(iv) *Genetic analysis of mutants: crosses by confrontation.* As previously mentioned, crosses made by confrontation develop perithecia along a narrow zone of nuclear and cytoplasmic mixing. Two genetic systems were used to identify perithecia from the two reciprocal crosses. The first system (Table 2, crosses: 1, 2, 3, 5, 7, 9) consists in marking one of the parental strains with the nuclear mutation *193*. The protoperithecia produced by the *193* strain yield, after fertilization, non-pigmented perithecia whereas those produced by *193*$^+$ yield black perithecia. The second system (Table 2, crosses 4, 6, 8, 10) consists in the isolation of perithecia from each side of the line of 'barrage' which develops between incompatible strains. The incompatibility system used here was the *s*/*S* interaction (Rizet, 1952). The germination phenotype of spores isolated from the two kinds of perithecia in crosses between cytoplasmically mutant and wild-type strains is given in Table 2. In perithecia developed from protoperithecia formed by the cytoplasmically wild-type parental strain, all spores showed a wild-type germination and all colonies were of the wild-type phenotype (Table 2; lines 1A, 2A, 3B, 4B, 5A, 7A, 9A). In perithecia developed from protoperithecia formed by the cytoplasmically mutant strain, the result depended on the system used. In the confrontation involving the alleles *193*/*193*$^+$ (compatible confrontations), most spores showed wild-type germination (Table 2, lines 1B, 2B, 3A, 5B, 7B, 9B). This result is not affected by the nuclear markers since the various combinations of the *mt* and *193* markers used in the crosses involving mutant (*64*) yielded the same results (Table 2; crosses 1, 2, 3). Lastly in the confrontations involving the *S*/*s* alleles (incompatible confrontations), all the spores showed the mutant type of germination and all the colonies had the mutant phenotype (Table 2; lines 4A, 6, 8, 10). This discrepancy can be related to the fact that vegetative incompatibility in *Podospora*, although not preventing anastomoses between two mycelia, leads very rapidly to the death of anastomosed cells (Beisson-Schecroun, 1962). Thus as in *Aspergillus* (Caten, 1972), incompatibility establishes a barrier to the cytoplasmic mixing of the two confronted strains.

It can be concluded that whenever cytoplasmic mixing occurs, as in compatible confrontations, the wild-type cytoplasmic phenotype is expressed, while in incompatible confrontations, which prevent cytoplasmic mixing, the mutant phenotype is retained.

4. DISCUSSION

The results presented here show that protoplasts from *Podospora anserina* can be used to overcome the absence of vegetative spores such as conidia. Their efficiency for obtaining the change (*s*) to (s^S) and for the isolation of cytoplasmically inherited mutants indicates that protoplasts permit the analysis of the cytoplasm which no other method has so far achieved. This may be partly explained by the small size of the protoplasts (3–10 μm in diameter) which receive at the moment of their formation a random and very small amount of cytoplasmic components and organelles. The four mutants show a maternal pattern of inheritance when crosses with wild-type strains are made by spermatization. When the same crosses are made by confrontation, provided that cytoplasmic mixing occurs, nearly all the progeny are of the wild-type phenotype. This either indicates dominance or that the wild-type cytoplasmic factors are strongly selected. This supports the arguments presented above in favour of the usefulness of protoplasts for the selection of cytoplasmic mutants. Although they are indistinguishable in phenotype, we have genetic evidence that these four mutations are not identical, since we obtained some wild-type progeny in mutant by mutant crosses (Belcour & Begel, in preparation).

We are now trying to identify the cellular constituent affected by these mutations, and a preliminary analysis of the cytochromes reveals abnormalities which suggest that the mutations are mitochondrial like most of the cytoplasmic mutants in *Neurospora crassa*. Their most striking phenotypic trait is the block in mycelial growth that occurs a few hours after spore germination. It is of interest to know whether this physiological effect on an important stage of development is controlled by the mitochondria.

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