Genet. Res., Camb. (1967), **10**, *pp.* 185–194
Printed in Great Britain

Heterokaryon studies of the cytoplasmic mutant SG in *Neurospora**

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(*Received* 24 *April* 1967)

1. INTRODUCTION

The cytoplasmic mutant SG (slow growth) in *Neurospora crassa* was first described by Srb (1958). It is characterized by a reduction in the growth rate of the young mycelium arising from conidia or ascospores. The growth rate, however, gradually increases to normal by 60 to 100 hours after spore germination. Strains with the SG trait are easily distinguishable from normal both on plates and in growth tubes. The SG trait is stable in vegetative culture, is maternally inherited and is expressed in all nuclear genotypes tested so far (Srb, 1958, 1963).

Infanger (1963) studied the mixing and subsequent interaction of normal and SG cytoplasms in heterokaryons. By making vegetative isolates from heterokaryons between a normal and an SG strain she was able to recover both the original combinations of nucleus and cytoplasm and the two recombinant types, and occasionally an altered SG type called 'slow SG'. The initial growth from slow SG spores was significantly slower than that of SG, and the growth lag lasted almost twice as long. Slow SG colonies on plates were readily distinguishable from those of either normal or SG. Usually slow SG arose from colonies which had a normal or SG phenotype when first isolated but which changed to slow SG upon sub-culture. Like the original SG, slow SG was stable in vegetative transfer and showed maternal inheritance in crosses. Since it could be recovered with the nucleus of either the original SG or the normal strain used in the heterokaryon, slow SG was not nuclear in origin. Infanger proposed that slow SG resulted from an interaction of normal and SG cytoplasms.

This paper presents the results of further studies of SG and slow SG. In addition the effects of acriflavine on SG are reported.

2. MATERIALS AND METHODS

(i) *Strains*

All work was done with strains of *N. sitophila*. The wild-type strains 540-34A and 2a sit, mating types A and a respectively, originated from the stocks of H. L. K.

* Supported by Grant GM 12953 from the National Institutes of Health. The senior author held a traineeship supported under Grant T1 GM 1035 from the National Institutes of Health, U.S. Public Health Service.

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Whitehouse. Strain N-6A, carrying a nuclear gene *al* for albino conidia, was isolated after β-propiolactone treatment of 540-34A. The SG mutation, originally isolated in *N. crassa*, was transferred to *N. sitophila* through ten backcrosses using SG as a protoperithecial parent throughout (Srb, 1958). The resultant strain was labelled SG-2A and was indistinguishable from wild-type *N. sitophila* except for the SG trait. Several cytoplasmic 'recombinant' strains were recovered from vegetative isolates of heterokaryons between N-6A and SG-2A. They were assumed to be homokaryotic when after several subcultures they failed to show segregation for conidial colour.

(ii) *Media*

The minimal medium of Beadle & Tatum (1945) was used. Complete medium was prepared by adding 0·25 % Difco yeast extract and 0·75 % Difco malt extract to the minimal medium and reducing the concentration of sucrose to 1 %. These media were solidified with either 2 % or 4 % agar. 1 % L-sorbose in combination with only 0·5 % sucrose was added to media on which compact, colonial growth was desired.

Crosses were made on Difco cornmeal agar medium.

In certain experiments acriflavine (British Drug Houses Ltd., Poole, England) was added to the medium before autoclaving.

(iii) *Plating conidia*

Conidia at least 5 days old were suspended in sterile distilled water and spread on agar plates. SG colonies were readily distinguishable from normal, and albino colonies from orange (wild-type), when the following incubation scheme was used: 46–48 hours at 35°C., followed by 2–4 hours at room temperature (*ca.* 25°C.) under a bright incandescent light and then 12–18 hours at 18°C.

(iv) *Crosses*

The protoperithecial (maternal) parent of a cross was inoculated on a slant of cornmeal agar and incubated at least 10 days at 25°C. A heavy conidial suspension of the other (paternal) parent was then sprayed over the slant. The crosses were kept at 25°C. until ascospores were discharged. Individual ascospores were isolated at random, heat activated at 60°C. for 30 min. and incubated at either 35°C. or 28°C.

(v) *Heterokaryon formation*

In all cases the two heterokaryon components were SG-2A (SG growth pattern, orange conidia) and N-6A (normal growth pattern, white conidia), both of mating type A. One heterokaryon partner was inoculated to complete medium with 4 % agar and incubated at 28°C. After 8–10 hours a small agar block with a hyphal tip about 0·75 mm. long was cut out, transferred to fresh medium and incubated at

18°C. When the tip had grown off the block to a length of several millimetres (10–12 hours), the block was removed and the remaining hypha stripped of side branches using a warmed microspatula. This stripped hypha is the 'main' hypha. A block carrying a similar hyphal tip of the other heterokaryon partner was positioned so that as this tip grew forward it would cross the main hypha. The pair was then incubated at a constant temperature of either 28°C. or 18°C. or else at 28°C. for the first 4 hours and then at 18°C. for the rest of the time. Periodically the pair was examined to note the time at which the second hypha crossed the main hypha and to remove the side branches which arose.

After various periods of time the points of intersection were cut out, transferred to small tubes with about 1 ml. of complete medium and allowed to grow to conidiation at 28°C. One millimetre segments along the main hypha at varying distances from the intersection toward the tip were also removed and transferred to culture tubes. The conidia from these slant cultures were plated on a sorbose medium and colonies scored for nuclear and cytoplasmic type on the basis of their morphology on plates. These conidia and the colonies produced by them are termed the first conidial generation. When pieces of these colonies were transferred to fresh slants of complete medium, their conidia formed a second conidial generation and so on. Normally twenty to fifty first-generation colonies were scored. If segregation for colour was found, two or three colonies of the same colour as the second heterokaryon partner or of an intermediate light orange were selected and carried through two more conidial generations.

3. RESULTS

(i) *Analysis of Heterokaryons*

The movement of nuclei from the second hypha into the established hypha was detected by the conidial colour marker in the progeny of the segments taken along the main hypha. Cytoplasmic migration was similarlly indicated by the presence of the cytoplasmic marker of the second hypha. In all pairs the nuclear marker was the allelic pair *al* vs. al^+ and the cytoplasmic marker was normal vs. SG.

In these experiments nuclear migration was substantial. Of eighty-six pairings at constant temperature with SG-2A as the main hypha and N-6A as the second hypha twenty-four (28%) showed nuclear migration. In the converse situation twenty-six out of sixty-five pairings (40%) showed migration. Had the sample of conidia (20–50) from each segment been larger, the estimate of the frequency of migration might have been larger. As can be seen in Table 1, nuclear migration was not uniform. These data suggest that nuclear migration is not a simple diffusion process and may occur in waves or pulses.

Of the fifty pairings showing nuclear migration only three showed signs of infused cytoplasm along the main hypha. In all three the main hypha was SG-2A, and the infused cytoplasm was detected as slow SG. Slow SG is considered a consequence of the mixing of normal and SG cytoplasms (Infanger, 1963). Typically slow SG did not appear till the second conidial generation; the colonies which yielded

slow SG were phenotypically SG in the first generation. The three pairs are shown in Table 2.

Table 1. *Distribution of infused nuclei along the main hypha in a sample of pairings*

		Percentage of orange colonies from segments on main hypha (total number of colonies)			
Pairings	Intersect.	3·3 mm.	6·6 mm.	9·9 mm.	13·2 mm.
a	0 (14)	1 (71)	0 (77)	12 (43)	10 (31)
b	0 (32)	12 (59)	15 (128)		
c	24 (19)	0 (22)	0 (43)	8 (40)	
d	13 (111)	3 (36)	18 (162)		

In the four pairings above the main hypha was cytoplasmically normal but carried the *al* gene (N-6A). The lengths listed at the top of the columns represent the distance from the intersection at which the 1 mm. segment was removed.

Lowering the temperature from 28°C. to 18°C. about 4 hours after the second hypha was put near the main hypha was found to enhance nuclear migration considerably. Of thirty pairings of N-6A as the main hypha and SG-2A as the second hypha made using this temperature change, twenty-five (83%) showed nuclear

Table 2. *Pairings showing cytoplasmic migration under constant temperature incubation*

Pairing	Intersect.	Segments along main hypha		
a	SG	*sl*(SG)	*sl*(SG)	SG
b	SG	*nh*(SG)	*nh*(SG)	*sl*(SG)
c	*N*	SG	*sl* (SG)	

Each line represents the main hypha of a pair; the point of intersection is at the left end. Positions of segments taken along the main hypha at 3·3-mm. intervals are shown as dots. The cytoplasm of the segment after three conidial generations is listed above the dot, except where it was seen not to be heterokaryotic after the first conidial generation. Symbols in parentheses show the cytoplasm of the segment in the first conidial generation (see text). *nh* = not heterokaryotic, second and third conidial generations not examined, *sl* = slow SG, *N* = normal growing.

migration, a percentage more than twice that found with a constant temperature. Cytoplasmic migration was also enhanced. Seven of the twenty-five pairs showing nuclear migration also showed cytoplasmic migration, scored as the appearance of slow SG types along the main hypha. These seven pairs are represented in Table 3. Pairs d, e and g yielded slow SG at a considerable distance from the intersection with no demonstrable SG cytoplasm in between. It is as though the SG factor were moving down the hypha in packets or waves.

Table 3. *Pairings showing cytoplasmic migration when there is a drop in incubation temperature*

Pairings	Intersect.	Segments along main hypha		
a	*N*	*sl*(SG)	*sl*(N)	*sl*(N)
b	*N*	*sl*(N-SG)	*sl*(N)	
c	*N*	*nh*(N)	*sl*(N-SG)	
d	*N*	*N*	*sl*(N)	*sl*(SG)
e	*N*	*nh*(N)	*N*	*sl*(SG)
f	*nh*	*N*	*N*	*sl*(N)
g	*nh*	*N*	*sl*(N)	*sl*(SG)

See Table 2 for explanation of symbols and format. The symbol N-SG designates a phenotype which had characteristics of both normal and SG types on plates.

(ii) *Cytoplasmic heterogeneity in hyphal segments*

Except for two dubious cases, Infanger (1963) recovered only one cytoplasmic type from any hyphal segment of a heterokaryon between a normal and SG strain. In order to confirm this result, thirty-two segments—both intersect and non-intersect—were chosen, their conidia plated and a sample of ten to fifty colonies derived from each segment was cultured to the third conidial generation. Experience has shown that within three conidial generations any potentially slow

Table 4. *Cytoplasmic heterogeneity among conidia of individual heterokaryotic segments*

Segment	Cytoplasm of segment	Position of segment	No. of isolates of segment tested	No. of isolates of cytoplasmic type		
				N	SG	Slow SG
a	*SG* → *slSG*	intersect.	45		44	1
b	*SG* → *slSG*	intersect.	50			50
c	*N* → *slSG*	intersect.	50	5		45
d	*N* → *slSG*	intersect.	50	19		31
e	*SG*	intersect.	35		35	
f	N	intersect.	30	30		
g	*SG* → *slSG*	on *main* (SG)	30			30
h	*SG* → *slSG*	on *main* (SG)	30		1	29
i	*N* → *slSG*	on *main* (N)	20			20
j	*NSG* → *slSG*	on *main* (N)	20	1		19
k	*SG*	on *main* (SG)	19		19	
l	*SG*	on *main* (SG)	9		8	1
m	*N*	on *main* (N)	14	13	1	
n	*N*	on *main* (N)	13	12	1	
o	*N*	on *main* (N)	18	14	1	3

These results are based solely on observations of colonies from conidia plated on complete medium with sorbose. All isolates were carried through at least three conidial generations. Symbols: *N*, normal growing types; *slSG*, slow SG; *main*, the main hypha. The arrows in the second column indicate a change in cytoplasmic attribute through successive conidial generations.

https://doi.org/10.1017/S0016672300010910 Published online by Cambridge University Press

SG types will be detected. These platings yielded only one cytoplasmic type from each hyphal segment in the majority of cases (twenty out of thirty-two). Occasionally, however, two cytoplasmic types were recovered. Some of the results are shown in Table 4. The most common class of mixed segments yielded normal and slow SG. More rarely, the other possible combinations of cytoplasms were found in single segments: e.g. SG and normal (segments m, n); slow SG and SG (segments h, l); and all three types (segment o).

Normal or slow SG strains recovered from a heterokaryon behave as expected in a cross; that is, if they are used as protoperithecial parent, all the progeny will be normal or slow SG respectively. No exceptions have been found. On the other hand, SG recovered from heterokaryons is less predictable. On occasion an SG type recovered from a heterokaryon between normal and SG, though quite stable in vegetative culture, will yield some or all slow SG progeny when used as the protoperithecial parent in a cross. This type should be classed as slow SG. Therefore, a critical test of the identity of different cytoplasmic types derived from a single hyphal segment is their transmission in crosses. A limited number of crosses between strains recovered from cytoplasmically heterogeneous segments and normal growing strains has been made. Results of these crosses have thus far confirmed the identity and stability of the different cytoplasms from several heterogeneous segments.

(iii) *The effect of acriflavine*

Since SG was originally isolated from a medium containing 0·125 mg./ml. acriflavine (Srb, 1958), it was of interest to examine its behaviour toward the dye. A solid medium with 1·25 μg./ml. acriflavine completely prevented the growth of SG and slow SG conidia plated on it but had no obvious effect on the conidia of normal strains. SG strains, whether with the original SG-2A nucleus or recombined with the nucleus of N-6A, are susceptible. This sharp distinction between normal and SG is found only at certain concentrations of acriflavine. Low concentrations permit growth of some SG conidia while high concentrations affect both normal and SG conidia.

The conidia of phenotypically normal types which change to slow SG in later conidial generations show an immunity to acriflavine. This immunity is lost, however, once the strain has changed to slow SG. If such conidia are assumed to have both normal and SG cytoplasm, the normal cytoplasm may be dominant and 'protect' the SG cytoplasm from the dye's effect.

Ascospores behave like conidia. Ascospores from a cross of SG as protoperithecial parent and normal as the conidial parent all failed to grow on a solid medium with 1·25 μg./ml. acriflavine, but those of the reciprocal cross were insensitive to the dye. The response of vegetative hyphae was much more variable. Preliminary investigations suggest they are less sensitive than spores and that this sensitivity depends on the physiological age of the hyphae.

Only a limited amount of work has been done on the physiology of the acriflavine

effect. The sensitivity of SG conidia in liquid acriflavine medium seems directly dependent on the rate of growth of the culture. Table 5 points up the striking difference in the dry weight of the SG strain in shaken and unshaken culture flasks containing acriflavine medium. Since conditions of light and temperature were the same for both, this difference can be due to the enhancement of growth rate in the shaken, and therefore better aerated, flasks. The acriflavine effect is apparently both an inhibition and a killing. Conidia of normal and SG strains were treated with an effective dose of acriflavine for varying times and their viability noted. If the conidia were not exposed to the acriflavine for too long, they could recover; but prolonged exposure led to death.

Table 5. *A comparison of growth of an SG and an N strain in shaken and unshaken flasks*

Strain	Medium	Dry weight (mg.)	
		Shaken culture	Still culture
SG-2A	Complete	79·8	44·5
	Complete + 2 × bc	59·9	31·3
	Complete + 3 × bc	1·8	17·9
	Complete + 4 × bc	0·0*	8·9
N-6A	Complete	98·2	51·1
	Complete + 2 × bc	95·0	37·8
	Complete + 3 × bc	92·8	43·1
	Complete + 4 × bc	91·4	45·2

Each flask was inoculated with a 0·5 ml. aliquot of a heavy conidial suspension in water and incubated at 35°C. for 70 hours in a water bath with and without agitation. There was a subsequent stay at 3°C., a temperature which essentially prevents growth, for 50 hours before the mycelial pads were harvested. The symbol 'bc' refers to the base concentration of acriflavine (1·25 μg./ml.). All dry weight measurements are an average of two flasks except the one with an asterisk where one flask was counted.

4. DISCUSSION

(i) *The physical nature of SG*

Two observations suggest that SG is particulate. First, the SG trait can be found in the hypha at some distance from its point of entry but with no evidence of SG cytoplasm in between. Thus the SG cytoplasmic factor appears to travel down the established normal hypha in spurts or waves. If SG were the result of a diffuse steady state, such a discontinuity would be unlikely. Second, cytoplasmic heterogeneity in individual hyphal segments implies that the normal and SG cytoplasms can exist together and later segregate unaltered. This segregation is also difficult to interpret on a non-particulate basis but can be readily explained as a reshuffling of discrete particles.

The low incidence of cytoplasmic migration compared with nuclear migration can be interpreted at this time in various ways. One possibility is a preferential transport of nuclei over cytoplasm during protoplasmic migration. Such preferential

transport is hard to imagine, however, unless the SG determinants are attached to structures in the cytoplasm which do not move readily in protoplasmic streaming. Such structures can exist; Buller (1933) has shown that vacuoles in *Gelasinospora*, a close relative of *Neurospora*, remain attached to the cell wall unless the flow of protoplasm is intense. A second possible interpretation is that the relative amount of protoplasm entering a cell necessary to establish a new cytoplasmic state is much greater than that needed to allow the entrance and expression of the foreign nuclei.

Infanger (1963) suggested that slow SG is the result of an interaction between normal and SG cytoplasms. Slow SG usually arises from strains which in the first or even second conidial generation are phenotypically normal or SG on plates. In some way normal or SG is converted to slow SG, and this requires active hyphal growth. If hyphal growth is maintained, a total conversion from normal to slow SG may occur within the length of one growth tube (30 cm.). If growth during this time is interrupted by conidiation the conversion process ceases within the conidia formed and does not resume until they geminate. This interpolation of conidiation is inevitable in small tube culture because of the small amount of medium present, and results in the 'conidial generations' described earlier in this paper. Small tube culture consequently permits a crude analysis of the steps in the conversion process. Conversion often proceeds from normal to an intermediate SG habit and finally to slow SG. Presumably both normal and SG cytoplasms were initially present but the former was soon eliminated. This suggests a competition between the two cytoplasms which may represent a higher multiplication rate of SG cytoplasm or even its actual suppression of normal cytoplasm.

All available data suggest that once initiated the process of conversion continues until the culture is slow SG. Although slow SG types are significantly slower than the parental SG, they may differ from one another. At least two of the slow SG's differ markedly in the length and intensity of the growth lag after spore gemination. The difference has been maintained in vegetative culture. In crosses these differences tend not to be maintained and instead a spectrum of slow SG types is found among the progeny.

SG was originally isolated in *N. crassa*. The cytoplasmic makeup of the original isolate can be represented as (SG, cra) where the symbol SG denotes the SG determinants and 'cra' the remainder of the cytoplasm of *N. crassa*. Within this remainder there are likely to be other stable, autonomous, hereditary units. Because *Neurospora* shows maternal inheritance for certain traits, the protoperithecial parent probably contributes the bulk, if not all, the cytoplasm to the progeny of a cross. Since in the transfer of SG from *N. crassa* to *N. sitophila* the SG parent was always the protoperithecial parent, the resultant strain SG-2A may have other stable *N. crassa* cytoplasmic determinants in addition to the SG even though this strain is *N. sitophila* in nucleotype. This is supported by the work of Reich & Luck (1966). They found that strain SG-2A (referred to as 6-486-2a in their paper) contains mitochondrial DNA like that in *N. crassa*, and not like that in wild-type *N. sitophila*. When SG-2A is combined with a wild-type *N. sitophila* strain in a heterokaryon, a mixing of the two cytoplasms of a kind not found in crosses may occur, followed by

recombinations among cytoplasmic determinants. Since SG-2A's cytoplasm is symbolized as (SG, cra), wild-type *N. sitophila* cytoplasm can be written as (N, sit) where 'sit' is the remainder of cytoplasm other than the normal vs. SG alternative. Slow SG perhaps represents the recombinant (SG, sit), that is SG with at least part of the true *N. sitophila* cytoplasm.

If such an hypothesis is true, (N, cra) types should also be recoverable. They would not yield slow SG in a heterokaryon with SG-2A. No such types have been found, but an exhaustive search has not been made. Infanger, however, mentions recovering normal type from a heterokaryon between normal and SG which 'may have been altered cytoplasmically'. This strain was cytoplasmically normal in crosses, but when used as the protoperithecial parent, the progeny had low viability. This may be indicative of an unfavourable association of N (normal) and cra determinants.

(ii) *The acriflavine effect*

Ephrussi and co-workers (1949) showed that acriflavine can cause a complete conversion of normal to petite yeast. Apparently the dye affects the cytochrome system, particularly the synthesis of cytochrome oxidase (Ephrussi, 1956). Since cytochrome oxidase is located in the mitochondria and the petite mutation appears to be the consequence of aberrant mitochondria (Yotsuyanagi, 1962; Tuppy & Wildner, 1965), it is very possible acriflavine affects these organelles directly.

The conversion from normal to petite requires active growth of the yeast. By comparison the suppressive effect of acriflavine on SG also seems to require active growth. After 5 hours in an acriflavine medium at 35°C. SG conidia are unaffected, but by 10 hours most have been killed. This interval of maximum killing corresponds with active germination and growth from the conidia. There is a parallel in the behaviour of SG hyphal tips in acriflavine. They grow normally for a while, produce a sparse though visible mycelium and then die. It is as though the acriflavine is preventing the multiplication of some vital entity. The similarity between acriflavine's effect on petite and SG suggests that the SG determinants could be associated with or an integral part of the mitochondria. The autonomy of mitochondria in *Neurospora* is still in debate, but the recent discovery of DNA in the mitochondria of *N. crassa* (Luck & Reich, 1964) and the maintenance of mitochondrial DNA peculiarities through crosses (Reich & Luck, 1966) strongly suggest this autonomy.

SG's phenotype can now be defined in both terms of growth habit and acriflavine sensitivity. Acriflavine sensitivity may be the more refined criterion if the conditions under which sensitivity are measured are carefully standardized.

SUMMARY

Heterokaryons were made between a cytoplasmic variant of *Neurospora* called SG, which shows a slow growth rate, and normal strains. Fusions were made in such a way that the fate of the protoplasmic mixes in individual hyphae could be

followed. A semi-quantitative analysis of nuclear and cytoplasmic migration along established hyphae was possible. Apparently, components of SG and normal cytoplasms are able to associate intimately in a single hyphal segment and later segregate intact. More often, however, an interaction of cytoplasms occurs which results in a new stable cytoplasmic type called slow SG. It is postulated the cytoplasmic determinants of SG are particulate and that the slow SG strains are the result of reassortment of the SG factor with other autonomous cytoplasmic entities. Acriflavine in the proper concentration inhibits and kills SG strains on solid medium without affecting normal strains. The acriflavine is most effective against germinating spores. The SG phenotype is redefined on the basis of this sensitivity to acriflavine.

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