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Suppressors of 'blue' mutations in yeast

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SUMMARY

Mutants of *Saccharomyces cerevisiae* whose colonies were blue when grown on nutrient agar medium containing methylene blue reverted to wild-type with white-colony phenotype at high frequency. This reversion was controlled by nuclear gene suppressors in some mutants, and by cytoplasmic suppressors in others. Each of the latter suppressed several independetly segregating blue mutants. These suppressors could be divided into two classes: suppression by petite mutations which behaved as recessives, and suppression by a cytoplasmic factor in respiration-sufficient cells which behaved as dominant over wild-type but might also be a mutation of *rho*. A relationship between blue mutation and temperature sensitivity was suggested.

1. INTRODUCTION

Mutants of yeast whose colonies were of various shades and intensity of blue when grown on agar medium containing methylene blue were first observed by Costello (1965). The stable blue mutants have been subsequently shown to take up more dye than wild-type and to exhibit single nuclear gene inheritance (Mitchell & Bevan, 1973). It was also observed that even the comparatively stable blue mutants had a high frequency of reversion to wild-type. This reversion could either be caused by genetic suppression (Gorini & Beckwith, 1966), or by a much higher reverse mutation frequency at the site of mutation than is normally found. Other investigators have reported such a high degree of instability in *Neurospora crassa* (Barnet & De Serres, 1963) and in yeast (Lindegren *et al.* 1965; Nasim, 1967; Nasim & Grant, 1973).

The investigations described below were carried out to determine the genetic nature of the revertants of several dark-coloured blue mutants. Although the description of a colony colour as blue may denote any shade and intensity of colour in the blue to green range we have divided blue phenotypes into dark coloured (D), medium coloured (M), light coloured (L) and pale coloured (P) groups for convenience. Pale colour is so light that it can usually be distinguished only by comparison with wild-type.

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2. MATERIALS AND METHODS

(i) *Strains and media used*

The following strains were used: haploid wild-type N1 (Oxford Stock Collection), from which were derived the non-allelic dark-blue mutants B18, B23, B28, B29 and B33 (Mitchell & Bevan, 1973). Revertant strains of these blue mutants 18R1 to 18R5, 28R2 and 28R3, 29R1 and 29R2, 33R1 to 33R3 were derived from B18, B28, B29 and B33 respectively by plating out these strains and selecting white or pale-coloured colonies each of which were respiration-sufficient. Petite revertant strains 23C1 and 23C2, 28C1 and 28C2, 33C1 and 33C2 were derived by acriflavine treatment of B23, B28 and B33 respectively.

The following media were used: yeast complete agar medium (Cox & Bevan, 1962) containing 0·003 % methylene blue; glycerol agar medium made as yeast complete agar medium but with 2·5 % glycerol in place of 4 % glucose; and acetate agar medium (Fowell, 1952).

(ii) *Measurement of reversion frequency*

This was found by growing 800–1800 colonies on medium containing 0·003 % methylene blue and by counting the number of white and pale-coloured colonies arising.

(iii) *Acriflavine treatments*

Yeast cells were inoculated into bubbler tubes containing 10 ml of liquid yeast complete medium and varying quantities of acriflavine. These cultures were aerated and grown at 28 °C till the end of log phase, i.e. until there were about 10^6 cells per ml, and appropriate dilutions were plated on to yeast complete agar medium.

(iv) *Genetic crosses*

Cells were mated using a mass mating technique followed by streaking out the mating mixture and separating the mating figures at the appropriate time using a Singer micromanipulator (Barer & Saunders-Singer, 1948), and a microloop *c.* 70 μm diameter made on a de Fonbrunne microforge. The open-plate method of micromanipulation (Bevan & Woods, 1963) was used. Diploids were then separated from the mating figures, grown up on yeast complete agar medium at 28 °C, and then spored on acetate medium at 24 °C. Tetrads of ascospores were then separated by micromanipulation after the asci walls had been dissolved in mushroom enzyme (Bevan & Costello, 1964).

(v) *Assay for petite*

The petite phenotype was tested either by replicating colonies on to glycerol agar and observing which could not grow, or by the tetrazolium overlay technique (Ogur, St John & Nagai, 1957).

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3. RESULTS

(i) *Production and frequency of revertants from blue to white phenotype*

Table 1 shows that reversion frequency from blue to white colony phenotype was very high, reaching 13 % in strain B28. It was also noticed that all the petite colonies arising from strains B23, B28 and B33 possessed a white colony phenotype except 3 out of a total of 257 observed, whilst the converse was true for B18 and B29 where only one petite out of 33 had a white colony phenotype. These results imply that the absence of functional mitochondria suppress some of the blue mutants but not others.

Table 1. *White revertant segregants from some blue-mutant strains*

Mutant used	Total colonies plated	Respiration-sufficient white or light-coloured colonies	Respiration-sufficient dark-coloured blue colonies	Respiration-deficient white or light-coloured colonies	Respiration-deficient dark-coloured blue colonies
B18	990	55	919	0	16
B23	823	1	761	61	0
B28	1280	8	1117	154	1
B29	1203	2	1183	1	17
B33	1726	8	1677	39	2

Table 2. *Production of petites using acriflavine treatment on blue-mutant strains*

Mutant	Acriflavine conc. (μg/ml)	Duration of application (h)	Blue-colony phenotype		White-colony phenotype	
			RS	RD	RS	RD
B18	0	5	568	28	4	0
	2·5	5	344	67	0	1
B23	0	20	540	0	1	12
	0·25	20	492	1	2	489
B28	0	20	514	1	1	30
	0·25	20	516	1	3	409
B29	0	5	186	6	1	1
	2·5	5	159	42	3	2
B33	0	24	511	2	0	5
	0·25	24	457	5	3	10
	0·5	22	25	0	0	608

RS = respiration-sufficient. RD = respiration-deficient.

(ii) *Effect of acriflavine on strains B18, B23, B28, B29 and B33*

To confirm the conclusions about the effects of petite mutation on different blue mutants five strains – B18, B23, B28, B29 and B33 – were treated with acriflavine. The results, Table 2, confirm the observations from Table 1, that the absence of functional mitochondria in nearly all cases suppressed the blue phenotypes of B23, B28 and B33 but not of B18 and B29.

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(iii) *Crosses between wild-type N1 and revertant strains of blue mutants*

These crosses were carried out to determine whether revertants from several blue mutants were caused by true reversions of the original mutation or by suppressor mutations, and in the latter case, to determine the mode of inheritance of the suppressors.

Table 3. *Wild-type N1 strain crossed with revertant strains of blue-mutant strains B18 and B29*

Cross	Revertant phenotype	4W	2W:2P	Tetrad data 3W:1B	2W:1P:1B	2W:2B	No. of incomplete tetrads
$18R_1 \times N1$	W	4	—	1	—	—	1*
$18R_2 \times N1$	W	4	—	—	—	—	4*
$18R_3 \times N1$	P	—	2	—	4	1	2*
$18R_4 \times N1$	P	—	2	—	2	—	2
$18R_5 \times N1$	P	—	2	—	7	1	6*
$29R_1 \times N1$	P	—	1	—	7	1	2*
$29R_2 \times N1$	W	1	—	7	—	1	—

W, B, and P: white, dark or medium blue, and pale-coloured colony phenotype respectively.
R: revertant phenotype.
* Dark-blue spore colonies occurred in these incomplete tetrads.

(*a*) *Blue mutants in which suppressors showed normal mendelian inheritance*

Revertants of B18 and B29 were crossed with wild-type N1 (Table 3) and showed the type of inheritance expected if the revertants contained independently segregating nuclear gene suppressors, i.e. when white revertant strains of B18 and B29 were crossed with wild-type strain N1 three types of tetrad ratios of spore colonies were found: 2 dark blue:2 white; 1 dark blue:3 white; and 0 blue:4 white. Similarly, when pale-coloured revertants were analysed the spore colony ratios were: 2 dark blue:2 white; 1 dark blue:1 pale coloured:2 white; and 2 pale coloured:2 white. The tetrads containing 1 dark blue:3 white, or 2 white:1 dark blue:1 pale-coloured spore colonies, were the most common.

On the assumption that suppression was by independently segregating nuclear genes each of the white spore colonies from a 2 blue:2 white tetrad should have contained the suppressor gene and no blue mutant gene, whilst the pale-coloured spore colony from tetrad type 1 dark blue:1 pale coloured:2 white should have contained both a suppressor gene and a blue mutant gene. Thus, when a white spore colony of this type was back-crossed with the original blue strain, and when such a pale-coloured spore colony was crossed to wild-type, both resulting diploids should have yielded tetrad ratios of 2 dark blue:2 white, 1 dark blue:1 pale coloured:2 white, and 2 pale coloured:2 white, with a preponderance falling into the 1 dark blue:1 pale coloured:2 white class. Second-generation crosses involving B18 and its suppressors (Table 6) show that such experimental results were obtained and agreed well with those expected on the hypothesis of the independent segregation of a nuclear gene suppressor present in each revertant.

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(b) Blue mutants with suppressors showing non-mendelian inheritance

(A) *Petites derived from B23, B28 and B33.* If petite mutation was responsible for suppressing blue phenotype in B23, B28 and B33 as suggested by the data in Tables 1 and 2 then crossing white petite colonies derived from these mutants with wild-type should yield white respiration-sufficient diploids which on tetrad analysis should segregate 2 blue:2 white respiration-sufficient spore colonies. Table 4 shows that such results were obtained when the revertant petites 23C1, 23C2, 28C1, 28C2, 33C1 and 33C2, derived by acriflavine treatments of respectively

Table 4. *Acriflavine-induced petite white revertant strains crossed with wild-type N1*

Cross	Diploid phenotype	Tetrad data	
		2B:2W	1B:3B
23C(1) × N1	W	6	1
23C(2) × N1	W	4	—
28C(1) × N1	W	9	1
28C(2) × N1	W	3	2
33C(1) × N1	W	5	—
33C(2) × N1	W	6	—

W and B: white and blue spore colony phenotype respectively.

Table 5. *Wild-type N1 strain crossed with respiration-sufficient revertants of blue-mutant strains B28 and B33*

Cross	Revertant phenotype	4W	Tetrad data		No. of incomplete tetrads
			2W:2P	3W:1PC	
$28R_2$ × N1	P	—	9	—	3*
$28R_3$ × N1	P	—	6	—	5*
$33R_1$ × N1	W	5	2	2	2*
$33R_2$ × N1	P	—	7	3	0
$33R_3$ × N1	P	—	8	1	3†

W, B and P: white, dark-blue and pale-coloured colony phenotype respectively.
R: revertant blue-mutant phenotype.
* No dark-blue phenotype spore colonies in these incomplete tetrads.
† One dark-blue spore colony occurred in these incomplete tetrads.

B23, B28 and B33, were crossed with wild-type strain N1. Thus in these petite revertants it would appear that elimination of all or part of the mitochondrial genome had resulted in suppression of the blue mutation and in the formation of petite phenotype.

(B) *Respiration-sufficient revertants of B28 and B33.* When respiration-sufficient revertants of B28 and B33 were crossed with wild-type the diploids had white colonies as expected, but yielded tetrads with only white or pale-coloured spore colonies on analysis; the dark-blue phenotype of the parental blue mutant was only once recovered in an incomplete tetrad (Table 5).

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These results present three possibilities: either true reverse mutation had occurred at the site of the blue mutation, or an extremely closely linked suppressor was present, or suppression was by a dominant cytoplasmic factor.

These possibilities were differentiated in second-generation crosses, the results of which are shown in Table 6. If reverse mutations or a very closely linked suppressor mutations were responsible for the revertants, all the spores derived

Table 6. *Crosses involving spore colonies derived from wild-type N1 crossed with white revertants of the blue-mutant strains B18, B28 and B33 (2nd-generation crosses)*

Original cross	Tetrad selected	Selected spore colony pheno-type	Cross carried out	Diploid pheno-type	No. of incom-plete tetrads	Full tetrad spore colony phenotypes
$18R_3 \times N1$	2W:2B	W	W × B18	W	3	1 (2W:2B), 7 (2W:1P:1B) 1 (4W)
$18R_5 \times N1$	2W:1P:1B	P	P × N1	W	6	2 (2W:2B), 4 (2W:1P:1B)
$28R_2 \times N1$	2W:2P	P	P × B28	P	2	10 (4P)
$28R_3 \times N1$	2W:2P	W	W × B28	W	1	4 (2W:2P), 1 (2W:1P:1B), 9 (2W:2B)
$33R_3 \times N1$	2W:2P	P	P × B33	P	3	6 (4P)
$33R_3 \times N1$	2W:2P	P	P × B33	P	3	6 (4P)
$33R_3 \times N1$	2W:2P	W	W × B33	W	5	6 (2W:2P)
$33R_3 \times N1$	2W:2P	W	W × B33	W	2	9 (2W:2P)

W, B and P: white, dark-blue and pale-coloured colony phenotype respectively.
R: revertant blue-mutant phenotype.

Table 7. *Spore analysis of segregants of suppressed diploids $B33 \times 33R_3$ and $B28 \times 28R_2$*

Diploid used	Phenotype of segregant	No. of spores with phenotypes shown W	P	M	D
$28R_2 \times B28$	M	0	15	12	10
$33R_3 \times B33$	P	22	14	0	0
$33R_3 \times B33$	M sectored	0	13	2	6
$33R_3 \times B33$	M	0	12	0	4

W, P, M and D: white, pale, medium and dark-coloured colony phenotype respectively.
R: revertant blue-mutant phenotype.

from the wild-type × revertant crosses should be wild-type and yield 2 blue:2 white spore colonies when crossed back to the original blue mutant. However, if a cytoplasmic suppressor was responsible, all the spore colonies from the wild-type × revertant crosses should have contained this suppressor, and when these spore colonies were crossed back to the original blue mutant all the resultant spore colonies should have been suppressed blue mutants (pale coloured) or wild-type.

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The latter results were obtained in five out of six cases as shown in Table 6, indicating dominant cytoplasmic suppression. It would appear that the exceptional case could be explained if the suppressor had been partially lost, resulting in 9 out of the 15 tetrads analysed giving 2 blue:2 white spore colonies.

(C) *Somatic segregation of blue phenotype colonies from respiration-sufficient revertants 28R2 and 33R3.* It was hoped to confirm cytoplasmic suppression of B28 and B33 by demonstrating somatic segregation of dark-blue colonies either from the haploid revertants themselves or from pale-coloured diploids synthesized by crossing the revertant back to the original blue mutant.

No segregation was found amongst at least 10^3 colonies plated out from the haploids. However, the diploids segregated pale- to medium-coloured colonies some of which were sectored. Analysis of these segregants (Table 7) showed that the darker-coloured segregants yielded more dark-coloured spore colonies in their tetrads than did the pale-coloured segregants as would be expected if somatic segregation of the suppressor had occurred in the diploid.

Table 8. *Crosses of strains $33R_3$ and $28R_2$ with B33 and B23*

Cross	Diploid phenotype	Tetrad data 1W:3P	2W:2P	4P
$28R_2 \times B23$	W	7	—	2
$28R_2 \times B33$	W	5	—	1
$33R_3 \times B23$	P	4	2	4

W and P: white and pale-coloured colony phenotype respectively.
R: revertant blue-mutant phenotype.

(D) *Crosses between 28R2 × B23, 28R2 × B33 and 33R3 × B23.* The similarity in the genetics of suppression of B23, B28 and B33 by petite suggested that perhaps the dominant cytoplasmic suppressor was also similar for all three mutants. The results (Table 8) support this theory as only white or pale-coloured spore colonies were recovered from the crosses described; the dark-blue parental phenotype never appeared, as would be expected if the same cytoplasmic factor was responsible for the dominant suppression in all three mutants.

4. DISCUSSION

Blue mutants could be divided into two classes according to the suppression found. In the first group suppressors showed independent nuclear gene segregation and were characteristic of B18 and B29. The high reversion frequency was therefore unexpected in B18 but might be explained if that particular mutant strain had an increased overall mutation frequency as is known to occur in DNA polymerase mutants (Hall & Brammar, 1973).

The second group, into which B23, B28 and B33 fell, showed non-mendelian inheritance of suppression. The results in Tables 1, 2 and 4 could be explained most simply if the white petites carried the blue mutation suppressed and if the presence

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of functional *rho* particles received from the parent wild-type strain N1 permitted the expression of blue mutation in the resultant spore colonies.

The occasional petite with a blue-colony phenotype could be explained in the blue strains B23, B28 and B33 if these petites retained some mitochondrial DNA which was responsible for permitting the expression of the blue-mutant phenotype. The occurrence of residual mitochondrial DNA in petites has been well documented (Monolou, Jacob & Slonimski, 1966; Nagley & Linane, 1972).

When spontaneous respiration-sufficient revertants of B28 and B33 were tested in genetic crosses (Tables 5, 6), there was evidence, supported by somatic segregation (Table 7), that suppression was caused by a dominant cytoplasmic element.

The observations that (*a*) the same factor appeared to suppress B23, B28 and B33 (Table 8) and (*b*) acriflavine appeared marginally to increase the frequency of these respiration-sufficient revertants (Table 2) suggest that the dominant cytoplasmic suppressor might be a mutated *rho* factor, but one which did not cause petite phenotype. This is the exact converse of the suggested cause of the rare blue petites derived from B23, B28 and B33. It is therefore implicit in these explanations that the suppressor genes for B23, B28 and B33 are carried on the mitochondrial DNA, but at different loci to those responsible for respiration sufficiency.

The involvement of mitochondria in cytoplasmic suppression is not surprising as they have been implicated in many (Mitchell & Mitchell, 1953; Nagley & Linane, 1972; Handwerker, Schweyen, Wolf & Kaudewitz, 1973) but not all (Jinks, 1959; Cox, 1965) examples of extra-nuclear suppression in fungi. The striking similarity in the genetics of the cytoplasmic suppression of temperature sensitivity (Handwerker *et al.* 1973) to the dominant cytoplasmic suppression of our mutants B23, B28 and B33 suggests that blue mutation and temperature sensitivity may be related. This theory is further reinforced by the observations that blue mutations were partially lethal and possibly involved changes in the cell wall or membrane (Mitchell & Bevan, 1974), and that the intensity of the blue colour was reduced when growth was at 24 °C rather than 28 °C (Mitchell, 1969).

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