ISOLATION OF SUPPRESSIVE SENSITIVE MUTANTS FROM KILLER AND NEUTRAL STRAINS OF *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Dominant sensitive mutants were isolated from a killer and a neutral strain of *Saccharomyces cerevisiae* by treatment with nitrosomethylurethane. Genetic studies suggest that these sensitives arose by mutation of the wild-type cytoplasmic genetic determinants (k) or (n) to a mutant form (s). (s) determinants lack wild-type (k) and (n) activity but are retained in the cell and suppress the replication or activity of the wild-type determinants, converting killer and neutral cells to the sensitive phenotype. These mutants show an obvious similarity in behavior to suppressive petite mutants of yeast.

STRAINS of the yeast *Saccharomyces cerevisiae* show one of three phenotypes with respect to the killer character; killer, sensitive, or neutral (BEVAN and MAKOWER 1963). Killer strains produce an extracellular protein, the "killer factor", which kills sensitive cells (WOODS and BEVAN 1968; BUSSEY 1972; BUSSEY and SHERMAN 1973). Neutral cells are not killed by killer cells, nor do they kill sensitive cells. The killer phenotype is determined by the presence of cytoplasmic genetic determinants (k), which are maintained in the cell only in the presence of the dominant nuclear genes *M* and Pet_s; hence the genotype of killer cells is *M* Pet_s (k). The neutral phenotype is conferred by the presence of cytoplasmic genetic determinants (n), which again are maintained in cells only in the presence of the dominant nuclear genes *M* and Pet_s. The genotype of neutral cells is therefore *M* Pet_s (n). Loss of functional determinants from killer and neutral cells results in a *cytoplasmic sensitive* strain *M* Pet_s (o), whereas mutation in either nuclear gene results in a *genic sensitive m* Pet_s (o) or *M* pet_s (o). (SOMERS and BEVAN 1969; BEVAN and SOMERS 1969; BEVAN, SOMERS and THEIVENDIRARAJAH 1969; FINK and STYLES 1972). Hence all spore cultures derived from a cross between a cytoplasmic sensitive strain and a killer or neutral strain have the killer or neutral phenotype respectively. By analogy with the well-known petite system in yeast recently reviewed by PREER (1971), the behavior of cytoplasmic sensitive mutants in crosses with killer and neutral strains is comparable to the behavior of neutral petite mutants in crosses with respiratory-sufficient strains.

The present study reports on a new class of cytoplasmic sensitive mutant isolated from killer and neutral strains of yeast. This sensitive mutation is domi-

nant to the killer and neutral phenotype, and the crosses described here show that this mutation behaves in an analogous manner to the suppressive petite mutation in yeast (EPHRUSSI, MARGERIE-HOTTINGUER and ROMAN 1955). The mutants were isolated by treatment of killer and neutral cells with nitrosomethylurethane, an agent which was shown by SCHWAIER, NASHED and ZIMMERMAN (1968) to be an extremely effective cytoplasmic mutagen in yeast.

MATERIALS AND METHODS

Yeast strains: The genotypes of the strains used in this study are shown in Table 1. The strains were derived from various crosses between strains K12, N2 and S13 of DR. E. A. BEVAN, and S2021B and S288C-26 of DR. R. MORTIMER. Strains K19 and N5 were used for mutant induction, strains K12, N12, S14 and S18 were used in first-generation crosses with the mutants, and the remaining strains were used for second generation crosses. Strains K12 and S14 were also used for phenotype tests.

Media and culture conditions: Minimal agar medium (MA) was that of HALVORSON (1958), pH 4.7, supplemented with 2% agar and with 12.5 μg/ml amino acids where necessary. YEPD: HALVORSON's medium with 0.5% yeast extract, 0.5% peptone, 2% glucose and 2% agar. YEPG: as YEPD with 2% glycerol substituted for glucose. MB: YEPD supplemented with 0.003% methylene blue. Sporulation medium contained 0.3% sodium acetate, 0.02% raffinose, and 1.5% agar. MB and sporulation plates were incubated at 20°C; other incubations were at 30°C unless stated otherwise.

Mutant induction and initial characterization: Cultures of K19 and N5 were starved overnight in water at 20°C, harvested by centrifugation and resuspended at 1×10^5 cells/ml in 1/15 M potassium phosphate buffer, pH 7.0, containing nitrosomethylurethane (NMU) to a final concentration of 20 mM. After standing at 20°C for 30 minutes, the treatment was terminated by dilution into buffer and plating onto YEPD. Survival was between 37–41%. Resulting colonies were checked for respiratory sufficiency by replica plating onto YEPG and scoring for growth after 5 days of incubation. Colonies were tested for the killer, neutral or sensitive phenotype by

TABLE 1

Yeast strains

Strain*	Genotype†
K19	*a trp5 leu1 M*(k)
N5	*a his4 M*(n)
K12	*α ade2.5 M*(k)
N12	*α ade2.5 M*(n)
S14	*α ade2.5 M*(o)
S18	*α ade2.5 m*(o)
K25	*α his5 M*(k)
K24	*a his5 M*(k)
K20	*a trp5 M*(k)
N5	*a his4 M*(n)
N10	*α his4 M*(n)
N17	*α his5 M*(n)
N6	*a trp5 M*(n)

* In our stocks, the letter K, N, or S followed by a number refers to the stock number of the killer, neutral or sensitive strain, respectively. The letter K, N, or S alone refers to the killer, neutral or sensitive phenotype, respectively.

† All strains have the genotype Pet_s, and all are respiratory sufficient.

replica plating onto MB agar previously spread with (a) a background lawn of S14 sensitive cells and (b) a background lawn of K12 killer cells. After 48 hours incubation, killer colonies are identified on the sensitive background by a surrounding zone of clear agar where the background cells have been killed. Sensitive colonies are identified on the killer background plates; the sensitive cells at the base of each colony are killed by the background cells and hence are stained deep blue (LINDEGREN 1949). Neutral colonies give no reaction on either background. Suspected mutant colonies identified by this preliminary mass screening were purified by two successive single cell isolations, and retested for phenotype individually as described below.

Phenotype testing individual cultures: Individual cultures were tested for the killer phenotype by streaking with a loop onto S14 sensitive background MB plates and identified as in (a) above. Cultures were tested for the sensitive phenotype by spreading the culture to be tested *as a background lawn* to a killer streak on MB medium. After 48 hours incubation sensitive cultures are detected by the presence of a clear zone of killing around the killer streak. This method is of particular value because it reveals cultures which consist of a mixture of killer and sensitive, or neutral and sensitive cells; in these cases resistant growth occurs within the zone of killing, either in the form of individual microcolonies or as a thin lawn of growth. Again, neutral cultures give no reaction in either test.

Crossing procedures: Strains were mass mated on solid medium by mixing a small loop of cells of each parent on YEPD, incubating for 3–4 hours, and then streaking out a very small inoculum of cells onto MA. After 48 hours of incubation diploid cultures were tested for phenotype, and samples were transferred to sporulation medium and incubated for at least 48 hours. Ascus walls were dissolved by treatment for 15 minutes in glusulase enzyme (Endo Laboratories) and single spores were isolated with a de Fonbrunne micromanipulator. Diploid subcultures were carried out by restreaking a small sample of cells onto fresh MA and incubating for 48 hours before phenotype testing for the second time.

RESULTS

Preliminary crosses: NMU treatment of killer strain K19 and neutral strain N5 yielded 5 and 12 sensitive mutants, respectively, from a total of 10,568 and 14,720 colonies screened. No spontaneous sensitive mutants were obtained from control platings of K19 (7,320 colonies screened) or N5 (4,081 colonies screened). The results of the first-generation crosses of the sensitive mutants to killer strain K12 and neutral strain N12 are summarized in Table 2. Mutant K19.1 behaved as a normal genic sensitive, giving 2K : 2S and 2N : 2S spore ratios after crossing with K12 and N12 respectively. This mutant was not investigated further. Two mutants from K19 and ten mutants from N5 behaved as normal cytoplasmic sensitives, giving 4K : 0S and 4N : 0S spore ratios after crossing with K12 and N12, respectively. The total number of tetrads analyzed have been summed for simplicity of presentation in Table 2, and these mutants have not been investigated further. The remaining four sensitive mutants were of an apparently new type in that the diploids produced were of mixed killer and sensitive (K+S)* or neutral and sensitive (N+S) phenotype, or in one instance a purely S phenotype. Diploids of mixed phenotype attained a purely S phenotype after 2–5 subculture transfers. Further, a predominance of *sensitive* spore cultures was obtained after sporulation of the original diploid cultures. These results are shown in full in Table 2.

* Cultures of mixed killer and sensitive cells can be maintained under the experimental conditions used here, since the killer toxin is unstable above 25°C (WOODS and BEVAN 1968). Even at 20°C, killing does not seem to occur on a one-to-one cell basis; a single killer cell is usually unable to kill an adjacent sensitive cell before the latter divides (author's unpublished observation).

TABLE 2

Preliminary crosses between sensitive mutants and killer strain K12 and neutral strain N12

	Crossed with K12			Crossed with N12		
Mutant	Diploid phenotype	Number of tetrads analyzed	Spore ratios	Diploid phenotype	Number of tetrads analyzed	Spore ratios
K19.1	K	5	2K:2S	N	4	2N:2S
K19.2, K19.3	K	9	4K:0S	N	6	4N:0S
N5.1–N5.10	K	77	4K:0S	N	24	4N:0S
K19.4	K+S	2	4K:0S	N+S	1	1N:3S
		1	3K:1S		4	0N:4S
		2	1K+S:3S			
		2	0K:4S			
K19.5	K+S	1	2K:2S	N+S	3	0N:4S
		3	0K:4S			
N5.11	K+S	4	0K:4S	S	3	0N:4S
N5.12	K+S	4	0K:4S	N+S	1	2N:2S
					1	1N:3S
					1	0N:4S

All tetrads showed 2:2 segregation for auxotrophic markers. All diploid and spore cultures were respiratory-sufficient.

Two basic alternative hypotheses of the genetic basis of this new type of sensitive mutant can readily be invoked. The first is that these mutants arose as a result of mutation of cytoplasmic determinants, possibly the (k) and (n) determinants themselves, to a suppressive form (s). (s) determinants do not have wild-type (k) and (n) activity (killer factor production and/or immunity to killer factor), but are retained in the cell, and suppress the replication or activity of the wild-type determinants. Thus the phenotype of the resulting mutant colonies appears to be sensitive. When the mutants are crossed with a killer or neutral strain, the (s) determinants are introduced into the zygote by the mutant parent, and suppress the replication or activity of the (k) or (n) determinants introduced by the other parent. Thus the diploid culture becomes converted to the sensitive phenotype during vegetative growth. This model predicts that, after sporulation of the diploid, all four spores of a tetrad will receive the mutant (s) cytoplasmic determinants, giving rise to spore cultures of mixed or purely sensitive phenotype, as was found to be the case. The presence of these (s) determinants in all four spores of each tetrad should be revealed by second-generation crosses with killer and neutral strains.

An alternative hypothesis is that the original event was a dominant nuclear gene mutation which prevented or slowed the replication of (k) and (n) determinants. The resulting mutant colony would consist of a majority of cells lacking cytoplasmic determinants and having the sensitive phenotype. Crossing with a killer or neutral strain would reintroduce cytoplasmic determinants into the original diploid cell, but the dominant gene mutation would prevent or slow their

replication, resulting in a diploid culture containing sensitive cells. This model again allows the possibility that all four spores of a tetrad would have the sensitive phenotype, but it is distinguishable from the first hypothesis in that only two of the four spores of each tetrad would receive the dominant gene mutation. The hypotheses are therefore distinguishable by second-generation crosses with killer and neutral strains.

Second generation crosses with killer and neutral strains: Two of the four sensitive mutants, K19.4 and N5.11, were investigated further by second-generation crosses. Cells of purely sensitive spore-derived cultures of two tetrads from each of first-generation cross K19.4 × K12, K19.4 × N12, N5.11 × K12 and N5.11 × N12 were crossed with an appropriate killer and neutral strain (of opposite mating type and with complementary auxotrophic markers). All diploid cultures resulting from these 64 crosses were tested for phenotype after two days of incubation on MA, and again after one subculturing transfer. All 64 diploids were found to have a purely sensitive or mixed phenotype either at the first testing or after one subculture transfer. Details of the results of some of these second-generation crosses (between cells of sensitive spore-derived cultures of one tetrad from first-generation crosses K19.4 × K12 and K19.4 × N12 with killer and neutral strains) are given in Table 3. These results indicate that the genetic basis of the mutation, at least in strains K19.4 and N5.11, is inherited cytoplasmically.

Crosses with M(o) *and* m(o) *sensitive strains:* If the proposed mutant (s) determinants are derived by mutation from (k) and (n) determinants them-

TABLE 3

Second-generation crosses between sensitive spore-derived cells of one tetrad from K19.4 × K12 and one tetrad from K19.4 × N12 with killer and neutral strains

Tetrad from first-generation cross	Sensitive spore culture	Crossed with strain	Phenotype of diploid original culture	subculture
K19.4 × K12	4a	K24	K	S
	4b	K25	S	S
	4c	K24	S	S
	4d	K25	S	S
K19.4 × K12	4a	N5	N+S	S
	4b	N10	N+S	S
	4c	N5	S	S
	4d	N10	N+S	S
K19.4 × N12	3a	K25	K+S	S
	3b	K24	K+S	K+S
	3c	K24	K+S	S
	3d	K25	K+S	S
K19.4 × N12	3a	N10	N	N+S
	3b	N5	N+S	S
	3c	N5	S	S
	3d	N10	S	S

All diploid and spore cultures were respiratory-sufficient.

TABLE 4

Results of crosses between mutant K19.4 with M(o) *sensitive strain S14 and* m(o) *sensitive strain S18*

First-generation cross	Sensitive spore culture	Predicted genotypes of spores	Second-generation cross to killer strain M(k) Phenotype of diploid subcultures	Predicted genotype	Spore ratios obtained
	1a		S		——
	1b	all M(s)	S	all MM(s)	——
	1c		S		——
	1d		S		——
K19.4 × S14	2a		S		0K:4S
i.e. M(s) × M(o)	2b	all M(s)	S	all MM(s)	0K:4S
	2c		S		0K:4S
	2d		S		0K:4S
	3a		S		——
	3b	all M(s)	S	all MM(s)	——
	3c		S		——
	3d		S		——
	1a		K	Mm(k)	——
	1b	2M(s):2m(o)	S	MM(s)	——
	1c		K	Mm(k)	——
	1d		K+S	MM(s)	——
K19.4 × S18	2a		K	Mm(k)	2K:2S
i.e. M(s) × m(o)	2b	2M(s):2m(o)	S	MM(s)	0K:4S
	2c		S	MM(s)	0K:4S
	2d		K	Mm(k)	2K:2S
	3a		K	Mm(k)	2K:2S
	3b	2M(s):2m(o)	K	Mm(k)	2K:2S
	3c		S	MM(s)	0K:4S
	3d		S	MM(s)	no viable spores

All diploid and spore cultures were respiratory-sufficient.

selves, they would be expected to require the presence of the nuclear gene M for their maintenance in the cell. In order to test this prediction, mutants K19.4 and N5.11 were first each crossed with M(o) sensitive strain S14 and m(o) sensitive strain S18. Three tetrads were analyzed from each of these crosses, and each was found to consist of four sensitive spore-derived colonies as expected. Table 4 shows the predicted genotypes of the spore-derived cultures obtained from crosses K19.4 × M(o) sensitive and K19.4 × m(o) sensitive, together with the results of the subsequent second-generation crosses which were carried out between these spore cultures and appropriate killer strains of opposite mating type and complementary auxotrophic requirements. A parallel series of second-generation crosses was also carried out between the 24 spore cultures derived from first-generation crosses N5.11 × M(o) sensitive and N5.11 × m(o) sensitive, and exactly comparable results were obtained (not shown in the table). These results indicate that

the presence of the dominant nuclear gene *M* is required for the maintenance of the (s) determinants in the sensitive spore cultures, and hence support the hypothesis that the (s) determinants are a mutant form of the (k) and (n) determinants.

DISCUSSION

The results of these analyses support the hypothesis that suppressive sensitive strains contain mutant cytoplasmic determinants, (s), which suppress the replication or activity of (k) and (n) determinants, thereby converting the cells containing them to the sensitive phenotype. That the (s) determinants arose by mutation of the (k) and (n) determinants themselves is suggested by the finding that (s) determinants require the presence of the nuclear gene *M* for their maintenance in the cell. This mutation therefore appears to be analogous to the suppressive petite mutation in yeast, which is due to an alteration in the mitochondrial DNA such that its introduction into respiratory-sufficient strains suppresses the replication or activity of the wild-type mitochondrial DNA and converts the cells to the respiratory-deficient phenotype. It should be noted, however, that cells containing (s) determinants are not converted into petites, and this finding argues against the possibility that (k) (n) and (s) determinants are part of the mitochondrial DNA of yeast cells. The converse tests of the effects of the suppressive petite factor on (k) and (n) have not been carried out, although it is routinely observed that spontaneously-occurring petite mutants do not lose their original killer or neutral phenotypes. The finding that killer cells are not converted to the sensitive phenotype by ethidium bromide treatment (BEVAN, SOMERS and THEIVENDIRARAJAH 1969; FINK and STYLES 1972; AL-AIDROOS, BUSSEY and SOMERS 1973), an agent which can eliminate all detectable mitochondrial DNA from yeast cells (GOLDRING *et al.* 1970; NAGLEY and LINNANE 1970, 1972), also argues against the possibility that (k) and (n) determinants are part of the mitocondrial DNA.

The nature of the (k) and (n) determinants is at present unknown. BERRY and BEVAN (1972) have identified a species of double-stranded RNA in certain strains of *Saccharomyces cerevisiae*; however these authors were unable to correlate the presence of this RNA species with the killer phenotype. The possibility that sensitive strains could harbor inactive and phenotypically-undetectable cytoplasmic determinants cannot be ruled out, and if this is the case it may not be possible to identify the (k) and (n) determinants by a comparison between the nucleic acid species present in killer, neutral and sensitive strains. Suppressive petite mutants show detectable differences in the physical properties of the mitochondrial DNA compared with that of wild-type respiratory-sufficient cells (MOUNOLOU, JACOB and SLONIMSKI 1966; BERNARDI *et al.* 1968; MEHROTRA and MAHLER 1968; CARNEVALI, MORPURGO and TECCE 1969; NAGLEY and LINNANE 1972). It is conceivable that the (s) determinants of suppressive sensitive mutants might have detectable alterations from the wild-type (k) and (n) determinants; if this were the case, identification of the (k) and (n) determinants might be possible by comparing the nucleic acid species in *M*(k), *M*(n) and *M*(s) strains.

The possibility that (k) and (n) determinants are mutant forms of one another has been suggested by SOMERS and BEVAN (1969) on the grounds that both require the same nuclear genotype for their maintenance in the cell, and that they share a common property of conferring killer-factor immunity to a cell containing them. These present studies have shown a third similarity in that both (k) and (n) determinants can mutate to (s), which in turn can suppress both (k) and (n).

Suppressive petite mutants in yeast show different and characteristic "degrees of suppressivity", measured by the percentage of petite diploid colonies resulting from a cross between a petite and a wild-type strain (EPHRUSSI, MARGERIE-HOTTINGUER and ROMAN 1955; EPHRUSSI and GRANDCHAMP 1965). Comparable quantitative studies between different suppressive sensitive mutants have not yet been carried out, but preliminary indications based on the results of the diploid subculturing procedure described here are that the majority of diploid cultures approach or attain a purely sensitive phenotype after one or a few subculture transfers. However, more detailed studies are required in order to determine whether or not these suppressive sensitive mutants show any characteristic differences from each other.

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