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Genetics

Mutation of the non-Mendelian suppressor, ψ^+, in yeast by hypertonic media

(nonsense suppression/*Saccharomyces cerevisiae*/cytoplasmic inheritance)

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ABSTRACT **The ψ^+ extrachromosomal determinant in the yeast *Saccharomyces cerevisiae* suppresses certain UAA markers and increases the efficiency of suppression of UAA suppressors and certain frameshift suppressors. Although the exact nature of ψ^+ determinant is unknown, it is believed to be a self-replicating cytoplasmic factor affecting some component of the translational machinery. In this report we describe growth conditions for efficient mutation or elimination of the ψ^+ determinant. Incubation of ψ^+ cultures in hypertonic nutrient medium resulted in rapid conversion to a culture containing predominantly ψ^- cells during the growth cycle. The kinetics of ψ^+ to ψ^- conversion established that the occurrence of ψ^- cells was due to induction and not to selection of pre-existing ψ^- cells. The results suggest that the replication of the ψ^+ determinant is sensitive to hypertonic conditions.**

The non-Mendelian genetic determinant, ψ^+, was first shown to modify the expression of certain nonsense suppressors in the yeast *Saccharomyces cerevisiae* (1). Further studies established that the efficiency of suppression of certain UAA suppressors (2–4) and certain frameshift suppressors (5) is higher in ψ^+ strains as compared to ψ^- strains. Even in the absence of any known suppressors, the ψ^+ determinant suppresses some UAA markers, especially *trp5-48*, albeit weakly (4, 6). Tetrad and heterokaryon analyses have demonstrated that the ψ^+ determinant resides outside the nucleus (1, 7). Genetic analysis with other types of cytoplasmic mutants indicated that the ψ determinant is not associated with mitochondrial DNA (8) or with the double-stranded RNA that controls the "killer" phenotype (9). Although the molecular identity of ψ is unknown, it is believed to be a self-replicating cytoplasmic factor that either directly or indirectly affects the translational process.

In this investigation we report that high frequencies of ψ^- cells arise in ψ^+ cultures incubated in hypertonic media and that these mutants are formed by mutation of ψ^+ cells and not by selection of pre-existing ψ^- cells. It is suggested that the $\psi^+ \rightarrow \psi^-$ induction is due to the impaired replication of the ψ^+ determinant under hypertonic conditions.

MATERIALS AND METHODS

Genetic Nomenclature and Yeast Strains. The basic strains, listed in Table 1, were constructed for this investigation by standard yeast genetic procedures and contain various UAA markers previously described (10). Strain B-4818 was derived from a culture of D898-3C grown in hypertonic medium containing 2.5 M KCl. The suppressor *SUP16*, previously designated *SUQ5*, *SUPQ5*, and S_{Q5} (1), is located on the right arm of chromosome XVI and causes the insertion of serine residues at UAA sites (4). The genetic symbols ρ^+ and ρ^- denote, respectively, wild-type and mitochondrial mutants deficient in cytochromes *a*, a_3, and *b*.

Media. The routine nutrient medium contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose. The hypertonic media were prepared by supplementing the nutrient medium with potassium chloride or ethylene glycol. Glycerol medium consisted of 1% Bacto-yeast extract, 2% Bacto-peptone, and 3% (vol/vol) glycerol. Either 1% or 2% Noble agar was added if solidification was desired.

Population Analysis. Freshly grown cells were inoculated into hypertonic media to an initial titer of approximately 10^6 cells/ml. The cultures were incubated at 30°C with vigorous shaking. At various times samples were withdrawn, sonicated to disperse the cell clusters, and plated on nutrient medium after appropriate dilutions. In some experiments the cells were also plated on a nutrient medium containing 10% dextrose, a medium that intensifies the red coloration conferred by the *ade2-1* marker (11). The nutrient plates were incubated at 30°C and the number and types of the resulting colonies were scored after 3–5 days. At least 5000–8000 colonies were scored when the ψ^- cells were relatively rare at early incubation times. Cell densities allowing approximately 200 colonies per plate were used for most experiments, whereas higher densities, allowing 700–5000 colonies per plate, were used for examining the cell types during the first 4 hr of incubation of the cultures in ethylene glycol. The numbers of ψ^- red colonies could be accurately counted over the high background of white colonies.

In some experiments the growth in liquid hypertonic media was monitored by measuring the turbidity with a Klett–Summerson photoelectric colorimeter equipped with a no. 62 light filter.

Phenotypes. The degree of suppression in strains carrying various UAA markers can be conveniently estimated from the growth on omission media and from the degree of coloration conferred by the *ade2-1* marker. Although these properties can vary with different strains, the patterns usually observed are presented in Table 2. The ψ^+ *SUP16* and ψ^- *SUP16* strains can usually be distinguished with the *ade2-1* marker, and ψ^+ and ψ^- strains can usually be distinguished with the *trp5-48* marker. However, ψ^+ strains are not reliably distinguished from ψ^- *SUP16* strains by the degree of suppression of UAA markers; these two cell types can be differentiated by genetic analysis of crosses with standard ψ^+ and ψ^- strains.

Genetic Analysis. Standard genetic procedures of crossing, sporulation, and tetrad analysis were used to determine the genotypes of the variants that arose after incubation of the ψ^+ *SUP16* and ψ^+ *sup16*$^+$ strain in hypertonic media. The mutants were crossed to standard ψ^+ and ψ^- strains, and the segregation of the non-Mendelian ψ determinant and the *SUP16* suppressor were determined in the meiotic progeny as described below.

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Table 1. Basic strains

Strain	Relevant genotypes
D898-3C	ψ^+ α *SUP16 ade2-1 trp5-48 lys1-1 his5-2 ura4-11*
B-4818	ψ^- α *SUP16 ade2-1 trp5-48 lys1-1 his5-2 ura4-11*
D909-1C	ψ^+ **a** *ade2-1 trp5-48 lys1-1 his5-2 ura4-11*
D896-6D	ψ^- **a** *ade2-1 trp5-48 lys1-1 his5-2 lys2-1 met3*
D940-5C	ψ^+ **a** *ade2-1 trp5-48 lys1-1 his5-2 ura4-11*
D940-5D	ψ^+ **a** *ade2-1 trp5-48 lys1-1 his5-2 ura4-11*
D883-1B	ψ^- α *trp5-48 lys1-1 his5-2 leu1-12 met1 ilv3*
D912-12B	ψ^- α *SUP16 ade2-1 trp5-48 lys1-1 his5-2 met1*

RESULTS

Growth in Hypertonic Media. The main strain, D898-3C, used in this study was specifically constructed with markers that conveniently reveal the variants arising after growth in hypertonic media. After cultures incubated in nutrient media containing several concentrations of either KCl or ethylene glycol were examined, detailed analysis of the number and type of viable cells was undertaken with media made hypertonic with either 2.5 M KCl or 1.75 M ethylene glycol. Strain D898-3C was inoculated into the hypertonic media and samples were plated on nutrient medium after various times of incubation. Shown in Fig. 1 are the total numbers of viable cells in the KCl culture, indicating that there was an initial death phase, growth, and subsequently a second death phase. Viable cells in the initial death phase of the KCl culture fell to 3.5% of the original before growth proceeded. The second death phase occurred immediately after the stationary phase of growth. The growth pattern of the ethylene glycol culture, shown in Fig. 2, revealed an initial death phase resulting in only 55% inactivation of the original inoculum. Growth proceeded more rapidly and to a higher titer in the ethylene glycol culture compared to the KCl culture because of the lower molality.

Variants Arising in Hypertonic Media. Cells from the hypertonic cultures gave rise to colonies with a variety of different characteristics that are summarized in Table 3. Cells plated after short times of incubation gave rise to pink colonies similar to the colonies derived from the parental strain D893-3C. After further incubation, high proportions of the colonies were distinctly redder in color, indicating a lower suppression of the *ade2-1* marker. However, there was still suppression of the other UAA markers *trp5-48*, *his5-2*, and *lys1-1* in the redder variant. A genetic analysis was undertaken with 10 red variants that arose from the culture incubated in KCl medium. Nine of the variants were chosen from sectored colonies and thus are of independent origin; one variant B-4818 was derived from a typical entirely red colony. Each variant was crossed to the ψ^+ strain D909-1C and to the ψ^- strain D896-6D. The hybrids were sporulated and 7–12 tetrads from each cross were analyzed. All of the results from these two series of crosses can be summarized by one typical tetrad from each of the two series

Table 2. Patterns of suppression of UAA markers in ψ^+ and ψ^- strains, each with and without the *SUP16* suppressor

	Suppressible alleles			
	trp5-48	*his5-2*	*lys1-1*	*ade2-1*
ψ^+ *SUP16*	+++	+++	+++	+
ψ^- *SUP16*	+++	+	+	0
ψ^+	++	0	0	0
ψ^-	0	0	0	0

Levels of suppression of the nutritional markers are indicated by: +++, good growth by the first day; ++, slight growth by the first day and good growth by the third day; +, no growth on the first day and slight on the third day; and 0, no sign of growth by 3 days.

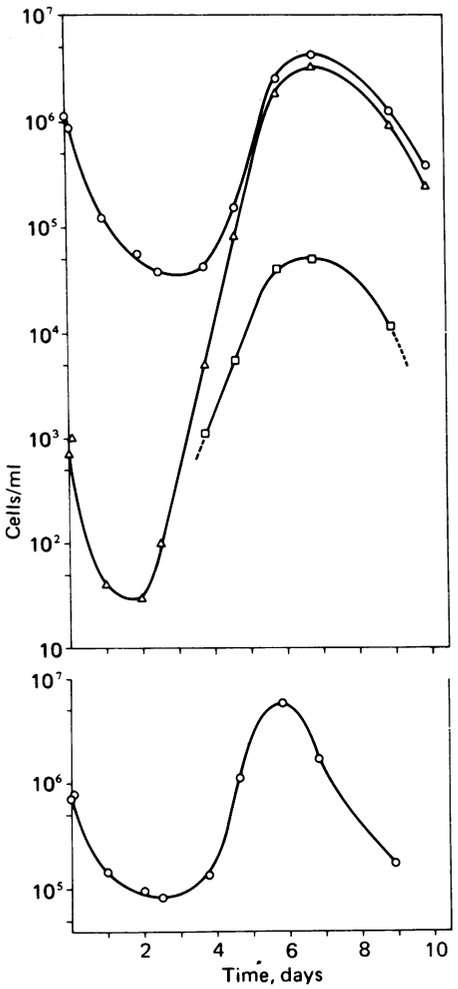

FIG. 1. (*Upper*) Total viability (○), ψ^- colonies (△), and ψ^-/ψ^+ sectored colonies (□) after incubation of the ψ^+ strain D898-3C in hypertonic medium containing 2.5 M KCl. (*Lower*) Viability of the ψ^- strain B-4818 after incubation in hypertonic medium containing 2.5 M KCl.

(Table 4). There was a clear 2:2 segregation for two levels of suppression in the pedigree of the B-4818 × D909-1C cross, indicating that the *SUP16* suppressor remained in the B-4818 variant. The analysis of the B-4818 × D896-6D cross also demonstrated that the *SUP16* suppressor was retained in the B-4818 variant, but the level of suppression of the *SUP16* segregants was distinctly lower than the level of the *SUP16* segregants from the B-4818 × D909-1C pedigree. Similar results were observed with 11 other tetrads from the B-4818 × D909-1C cross and with 10 other tetrads from the B-4818 × D896-6D cross, as well as with tetrads from analogous crosses with the nine other red variants. These results indicate that the lower efficiency of suppression in all of the red variants was due to $\psi^+ \rightarrow \psi^-$ mutations and not to mutation of the *SUP16* suppressor nor to formation of chromosomal antisuppressors. In addition, these and numerous other red variants from KCl and ethylene glycol cultures were tested for the pattern of suppression of the other UAA markers *trp5-48*, *his5-2*, and *lys1-1*; the suppressibility of the UAA markers was identical or similar to the suppressibility of ψ^- *SUP16* strains as presented in Table 2. Thus, we believe all variants having the same degree of red coloration probably are all ψ^- *SUP16* derivatives of the ψ^+ *SUP16* parental strain.

In addition to the ψ^- cells that gave rise to red colonies, approximately 25% of the cells throughout the growth cycle gave

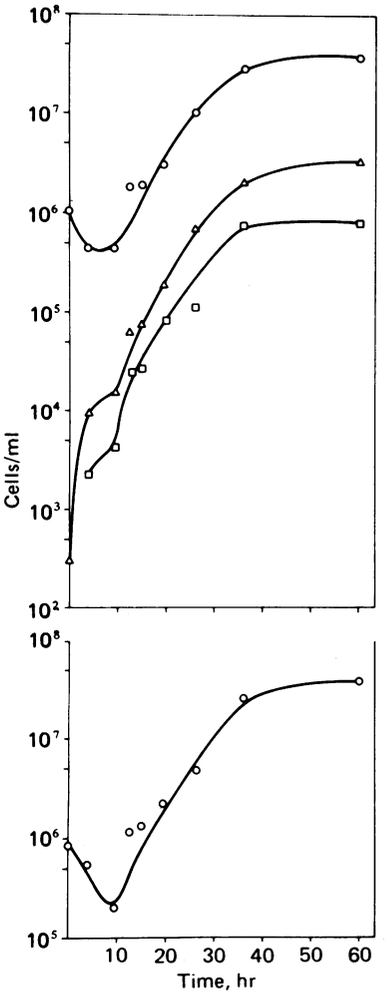

FIG 2. (*Upper*) Total viability (○), ψ^- colonies (△), and ψ^-/ψ^+ sectored colonies (□) after incubation of the strain D898-3C in hypertonic medium containing 1.75 M ethylene glycol. (*Lower*) Viability of the ψ^- strain B-4818 after incubation in hypertonic medium containing 1.75 M ethylene glycol.

rise to colonies that were smaller and whiter than the parental colonies. The lack of growth on glycerol medium and the absence of cytochromes *a*, a_3, and *b* in representative colonies indicate that these colonies are typical ρ^- mutants. When plated on nutrient medium containing 10% dextrose, which intensifies the red coloration, the ψ^+ ρ^- and ψ^- ρ^- mutants could usually be distinguished by the slightly orange color of the ψ^- ρ^- strains (Table 3).

The other major cell type, giving rise to large white colonies, appeared after 6 days of incubation in the KCl medium during the second death phase and constituted approximately 20% of the total population at 9–10 days of incubation. The large size of these colonies suggested that they arose by selection of pre-existing cells during the growth of the culture. Complete pedigree analysis of one of the variants and complementation tests with 10 others indicated that these variants contain secondary mutations in the adenine biosynthetic pathway. The secondary mutations that were uncovered included mutations at the *ade4*, *ade6*, *ade8*, and *ade11* loci. The selection of mutants having second mutations in the adenine biosynthetic pathway also occurs after prolonged incubation of *ade2* strains in normal nutrient medium because the secondary mutations prevent the formation of the red pigment, which slightly inhibits growth (12). The ψ^+ and ψ^- state can not be conveniently distinguished in the variants having a second *ade* mutation because red pigment produced by the *ade2-1* marker is absent.

Table 3. Colony characteristics of strain D898-3C and its derivatives, each containing the markers *α SUP16 ade2-1 trp5-48 lys1-1 his5-2 ura4-11*

Variable genotype	Colony morphology
ψ^+ ρ^+	Pink
ψ^- ρ^+	Red
ψ^+ ρ^-	Small white
ψ^- ρ^-	Small orange
ψ^+ ρ^+ *adex*	Large white
ψ^- ρ^+ *adex*	Large white

Table 4. Typical tetrads from the B-4818 × D909-1C cross and the B-4818 × D896-6D cross

Cross	Segregant	Genotype	Degree of suppression *trp5-48*	*his5-2*	*ade2-1*
B-4818 × D909-1C	A	ψ^+ *SUP16*	+++	+++	+
(ψ^- *SUP16* × ψ^+)	B	ψ^+ *SUP16*	+++	+++	+
	C	ψ^+ +	++	0	0
	D	ψ^+ +	++	0	0
B-4818 × D896-6D	A	ψ^- *SUP16*	+++	+	0
(ψ^- *SUP16* × ψ^-)	B	ψ^- *SUP16*	+++	+	0
	C	ψ^- +	0	0	0
	D	ψ^- +	0	0	0

Designations for levels of suppression are described in the legend for Table 2.

Induction of ψ^- Mutants from ψ^+ *SUP16* Strain. The frequencies of ψ^- cells after incubation of the ψ^+ strain in KCl and ethylene glycol media are presented in Figs. 1–3. In the KCl

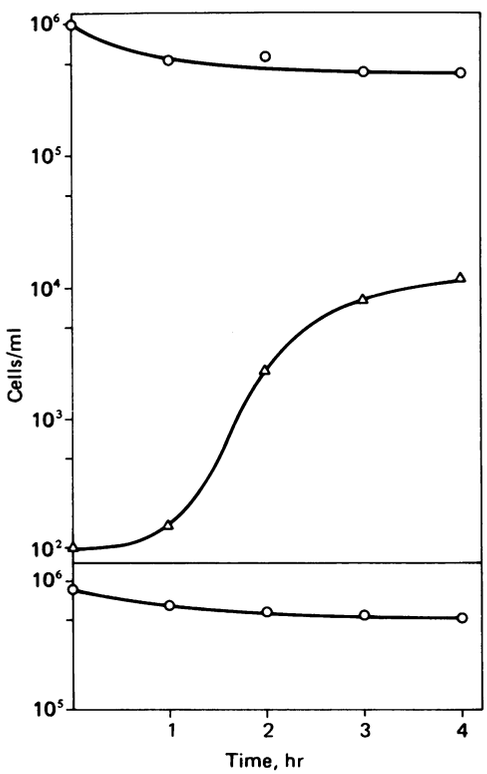

FIG. 3. (*Upper*) Total viability (○) and ψ^- colonies (△) after incubation of the ψ^+ strain D893-3C in hypertonic medium containing 1.75 M ethylene glycol. (*Lower*) Viability of the ψ^- strain B-4818 after incubation in hypertonic medium containing 1.75 M ethylene glycol.

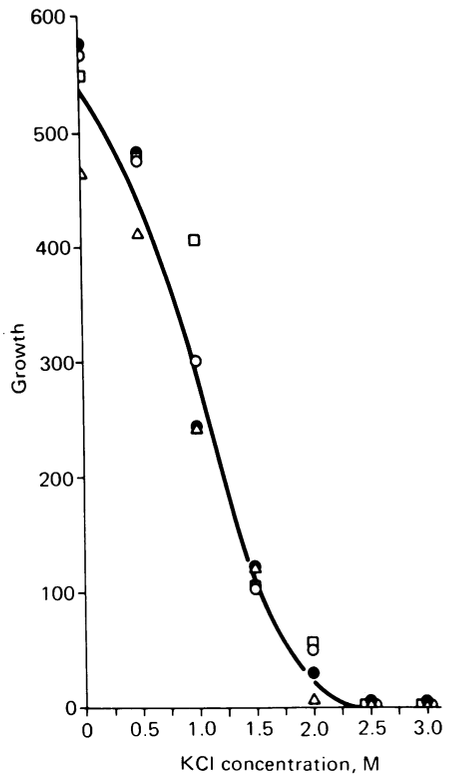

FIG. 4. Growth (Klett units) of the ψ^+ strain D898-3C after 2 days (●) and 3.5 days (○) and of the ψ^- strain B-4818 after 2 days (△) and 3.5 days (□) in nutrient medium containing various concentrations of KCl.

culture, both the ρ^+ and ρ^- colonies, but not the large white colonies containing second *ade* mutation, were scored for ψ^+ and ψ^-. The ρ^- colonies from the ethylene glycol culture were not scored for ψ^-. Thus, the frequencies of ψ^- variants are underestimated in Figs. 1–3. In addition to presenting the number of total viable cells and of ψ^- cells, sectored colonies containing both ψ^+ and ψ^- cells were scored and are presented separately. Because the samples were sonicated to disperse the cells, the sectored colonies probably arose from single cells undergoing $\psi^+ \rightarrow \psi^-$ mutations.

The mass conversion to predominantly ψ^- cells and the high frequencies of sectored colonies from the KCl culture suggested that the hypertonic condition induced $\psi^+ \rightarrow \psi^-$ mutations and that the ψ^- cells did not arise by selection of pre-existing ψ^- cells. This suggestion is substantiated from the comparison of growth of ψ^+ and ψ^- cultures in KCl media. The similarity of the growth patterns of the ψ^+ culture and of B-4818 (Fig. 1), a ψ^- variant derived from the ψ^+ strain D898-3C, indicated that both ψ^+ and ψ^- strains are equally inhibited in the 2.5 M KCl medium used in the induction experiment. In addition, as shown in Fig. 4, approximately equal sensitivities are observed over a wide range of KCl concentrations.

Similar results were observed with the ethylene glycol culture although the proportion of ψ^- cells did not rise as high as with the KCl culture (Fig. 2). As shown in Fig. 2, approximately equal growth patterns in the ethylene glycol medium were observed for the related ψ^+ and ψ^- strains, also suggesting that the ψ^- cells did not arise by selection. A detailed analysis of the initial death phase in the ethylene glycol medium, presented in Fig. 3, established unambiguously that the ψ^- variants were induced by the hypertonic condition; there was a 100-fold increase in the absolute number of ψ^- cells in the absence of growth of the ψ^+ culture during the 4-hr incubation period. In addition, there was no growth of the control ψ^- culture during this period (Fig. 3).

Induction of ψ^- Mutants from ψ^+ *sup*+ Strains. Although the kinetics of ψ^- induction was analyzed in detail with only strain D898-3C, high frequencies of ψ^- mutants arose from numerous other strains incubated in hypertonic media. The strains examined included other ψ^+ *SUP16* strains similar to D898-3C and ψ^+ strains that lacked the *SUP16* suppressor. Several ψ^+ strains containing the UAA markers *ade2-1*, *trp5-48*, *lys1-1*, and *his5-2* were incubated for 5 days in nutrient medium containing 2.5 M KCl. The cultures were diluted and plated on nutrient medium, and the resulting colonies were tested for the suppressibility of the UAA markers. From 20 to 70% of the colonies required tryptophan, suggesting that in these colonies the ψ^+ determinant, which suppresses *trp5-48* (Table 2), mutated to ψ^-. A genetic analysis of five of the presumptive ψ^- mutants derived from strains D940-5C and D940-5D (Table 1) was undertaken. Each of three of the presumptive ψ^- mutants was crossed to the following three tester strains described in Table 1: D883-1B (ψ^-); D898-3C (ψ^+ *SUP16*); and D912-12 (ψ^- *SUP16*). In addition, two other presumptive mutants were crossed to the D883-1B and D912-12B tester strains. The analysis of seven tetrads from each cross established that the five tryptophan-dependent mutants were indeed ψ^- mutants; scoring the segregants for *trp5-48* and other UAA markers indicated that the $\psi^- \times \psi^-$ crosses yield only ψ^- segregants, that the $\psi^- \times \psi^-$ *SUP16* cross yielded only ψ^- segregants and a 2:2 segregation of the *SUP16* suppressor, and that the $\psi^- \times \psi^+$ *SUP16* cross yielded only ψ^+ segregants and a 2:2 segregation of the *SUP16* suppressor. Thus, ψ^- mutants can be efficiently induced from ψ^+ strains whether or not they contain the *SUP16* suppressor.

Lack of Induction of ψ^- Mutant by Growth at Elevated Temperatures. Incubation at above optimum growth temperatures has been shown to be an effective means for eliminating certain cytoplasmic hereditary determinants and plasmids from yeast and other organisms (see ref. 13). Growth at the supraoptimum temperatures of 37°–40°C causes a high rate of $\rho^+ \rightarrow \rho^-$ mitochondrial mutations (14) and of KIL-k → KIL-o "killer mutations" (15) in yeast. To test if $\psi^+ \rightarrow \psi^-$ induction occurs by growth at high temperatures, we grew strain D898-3C at 39°C in a nutrient medium supplemented with ergosterol and Tween 80 (16); after 1 and 2 days, appropriate dilutions were plated on nutrient medium containing 10% dextrose and the resulting colonies were analyzed. Although over 99.9% of the colonies were ρ^-, no ψ^- colonies were observed among 2820 colonies from the 1-day culture nor among 3271 colonies from the 2-day culture. Thus growth at elevated temperatures is ineffective for inducing ψ^- mutations.

DISCUSSION

In this investigation we have demonstrated that exposure to hypertonic media induces high rates of $\psi^+ \rightarrow \psi^-$ mutations. It was concluded that the ψ^- mutants arise by mutation of ψ^+ cells and not by selection of pre-existing ψ^- cells for the following reasons: both ψ^+ and ψ^- strains are equally inactivated and equally inhibited by hypertonic media (Figs. 1–4); sectored colonies are observed at relatively high frequencies (Figs. 1 and 2) and these are believed to arise by single cells undergoing $\psi^+ \rightarrow \psi^-$ mutations; of most importance, there was over a 100-fold increase in the absolute number of ψ^- cells after 4 hr of incubation before ψ^+ and ψ^- cells began to divide (Fig. 3). We have reported previously that strains containing UAA and UAG suppressors, which insert tyrosine, and certain UAG suppressors, which insert serine or leucine, fail to grow on hypertonic medium; revertants lacking suppressor activity arise from these suppressors on hypertonic media (17). The occurrence of ψ^- mutants in hypertonic media is fundamentally different from

Table 5. Comparison of cytoplasmic mutations in yeast

Mutation	$\psi^+ \rightarrow \psi^-$	$\rho^+ \rightarrow \rho^-$	KIL-k → KIL-o
Mutant phenotype	Altered suppression	Loss of cytochromes a, a_3, and b	Loss of killer trait
Component lost	Unknown	Mitochondrial DNA	Double-stranded RNA
Nuclear genes	*PNM*$^-$	*pet*	*mak*
Agents	Hypertonicity	High temp., ethidium bromide, etc.	High temp., cycloheximide

the occurrence of mutants lacking suppressor activity that is caused by the selection against osmotic-sensitive strains containing certain suppressors (17). As noted previously (17) and confirmed in this investigation, strains containing the *SUP16* suppressor, a UAA suppressor which inserts serine, are not inhibited on hypertonic media.

The ψ^- mutants were distinguished from the ψ^+ original strain by the lack or decreased efficiency of suppression of the *ade2-1* marker in *SUP16* strains and by the lack of suppression of the *trp5-48* marker in *sup+* strains (Table 2). Genetic analysis of ten ψ^- *SUP16* strains and of five ψ^- *sup+* strains established that the lower efficiency of suppression was due to ψ^- mutations and not to the occurrence of chromosomal antisuppressors or to mutations of *SUP16* or other nuclear markers. The high rates of $\psi^+ \rightarrow \psi^-$ mutations and the lack of any obvious nuclear mutations by hypertonic media suggest that the ψ^+ determinant may be eliminated because its replication is sensitive to osmotic conditions. Mass $\rho^+ \rightarrow \rho^-$ mutations are associated with the complete loss of mitochondrial DNA (18), and mass KIL-k → KIL-o mutations are associated with the complete loss of a specific double-stranded RNA (19). However, since at least some ψ^- mutants can be reverted back to ψ^+, not all ψ^- mutants are expected to have a physical loss of the determinant (6).

Exposure to osmotic conditions is the first reported method of inducing high rates of ψ^- mutants. Growth at elevated temperatures, which is effective in inducing ρ^- (14) and KIL-o (15) mutants, did not induce ψ^- mutants. Numerous chemical agents, such as the acridines and ethidium bromide, induce high rates of ρ^- mutants (20). Cycloheximide induces high rates of KIL-o mutants (21). However, ψ^- mutants are not induced by cycloheximide (22) nor by several of the chemical agents highly active on mitochondrial DNA. There are genes that appear to be required for the maintenance of replication of the ψ^+ (9, 23), ρ^+ (24), and KIL-k (25) determinants and these cytoplasmic determinants cannot be retained in strains having mutations in these genes. One of the mutant genes, *pet18*, causes the loss of both the ρ^+ and KIL-k determinant but not the ψ^+ determinant (26). ψ^- mutants induced by hypertonic medium maintain the KIL-k determinant and the 2-μm circular DNA present in the parent strain (unpublished data). Thus there are no known common mutant genes and no known common chemical or physical treatments that produce high rates of ψ^- mutants along with high rates of either ρ^- or KIL-o mutants. A comparison of the means for eliminating the ψ^+, ρ^+, and KIL-k determinants is summarized in Table 5.

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