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Nuclear and cytoplasmic cross-resistance and correlated sensitivity to DNA intercalating drugs in a petite-negative yeast

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SUMMARY

Ethidium bromide and acriflavin-resistant mutants of petite-negative yeast *Kluyveromyces lactis* were prepared. One kind of nuclear mutation (EB^{R1}) gave resistance to ethidium bromide and correlated sensitivity towards acriflavin. Another nuclear mutation (EB^{R2}) did not affect 'natural' resistance of this yeast towards 15 μM acriflavin. Both nuclear mutations mapped at different *loci*, suggesting lack of linkage. Cytoplasmic mutants resistant to these two drugs were unstable when grown in complete media with dextrose, reverting to a wild-type resistance genotype. When grown in glycerol-containing media these mutants maintained their cytoplasmic drug-resistance conferring factors.

1. INTRODUCTION

Ethidium bromide (EB)* and acriflavin (Acri) are both intercalating drugs that share some interesting properties. Their structures are somewhat similar, each consisting of three rings, with a quaternary nitrogen; both are well known to inhibit mitochondrial DNA (mit-DNA) replication in yeast, which results in the formation of cytoplasmic respiratory-deficient 'petite' mutants in the so-called petite-positive yeasts (Marcovich, 1951; Bulder, 1964; Slonimski, Perrodin & Croft, 1968). While acriflavin induces this sort of mutation only in daughter cells (Ephrussi & Hottinguer, 1950), ethidium bromide affects both mother and daughter cells (Slonimski *et al.* 1968). It has also been reported that these drugs inhibit mit-DNA transcription in the yeast *Saccharomyces cerevisiae* (Fukuhara & Kuhawa, 1970). The yeast *Kluyveromyces lactis* was chosen for this work, since it is known to be a petite-negative yeast, and treatment with any of these drugs will not produce viable 'petite' cells (Bulder, 1964), allowing isolation and characterization of mutants resistant to EB and similar drugs. Such mutants are particularly useful tools for studying the genetics and biogenesis of mitochondria. Genetic analyses in this yeast are easily done, since its life cycle includes both haploid and diploid

* Abbreviations used: EB, ethidium bromide; Acri, acriflavin; mit-DNA, mitochondrial DNA; (R:S), (resistant:sensitive).

stages, making it possible to perform tetrad analysis by following the segregation pattern of mutants. Indeed *K. lactis* mutants resistant to EB and alkylguanidines which show both nuclear and cytoplasmic inheritance have been isolated (Brunner *et al.* 1973).

The effect of EB and Acri on the growth and properties of various *K. lactis* mutant strains were studied. These experiments indicate that several mechanisms are involved in the conferring of resistance to intercalating drugs. In some cases the mutation that confers resistance to EB induces correlated sensitivity to Acri, suggesting the existence of a common site of action.

2. MATERIALS AND METHODS

(i) *Yeast strains*

Haploid *Kluyveromyces lactis* (*Saccharomyces lactis*) strains WM27, WM37, WM66, W600B, Y14 and Y123 were obtained from Dr A. Herman; strains K2, K3, K7, K8 and K9 were a generous gift from Dr M. Tingle. All other mutant and tester (non-mutant) strains employed are segregants obtained from the above mentioned strains. Several of these segregants were obtained from Dr James R. Mattoon.

(ii) *Media*

Complete media – YPAD: 1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose and 80 mg/l adenine sulphate; YPAG: same as the above-described medium, but with 3% (v/v) glycerol, instead of dextrose; YPADG: YPAG plus 0·2% dextrose. Minimal media (Wickerham): 0·67% Bacto-yeast nitrogen base without amino acids plus 2% dextrose (SD) or 3% (v/v) glycerol (SG). Conjugation and sporulation media (solid ME): 5% Bacto-malt extract broth plus 3% Bacto-agar. To all other solid media, 2% Bacto-agar was added. Auxotrophic nutritional requirements were tested on solid minimal media (SD) supplemented with the adequate concentrations of amino acids and adenine. Aliquots of stock solutions of 10 mM EB and 5 mM Acri, protected from light and kept at 4 °C, were added to the media (YPAD, YPAG or YPADG) after autoclaving, so as to give the desired final concentration. For Acri, a mean molecular weight of 254 was considered.

(iii) *Chemicals*

Ethidium bromide, acriflavin HCl (euflavine), and adenine sulphate were purchased from Sigma Chemical Co.

(iv) *Mutant selection*

EB-resistant mutants were selected by plating approximately 10^7 cells on YPADG containing 25 or 50 μM EB. Mutants resistant to both EB and Acri were selected by plating the same number of cells on YPADG that contained both drugs at 15 μM concentration each and treating the plates with 120 μW/cm^2/sec of ultraviolet

light for 20 sec (this corresponds to half the lethal dose for this yeast). When colonies were of sufficient size (6–7 days after plating), mutants were selected and transferred to YPAD or YPAG. YPADG was used to isolate all mutants since they did not grow in YPAG at the concentration of the drugs employed.

(v) *Conjugation and tetrad analysis*

Diploids were obtained by mass mating conjugation on solid ME at 30 °C for 24 h, followed by plating on SD or SG medium for the isolation of individual clones. Sporulation was induced on solid ME at 20 °C. After 48 h incubation, 70–80 % of the population of most crosses had sporulated. Sporulated cells were digested for 15 min with 0·3 ml of a 1:1 dilution of Glusulase (Endo Laboratories Inc.) per ml of a weak cell suspension. Dissection was performed with a Leitz micromanipulator following the technique described by Mortimer & Hawthorne (1969).

(vi) *Incubation conditions*

All cultures were incubated at 30 °C. Growth was recorded at 48 and 72 h after plating.

3. RESULTS

(i) *Properties of mutants isolated from EB-containing media*

EB-resistant mutants were obtained as described under 'Materials and Methods'; clones were isolated, plated on YPAD or YPAG media and tested for resistance to EB and Acri with a Pepper inoculator; the results are presented in Table 1. All the parental strains tested (WM27, WM37, WM66, W600B, Y14, Y123, K2, K3, K7, K8 and K9) were found to be sensitive to 10 μM EB and resistant to 15 μM Acri in media that contained glycerol as a carbon source. The results of Table 1 show that 89 % (24 out of 27 strains) of the EB-resistant mutants that were isolated in the presence of this drug lost simultaneously their resistance to Acri.

Table 1. *Properties of* Kluyveromyces lactis *EB-resistant strains*

			No. of clones		
Strain	Genotype	No. of resistant clones isolated	Resistant to 10 μM EB	Resistant to 15 μM Acri	Sensitive to 15 μM Acri
WM37(R)	a, his	1	1	0	1
K2(R)	α, his	7	7	1	6
K3(R)	α, his	3	3	1	2
K7(R)	α, trp	2	2	1	1
K8(R)	a, met	5	5	0	5
KA5-6B(R)	a, ad 1	3	3	0	3
KA6-8A(R)	a, his	6	6	0	6
Total		27	27	3	24

Resistant clones were isolated from YPADG medium containing 25 or 50 μM ethidium bromide. When colonies were of sufficient size they were transferred to YPAD medium before testing its resistance to EB and Acri.

In order to see how this resistance was inherited, some of the EB-resistant Acri-sensitive mutants K8(4) and K2(7) were crossed with a tester strain and submitted to tetrad analysis. Simultaneously, the non-mutant parent strain K8 was also crossed with a tester strain. All the tetrads from the crosses of the mutants gave a 2:2 (R:S) segregation towards EB, whereas all the data on the segregation of the tetrads from the tester cross gave 0:4 (R:S) segregation towards this same drug (segregation patterns are not shown), indicating that the resistance was conferred by a nuclear mutation.

Table 2. *Resistance and sensitivity towards EB and Acri of several* Kluyveromyces lactis *segregants**

	Cross: mutant strain × tester strain		Diploid no.	No. of segregants which gave: EB^R $Acri^R$	EB^R $Acri^S$	EB^S $Acri^R$	EB^S $Acri^S$	Total no. of segregants
1	K8†	KA5-1B	KC5	0	0	20	0	20
2	K8†	KZ12-A23	KC6	0	0	36	0	36
3	K8(4)	KZ12-A23	KC7	0	14	14	0	28
4	K2(7)	KA7-11A	KC10	0	18	18	0	36
5	K2(7)	KZ10-B27	KC11	0	10	10	0	20
6	KA10SG-2A	KA9-17C	KC2SD	2	23	30	5‡	60
7	KA10SG-2B	KA9-17C	KC3SD	4	20	24	0	48
8	KA10SG-2C	KZ12-A11	KC1SD	14	14	12	0	40
9	KA10SG-2D	KZ12-A11	KC4SD	0	26	26	0	52

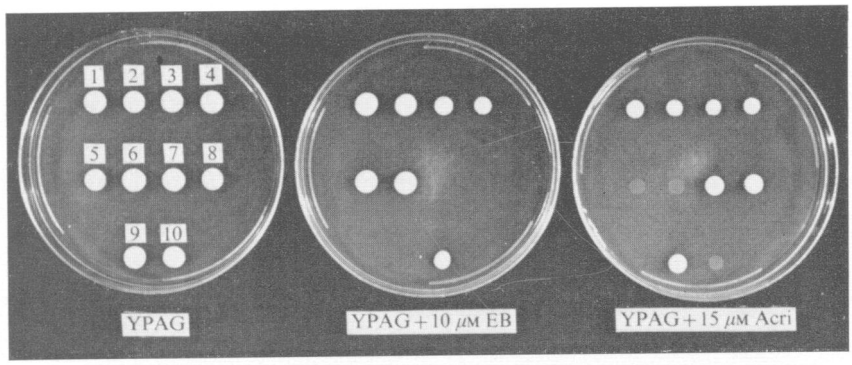
* All segregants were obtained by tetrad dissection.
† This parent was resistant to 15 μM Acri, but not to EB as all other tester strains (tester cross).
‡ These segregants did not grow well in glycerol, so that sensitivity to the drugs cannot be assessed.

When the segregants of these crosses were tested for their resistance towards 15 μM Acri, it was found that all the segregants which were resistant to EB had correlated sensitivity towards Acri and vice versa that all the Acri-resistant strains were sensitive to EB (Table 2, lines 1–5).

In order to see how an EB-resistant cytoplasmic mutation would affect the sensitivity towards Acri, previously isolated (Brunner *et al.* 1973) segregants (KA10-SG-2A, 2B, 2C and 2D) of an EB-resistant mutant that contained both nuclear and cytoplasmic determinants for resistance were crossed with tester strains. The isolated diploids were made to sporulate and thereafter submitted to tetrad analysis; segregants were replicated in media that contained 10 μM EB or 15 μM Acri in YPAG. If the overall results in Table 2, lines 6–9 are examined, it is observed that (1) almost all of the EB-sensitive segregants were resistant to Acri, behaving as the tester strains, (2) 80% of the EB-resistant segregants were sensitive to Acri, (3) 20% of the EB-resistant segregants were resistant to Acri.

(ii) *Instability of the cytoplasmic mutation in dextrose*

In order to see how the factors that confer cross-resistance to these drugs segregate, three tetrads with all four segregants of the last group mentioned above (EB-resistant, Acri-resistant) were further studied. When they were subcloned in the



Replica plates showing disappearance of the cytoplasmic resistance factors during dextrose subcloning. Tetrad KC1-8 subcloned 3 times in glycerol (1–4) and subcloned 3 times in dextrose (5–8). Controls are WM37, EB^{S} $Acri^{R}$ (9) and WM37(R), EB^{R1} $Acri^{S}$ (10).

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presence of dextrose as carbon source, it was found that two members of each tetrad became sensitive to Acri but remained resistant to EB (type 'A' segregant). On the other hand the other two members did the opposite, i.e. they became sensitive to EB and remained resistant to Acri (type 'B' segregants), thus behaving as the tester strains. The resistance to both drugs was preserved when the tetrads were subcloned in a medium that contained glycerol instead of dextrose (Plate 1).

When one of the type 'A' segregants (KC1-8A) was backcrossed with a tester strain, the resistance to both drugs was transmitted in a Mendelian pattern (2:2, R:S), and all but two of the EB-resistant segregants of this cross showed correlated sensitivity to Acri (Table 3). However, when a type 'B' segregant (KC1-8C) was backcrossed in the same manner, the resistance to the drugs was transmitted in a non-Mendelian (cytoplasmic) way. All the EB-resistant segregants were also resistant to Acri (Table 3).

Table 3. *Resistance towards EB and Acri of type 'A' and type 'B' mutants*

Cross: mutant × tester	Medium in which resistance was tested*	No. of tetrads that segregate† (R:S) 4:0	3:1	2:2	1:3	0:4	Resistance (%)
KC1-8A KA9-9B (type 'A')	10 μM EB	0	0	15	0	0	50
	15 μM EB	0	0	15	0	0	50
	15 μM Acri	0	2	13	0	0	53
KC1-8C KA9-9B (type 'B')	10 μM EB	12	1	1	0	5	70
	15 μM EB	1	0	1	0	17	8
	15 μM Acri	19	0	0	0	0	100

* Results were scored 48 h after plating and incubation at 30 °C.

† Total number of tetrads analysed was 15 for the type 'A' cross and 19 for the type 'B' cross. All auxotrophic markers segregated 2:2.

These results suggest that all the members of these tetrads possess a cytoplasmic inherited resistance factor when they are grown in glycerol. Apparently the factor which confers the resistance to both drugs disappears when the cells are grown in the presence of dextrose. This would account for the fact that two segregants of each tetrad became purely nuclear mutants (type 'A' segregants), and the other two turned out to be like the tester strains (type 'B' segregants). It was also clear that this nuclear mutation confers resistance to EB but also induces the disappearance of the 'natural resistance' of this yeast to Acri. Hereafter these nuclear mutants which show correlated sensitivity towards Acri will be referred to as EB^{R1}.

It must be noted that all the manipulations performed with KC1-8A were carried out using dextrose in order to get rid of the cytoplasmic factors which otherwise would mask the nuclear mutation. On the other hand, the results presented with KC1-8C were obtained in the presence of glycerol as the sole carbon source, since in dextrose media the cytoplasmic resistance-conferring factors tend to disappear.

Table 4. *Resistance segregation pattern of* Kluyveromyces lactis *mutants resistant to both EB and Acri*

Diploid no.	No. of tetrads that segregate (EB^R $Acri^R$:EB^S $Acri^R$) 4:0	3:1	2:2	1:3	0:4	Total no. of tetrads analysed
KD-1*	13	8	0	0	0	21
KD-3*	0	0	9	13	1	23
KD-4	0	3	2	11	1	17
KD-5	0	0	4	9	6	19
KD-6	0	0	15	0	0	15
KD-7	0	0	15	0	0	15
KD-8	0	0	3	10	2	15
KD-9	0	0	0	0	15	15
KD-10	0	0	4	10	1	15
KD-11	0	0	0	0	15	15

Results were scored after 48 h incubation at 30 °C.
All auxotrophic markers segregated 2:2.
* Transferences of these strains were made on glycerol containing media, the rest were made in dextrose media.

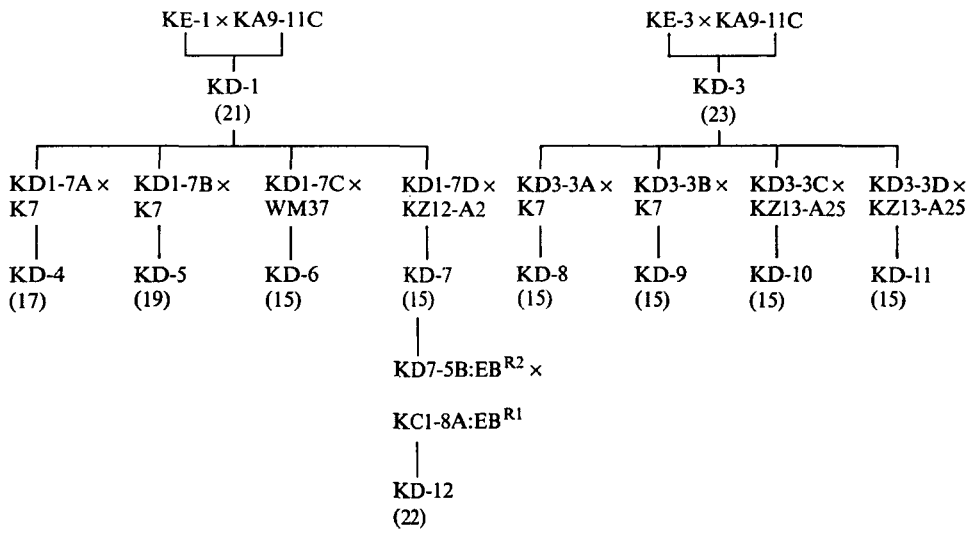

Fig. 1. Scheme representing the isolation and crossing of strains obtained from YPADG media with both EB and Acri. Figures in parentheses give the number of tetrads analysed. KD1-7 tetrad segregated 4:0 and KD3-3 tetrad segregated 2:2 (EB^R $Acri^R$:EB^S $Acri^R$).

(iii) *Properties of mutants isolated from media containing both EB and Acri*

Since it was observed that the cytoplasmic mutation that conferred resistance to EB also made the strain resistant to Acri (see Plate 1), strains resistant to both drugs were prepared; it was hoped that cytoplasmic mutations could be obtained by this procedure.

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Strains resistant to these drugs and which always were transferred in glycerol were isolated with the help of u.v. irradiation in YPADG media that contained 15 μM of each drug. Two mutants were isolated and transferred to YPAG. Thereafter they were crossed with a tester strain and a complete tetrad of each mutant cross was again backcrossed with tester strains. The segregation pattern of these crosses, together with a short scheme of the crosses made are presented in Table 4 and Fig. 1, respectively. One of these mutants (KE3) turned out to be purely cytoplasmic, while the other (KE1) possessed nuclear and cytoplasmic mutations; the latter resisted EB better than the former. This nuclear mutation (which we designated EB^{R2}) did not confer sensitivity to Acri.

(iv) *Linkage analysis of two EB resistance-conferring genes*

In order to see whether EB^{R1} and EB^{R2} genes were linked, a cross was made between these two kinds of mutants (diploid KD-12, Fig. 1); 22 complete tetrads were analysed giving 11 tetratypes, 4 parental ditypes and 7 non-parental ditypes (data not shown); from these results it is suggested that there is no linkage between these two genes.

(v) *Mitotic segregation of cytoplasmic EB resistance factors in diploids*

The segregation of resistance to EB in diploids derived from several crosses of a purified cytoplasmic EB-resistant mutant KD1-7A with two different sensitive tester strains is observed in Table 5. These results give further evidence of the cytoplasmic nature of this mutation and at the same time indicate that the carbon source which is utilized during vegetative growth of diploids

Table 5. *Segregation of cytoplasmic resistance to EB*

Cross*	Diploid media†	Survivors (%)‡	Resistant / Survivors (%)	Resistant / Total cells (%)	No. of total cells
WM37§ × W600B	SD	0·13	0	0	1500
KD1-7A[EB^R]‖ × KZ12-A2	SG	71·5	83·9	60·0	260
	SG	70·7	86·3	61·1	840
	SG	70·5	87·8	61·9	685
KD1-7A[EB^R] × KZ12-A2	SD	57·7	59·9	34·5	721
KD1-7A[EB^R] × KA6-9A	SG	85·5	79·8	68·3	876
KD1-7A[EB^R] × KA6-9A	SD	53·1	24·0	12·7	409

* Crosses were performed as described in Materials and Methods.

† Diploids were isolated and grown for 48 h in minimal medium supplemented with glucose (SD) or glycerol (SG). Approximately 200 cells were plated per plate in minimal medium SG, and SG + 10 μM EB. Plates were incubated for 48 h and colonies were counted.

‡ Two different populations were obtained in the medium containing EB: large colonies were considered and scored as 'resistant' and small colonies, not resistant; the sum of large and small colonies was considered as 'survivors'.

§ A cross of two tester strains was performed as control.

‖ Markers enclosed by brackets were shown to be cytoplasmically inherited (only resistant markers are shown).

can influence the segregation patterns. The effect of glucose in these results can be correlated with the instability of cytoplasmic EB-resistant haploids when grown in the presence of this carbon source (Plate 1).

4. DISCUSSION

We have observed that our strains show a 'natural resistance' to Acri at concentrations of the order of 15 μM in the presence of glycerol as a carbon source. In agreement with Luha, Sarcoe & Whittaker (1971), we find that a relatively high concentration of the drug (25 μM) is required for complete inhibition of growth.

When mutants resistant to EB were prepared and their resistance to Acri tested (Table 1), 89 % had already lost their resistance to Acri. These results suggest that a single mutation is responsible for this correlated sensitivity.

As these strains were sensitive to these drugs only when glycerol was used as a carbon source, it is logical to conclude that EB and Acri interfere with some mitochondrial function(s). However, as the mutation that confers resistance to EB and sensitivity to Acri (EB^{R1}) segregated in a Mendelian fashion (Table 2), it is also probable that the mutation involved is of nuclear origin.

When a complete tetrad from mutant KA10-SG (Table 2) that carried cytoplasmic and nuclear mutations that conferred resistance to EB was crossed to sensitive tester strains, 80 % of the progeny were simultaneously resistant to EB and sensitive to Acri, therefore showing a behaviour similar to those strains mentioned in Table 1. Nevertheless, segregants which were resistant to both drugs have also been isolated. When three complete tetrads from diploid KC1-SD (Table 2) that segregated 4:0 (EB^R $Acri^R$:EB^S $Acri^S$) were further analysed, it was found that subcloning of the three tetrads in a medium that contained dextrose as a carbon source induces a change in the resistance towards these drugs; two segregants of each tetrad remained resistant to EB but sensitive to Acri, while the other two became sensitive to EB and maintained their resistance to Acri, behaving as the tester strains. If the subcloning was carried out in a medium that contained glycerol instead of dextrose, the original pattern of resistance was maintained. Thus it is conceivable that two segregants of each tetrad carry the nuclear and cytoplasmic mutations, and the latter mutation confers resistance to both drugs, whereas the other two segregants from the tetrad carry only the cytoplasmic mutation. The presence of these nuclear and cytoplasmic mutations was further assessed by the backcrosses studies shown in Table 3. Another difference between the cytoplasmic and nuclear mutations is the fact that the cytoplasmic mutant is resistant to only 10 μM EB, while the nuclear mutant resists up to 15 μM EB.

The effect of glucose on the loss of the cytoplasmic mutation may be explained by some sort of catabolic repression. Glucose affects mitochondrial structure and function (Matile, Moor & Robinow, 1969), and diminishes the number of mitochondria and mit-DNA content per cell in yeast (Bleeg *et al.* 1972). Thus dextrose may cause a selection or preferential regeneration of 'normal' (non-resistant)

mitochondria over the mutated ones when the process of repression ceases, with the result that the cytoplasmic mutation is lost during glucose subcloning. Growth in glycerol, on the other hand, requires the presence of functional mitochondria, and although a continuous subcloning in the absence of the drug might eventually cause a selection of the non-mutated over the mutated mitochondria, this effect would probably require several generations before it could be observed. Intracellular selection of mitochondrial genomes has been previously proposed (Birky, 1973).

Some authors in order to maintain their cytoplasmic mutants, grow them in glycerol media in the presence of the drugs to which they are resistant, considering that under these conditions there is a selection of the mutant mitochondria over the sensitive ones (Birky, 1973; Shannon *et al.* 1973). However, one problem that arises in maintaining the resistance phenotype of our cytoplasmic mutants by growth in the presence of the drugs is the possibility that a new cytoplasmic or nuclear mutation is selected. This is very likely to occur since these drugs act directly on DNA and probably have a mutagenic effect. In fact, an attempt to purify one of our purely cytoplasmic resistant strains (KD1-7A), by subcloning on YPAG media containing 10 μM EB, resulted in the isolation of a new nuclear mutation (unpublished data).

From the results presented we can see that there are at least two kinds of nuclear mutations that confer resistance to EB. One of these mutations confers resistance to EB and correlated sensitivity to Acri (EB^{R1}), whereas the other does not affect the resistance towards Acri (EB^{R2}) and maps at a different *locus*. Recently Rank & Bech-Hansen (1973), have reported correlated sensitivity towards EB, Acri and other drugs in a nuclear mutant of *S. cerevisiae* resistant to oligomycin. One of our EB-resistant Acri-resistant mutants, KE1, possessed both nuclear and cytoplasmic mutations. The cytoplasmic mutation is indicated by the large number of tetrads segregating 4:0 or 3:1 (R:S) (Table 4, diploid KD-1). In addition, when dextrose-treated segregants of a 4:0 (R:S) tetrad were backcrossed the subsequent segregation patterns reveal that the stable nuclear gene was retained (Table 4, diploids KD-6 and KD-7). On the other hand, only a few of the segregants from diploid KD-4 and diploid KD-5 were resistant, indicating that most but not all of the cytoplasmic resistance factors have been eliminated during dextrose subcloning. Apparently the cytoplasmic resistance factor in mutant KE3 was much more labile (or in lower relative concentration), since less than half of the segregants from diploid KD-3 (Table 4) were resistant. Moreover, further dextrose subcloning of KD-3 segregants decreased still further the number of resistant progeny (diploids KD-8 and KD-10, Table 4). In fact, the original mutant, KE3, progressively lost resistance even when grown in glycerol medium.

EB and Acri produce an alteration in the respiratory enzymes and modify the cytochrome content of yeast (Bulder, 1964; Slonimski *et al.* 1968). In order to verify that indeed we were dealing with a mitochondrial alteration, the cytochrome content of various mutants was studied (Table 6). We observed that those strains sensitive to EB when grown in dextrose media in the presence of this drug showed a strong diminution in the content or loss of the content of cytochromes a, b and c_1,

whereas cytochrome *c* was notably increased. This same pattern was observed in a $\overline{EB}^{R1}$ mutant grown in dextrose media with Acri. Strains resistant to these drugs, whether they are nuclear or cytoplasmic mutants, showed only a small decrease in all cytochromes.

Table 6. *Cytochrome spectra of different ethidium bromide-resistant mutants*

Strain*	Drug added to medium†	Type of cytochrome spectrum‡
WM37: EB^S $Acri^R$	None	1
	10 μM EB	3
	10 μM Acri	2
KA10D-1A: EB^{R1} $Acri^S$	None	1
	10 μM EB	2
	10 μM Acri	3
KC1-8B: EB^S $Acri^R$ [EB^R $Acri^R$]	None	1
	10 μM EB	2
	10 μM Acri	2
KC1-8A: EB^{R1} $Acri^S$ [EB^R $Acri^R$]	None	1
	10 μM EB	2
	10 μM Acri	2
KD1-5B: EB^{R2} $Acri^R$	None	1
	10 μM EB	2
	10 μM Acri	2
KE-3: EB^S $Acri^R$ [EB^R]	None	1
	10 μM EB	2
	10 μM Acri	2
KE-1: EB^{R2} $Acri^R$ [EB^R]	None	1
	10 μM EB	2
	10 μM Acri	2

Strains were grown on YPAD plates with or without drugs for 24 h at 30 °C. Cells were collected with a spatula, mixed with a few grains of sodium dithionite and frozen in liquid nitrogen. The reduced cytochrome spectra was analysed with a Zeiss spectroscope according to the technique of Sherman (Sherman, 1964).

* Markers enclosed by brackets were shown to be cytoplasmically inherited (procedure suggested by Rank (Rank & Bech-Hansen, 1973)).

† Dextrose media were used in order to obtain cell growth of cells sensitive to the drugs in glycerol.

‡ Three types of cytochrome spectra were observed: (1) typical spectrum with bands for cytochromes *a*, *b*, *c*, and c_1 were all present; (2) all bands were present but showed a lower intensity; (3) bands for cytochromes *a*, *b* and c_1 were absent or almost absent, but the band for cytochrome *c* was highly intense.

We cannot as yet establish which cellular constituent has been changed in our mutants; the fact that a single mutation makes the cell resistant to EB and simultaneously provokes an increased sensitivity towards Acri strongly suggests that there is a common site of action for both drugs.

Ethidium bromide interferes with mit-DNA synthesis possibly by producing distortions in an inner mitochondrial attachment site (Perlman & Mahler, 1971). We might then consider the possibility that a mutation in this attachment site in-

hibits further binding of EB and simultaneously facilitates the binding of Acri in EB^{R1} mutants. This mutation could have also affected some cell or mitochondrial membrane component inhibiting the uptake of EB into the cell or mitochondria and favouring the introduction of Acri. Further studies should be done in order to localize and characterize the components affected by these mutations.

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