CYTOPLASMIC AND NUCLEAR INHERITANCE OF RESISTANCE TO ALKYLGUANIDINES AND ETHIDIUM BROMIDE IN A PETITE-NEGATIVE YEAST*

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SUMMARY

Mutants of the petite-negative yeast, Kluyveromyces lactis, resistant to the inhibitors of oxidative phosphorylation, decamethylenediguanidine and octylguanidine were isolated from medium containing ethidium bromide. All mutants were resistant to ethidium bromide; some mutants were resistant to both alkylguanidines, some to one, others to neither. Both nuclear and cytoplasmic inheritance of resistance to decamethylenediguanidine and ethidium bromide was demonstrated by tetrad analysis.

A fundamental problem in mitochondrial biogenesis concerns the function of mitochondrial DNA (mtDNA).** This problem involves two questions: 1. What are the gene-products of mtDNA? 2. What are the functions of these gene-products? Evidence exists for three types of gene-products: mitochondrial ribosomal RNA, mitochondrial tRNA and a few membrane proteins whose precise functions are not well understood. This study extends our knowledge of mtDNA function. It shows that ethidium bromide (EB)**, which has a much higher mutagenicity for mtDNA than for nuclear DNA, may be used to select non-Mendelian mutants of Kluyveromyces lactis, which are resistant to the oxidative phosphorylation inhibitor, decamethylenediguanidine (DMG)** and to EB itself. Other mutants resist EB and octylguanidine (OG)**, an inhibitor of phosphorylation at site I.

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** Abbreviations used: DMG - decamethylenediguanidine (Synthalin A), OG - octylguanidine, EB - ethidium bromide, mtDNA - mitochondrial DNA.

Experimental

Parental K. lactis strains were supplied by Dr. A. Herman. Properties of the strains are given in Table 1. The following media were used: YPD - 1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose; YPG - similar to YPD except dextrose is replaced by 3% (v/v) glycerol; YPDG - YPG plus 0.2% dextrose. The media were supplemented with adenine sulfate (80 mg/liter) and 1% agar (Ionagar No. 2, Colab Laboratories). EB was added before autoclaving; DMG and OG after autoclaving and cooling to about 50°. Nutritional requirements were determined as previously described (1). Sporulation was induced at 25° using ME agar (5% Difco malt extract and 1.5% Ionagar). Growth of all other cultures was at 30°. Mutants were selected by plating approximately 10^7 cells on YPDG containing 10 or 50μM EB.

Results

Mutagenesis and survival: Since K. lactis does not produce viable cytoplasmic petite mutants (2), survivors of EB treatment retain functional mtDNA and grow on YPG. With 10μM EB there were 2-5 survivors per 10^6 plated cells, with 50μM EB, 2-3 per 10^7.

Cross-resistance of mutants: Clones resistant to inhibitors of oxidative phosphorylation could be isolated directly from YPDG+EB plates. Table 2 shows that over 80% of the mutants isolated were resistant to DMG. All mutants were resistant to 10μM EB, but showed different degrees of DMG resistance.

Possible phosphorylation-site specifity of mutations: Studies with liver mitochondria indicate that different alkylguanidines are site-specific in inhibiting oxidative phosphorylation. DMG may act at site III (3) while OG may be specific for site I (4). Resistance of various mutants to DMG and OG is compared in Table 3. Some mutants were resistant to both alkylguanidines, others to only one. Mutants resistant to a single guanidine derivative may be altered in components specific to individual phosphorylation sites. Multiple resistance may involve a generalized change in cation affinity of mitochondrial (or cell) membranes.

TABLE 1

RESISTANCE OF NORMAL AND MUTANT STRAINS TO ALKYLGUANIDINES AND EB

Strain No.	Genotype	Concentration (μM) of compound tolerated DMG	EB
WM66	α ade	<15	<5
Y123	α	<15	5
WM37	a his	7.5	5
WM37R1	a his	60	15
W600B	α ade leu	7.5	5
W600BR1	α ade leu	45**	15
KA7-3A	α ade1	60	25*
KA7-8B	α ade1 his	60	10
KA5-4C	α ade2	7.5	5

*Partially inhibited. **Heterogeneous growth. Tests were made on YPG containing EB or DMG. WM66 and Y123 were inhibited by 240μM OG.

TABLE 2

CROSS-RESISTANCE OF MUTANTS TO DMG AND EB

Parental strain	No. of EB^r strains tested	No. tolerating DMG at given conc. 15μM	30μM	45μM
WM66	31	24	16	5
YI23	16	16	9	6
Total strains	47	40	25	11
%	100	85.1	53.2	23.4

Resistance was tested on YPG+DMG. Cell suspensions were replicated with a Pepper inocula replicator. Plates were incubated for 96 hours and scored daily.

TABLE 3

CROSS-RESISTANCE OF MUTANTS TO ALKYLGUANIDINES AND EB

Parental strain	No. of EB^r strains tested	Cross-resistance: No. of strains OG^sDMG^s	OG^rDMG^r	OG^rDMG^s	OG^sDMG^r
Y123	16	0	8	0	8
WM66	7	2	2	2	1
Total strains	23	2	10	2	9
%	100	8.7	43.5	8.7	39.1

Drug concentrations: OG, 240μM; DMG, 15μM; EB, 10μM

Inheritance of DMG and EB resistance: The existence of non-Mendelian, oligomycin-resistant yeast mutants with modified mitochondrial ATPase (5) suggests that one or more mtDNA gene-products are inner membrane proteins. Other components are probably determined by nuclear genes (6). To determine

TABLE 4

DMG AND EB RESISTANCE IN TETRADS FROM NORMAL AND MUTANT STRAINS

Diploid No.	No. of tetrads of type (R:S) 0:4	1:3	2:2	% Resistant segregants
A. EB Resistance				
KA5	12	1*	0	2
KA6	1	1	14	45.1
KA7	0	0	16	50.0
B. DMG Resistance				
KA5	13	0	0	0
KA6	15	1	0	1.6
KA7	7	7	2	17.2

*Very weakly resistant. YPG medium contained 10μM EB or 45μM DMG. KA5 was prepared by crossing sensitive strains W600B and WM37. In KA6, W600BR1 replaced W600B, and in KA7, WM37R1 replaced WM37. Diploids were maintained on YPD prior to sporulation. R:Resistant, S:Sensitive. Markers segregated 2:2.

TABLE 5

NON-MENDELIAN INHERITANCE OF DMG AND EB RESISTANCE

Diploid Strain No.	Inhibitor	No. of Tetrads	% 4:0 (R:S)
KA-10	DMG	24	100
	EB		100
KA-11	DMG	8	100
	EB		100

KA-10 and KA-11 were made by crossing sensitive strain KA5-4C with resistant strains KA7-3A and KA7-8B, respectively. Marker genes segregated normally. DMG was 60μM and EB 10μM. Other conditions as in Table 4. Some segregants gave somewhat heterogeneous growth on DMG.

if mtDNA controls the interaction of *K. lactis* with DMG, inheritance of DMG resistance was studied. Two complementary strains, W600B and WM37, were plated on YPDG+50μM EB and resistant clones isolated. One mutant from each strain was selected. The sensitive parental strains were used as testers and crossed with the mutants, WM37R1 and W600BR1. As a control, the two sensitive parental strains were crossed. Tetrad analyses of the resulting diploids are shown in Table 4.

All segregants from the control, KA5, were sensitive to 45μM DMG; only one showed very weak EB resistance. Tetrads from KA6 and KA7 contained segregants resistant to both drugs or to EB only. Although resistance to

10μM EB segregates with the 2:2 ratio typical of Mendelian genes, segregation of DMG resistance was unusual. Only one KA6 segregant was resistant to 45μM DMG, but 9 of 16 KA7 tetrads contained 1 or 2 DMG^r segregants. Therefore, in both KA6 and KA7 a nuclear gene segregated which was responsible for resistance to 10μM EB, but whose presence was not sufficient to insure resistance to 45μM DMG. The results with KA6 suggest that the DMG resistance of the parent, W600BR1, is unstable.

Since only 17% of KA7 segregants were resistant to 45μM DMG, DMG resistance is not solely a function of the nuclear gene responsible for resistance to 10μM EB. Since all DMG^r segregants were also EB^r, DMG resistance may require two genetic determinants which act independently. If the second determinant were a single, unlinked nuclear gene, one would expect to find 25% DMG^r segregants instead of the observed 17.2%. Moreover, the proportion of tetrads containing one DMG^r segregant is high, and suggests that DMG resistance depends upon a cytoplasmic (mitochondrial) gene. The high proportion of tetrads having no DMG^r segregants might then be a reflection of mitotic (somatic) segregation of DMG^r in KA7.

To test whether DMG resistance is controlled by a mitochondrial determinant, two of the DMG^r segregants, KA7-3A and KA7-8B, were crossed with a sensitive strain, KA5-4C. Several diploid clones were allowed to develop on drug-free YPD and replicated on YPD+60μM DMG. A heterogeneous growth pattern was obtained, with a number of rapidly growing colonies superimposed on a background of slowly-growing cells. This suggested that mitotic segregation of DMG^r was occurring. Therefore, a few cells from the faster-growing colonies on the YPG+DMG test plate were transferred to YPG+60μM DMG. These purified cultures were then allowed to sporulate on ME agar lacking DMG. In the resulting tetrads all segregants were resistant to both DMG and EB (Table 5). Therefore, DMG resistance in KA7-3A and KA7-8B was determined by a cytoplasmic determinant. When this determinant is present the strains are also resistant to EB, whether or not they contain the nuclear EB^r gene present in the original KA7 segregant.

Discussion

These results demonstrate the existence of an extrachromosomal genetic determinant, probably localized in mtDNA, which determines resistance to DMG and EB. The parallel inheritance of DMG^r and EB^r may indicate either that a single mitochondrial gene controls both traits or that two closely-linked genes are responsible. Although the chemical structures of DMG and EB differ greatly, the two compounds do have common features. Both molecules contain hydrophobic regions and regions of strong positive charge. Consequently, each compound has the potential for interaction either with nucleic acids or with negatively charged centers in membranes. The intercalation of EB into DNA is well known (7). DMG, on the other hand, might be acting as an analog of a polyamine such as spermine, spermidine or agmatine, substances ubiquitously associated with nucleic acids in living cells (8). Interaction of EB with membranes has been used as a fluorescent probe for detecting energy-linked conformational changes in mitochondria (9), while DMG inhibits oxidative phosphorylation (3). Therefore, it is reasonable to propose that cross-resistance to DMG and EB is caused by mutation in a single mitochondrial gene.

Possibly the mutation protects mitochondrial DNA directly by modifying a sequence or conformation which is especially vulnerable to these compounds, for example, an attachment site for DNA polymerase or DNA-dependent RNA polymerase. Alternatively, the gene-product affected by the mutation may be a membrane protein, associated with binding and/or transport of either type of organic cation. Genetic modification of cation binding to the mitochondrial membrane has recently been suggested by Bech-Hansen and Rank as possible explanation for cross resistance of Mendelian mutants of Saccharomyces cerevisiae to both EB and the cationic detergent, cetyltrimethylammonium bromide (10). In this connection the observation that DMG appears to undergo respiration-linked transport in yeast mitochondria (11) may be pertinent. Bech-Hansen and Rank also reported the isolation of EB-resistant, diploid strains of S. cerevisiae whose behavior suggested that EB resistance had a

cytoplasmic basis. Unfortunately resistance did not survive sporulation, so that only EB-sensitive progeny were obtained.

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