RESISTANCE AND SENSITIVITY TO ROTENONE IN *SACCHAROMYCES CEREVISIAE*: POSSIBILITY OF A GENETIC REEXAMINATION

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1. Introduction

In most yeast species respiration with NAD-linked substrates is insensitive to rotenone, however this peculiar property of the mitochondrial $NADH_2$-dehydrogenase does not prevent energy coupling at site I of phosphorylation [1–3]. *Saccharomyces cerevisiae* is also resistant to rotenone [4] and this has been related to the anomalous response to succinate on non-heme iron mitochondral proteins [5].

If resistance to rotenone in wild type cells is the consequence of a selective advantage for multiplication it should be possible with an appropriate sieve to isolate mutants sensitive to this drug. A few mutants of this kind have been obtained. In view of the possible mitochondrial site of action of rotenone and of the tight association of $NADH_2$-dehydrogenase with the inner membrane of the organelle [6–8], the control of resistance and sensitivity can be a priori operated by the mitochondral or the nuclear genome. The latter possibility turns out to be true.

2. Materials and methods

2.1. Strains

Two haploid grandes strains originated from Centre de Génétique Moléculaire, Gif-sur-Yvette, were used: 194-5C and iL126-1B. Their nuclear genotypes are: 194-5C: a ade6 his 4 ura 1, iL126-1B: α his 1; their mitochondrial genotypes are: 194-5C: $\rho^+\omega^+C^SE^S$; iL126-1B: $\rho^+\omega^-C^RE^R$. ρ^+ refers to the grande genotype ω^+/ω^- to the mitochondrial mating type [9], C^S/C^R and E^S/E^R refer, respectively, to sensitivity and resistance to chloramphenicol and erythromycin. These two strains are resistant to rotenone, all the mutants have been derived from them.

2.2. Media

The normal medium for growth on plates contains: 1% yeast extract, 1% peptone, 3% glycerol, 5×10^{-2} M Na-phosphate buffer, pH 6.8, 2% agar. According to necessity the following additions are made: 10^{-5}M rotenone from a 10^{-3}M stock solution in DMSO, chloramphenicol 2 g/ℓ or erythromycin 2 g/ℓ. Liquid cultures are performed in the following medium: 1% yeast extract, 0.12% $(NH_4)_2\,SO_4$, 5×10^{-2} M Na-phosphate buffer, pH 6.8, adenine 40 mg/ℓ, histidine 40 mg/ℓ, uracil 40 mg/ℓ. The carbon source can be 2% glucose, 2% galactose, 3% glycerol or 2% ethanol. Rotenone is eventually added (final concentration 10^{-5} M).

Cells are usually grown at 29°, liquid cultures are steadily shaken.

2.3. Isolation of mutants

Mutants sensitive to rotenone were isolated after UV irradiation of a suspension of 5×10^6 cells/ml (1 to 10% viability estimated on the glycerol medium which eliminates petites). Cells are plated on the glycerol medium, after 4 days colonies are replicated on the same medium containing rotenone. From the comparison of the two kinds of plates sensitive mutants are recovered from the mother-plate and

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subsequently tested for their homogeneity and their sensitivity both by plating out and by replica plating.

2.4. Genetic analysis

Crosses are performed between compatible haploid strains and prototroph diploid cells are isolated owing to their ability to grow on a minimal medium (yeast nitrogen base 6.7 g/ℓ, 2% glucose, 2% agar). These diploid populations are then tested for sensitivity and resistance to rotenone, chloramphenicol and erythromycin.

Sporulation from pure diploid cells is induced on plates containing 0.2 M K-acetate, 0.5% galactose, 5×10^{-2}M Na-phosphate buffer, pH 7. Haploid products of meiosis are analyzed either in tetrad by micromanipulation or at random.

2.5. Preparation of a mitochondral fraction

Cells grown in liquid cultures are harvested by centrifugation (1500 *g*) in the cold, washed once, and turned into protoplasts [10]. Protoplasts are washed twice, suspended in 10% saccharose, 6×10^{-2}M Tris-HCl, pH 7.7, 10^{-3} M EDTA and homogenized for 15 sec in an ultra-turrax. Intact cells, debris and nuclei are eliminated by centrifugation (2000 *g*) and the mitochondrial fraction is then separated from a supernate by centrifugation (20,000 *g*). The mitochondrial pellet is suspended in the same buffer.

2.6. Enzyme activities

$NADH_2$-oxidase, $NADH_2$-cytochrome *c* oxidoreductase, $NADH_2$-ferricyanide oxidoreductase, $NADH_2$-dichlorophenolindophenol dehydrogenase, succinate-cytochrome *c* oxidoreductase and succinate-ferricyanide oxidoreductase activities are examined according to Macker et al. [11]. Protein determinations are performed according to Stickland [12].

3. Results and discussion

3.1. Isolation of mutants sensitive to rotenone

Three independent mutagenesis and an examination of 9 400 colonies allowed the isolation of four sensitive mutants from 194-5C: 194-5C/RII/53, 194-5C/R12/21, 194-5C/R12/3 and 194-5C/R21.

The second and the third strains have been isolated by cloning of an heterogeneous colony, they are probably related although their growth patterns are different. The last mutant grows very slowly anyhow.

Three mutants have been obtained in one experiment out of 4620 colonies of iL126-1B. One, iL126-1B/R/III/3 appears to be really sensitive to rotenone, the two others are only slightly sensitive and only one has been kept for comparison, iL126-1B/R91.

Thus the frequency of appearance of a sensitive mutant in a haploid population is rather small. Moreover the same selection procedure has been applied on diploid UV-irradiated cells without any success although 14400 colonies were examined. These observations are consistent with a control of resistance and sensitivity by nuclear genes and not by the mitochondrial genome.

3.2. Phenotype of mutants sensitive to rotenone

Sensitive mutants have been classified in two types according to the pattern of inhibition of growth by rotenone in liquid cultures and to the sensitivity of $NADH_2$-oxidase activity of isolated mitochondrial fractions.

The first class consists of mutants in which growth is only slowed down by the drug and not completely inhibited (table 1 and fig. 1). The analysis of the $NADH_2$-oxidase activity of the mitochondrial fraction does not reveal any sensitivity to rotenone (table 2). Mutants 194-5C/R12/21, 194-5C/R12/3, 194-5C/R21, iL126-1B/R91 and iL126-1B/RIII/3 belong to this category.

The second class is represented by only one mutant: 194-5C/RII/53. In the presence of rotenone (10^{-5} M) mutant cells keep on dividing for some 3 generations although somewhat more slowly then growth stops completely (table 1 and fig. 1). The level of sensitivity has been examined: cells are only slightly sensitive and keep growing when the concentration of the drug is under 3×10^{-7} M, the same pattern of inhibition as above is observed when the concentration of rotenone reaches 3×10^{-7} M, 10^{-6} M or 3×10^{-6} M. To check *in vivo* the specificity of action, the effect of rotenone has been observed with

Table 1
Growth of mutants sensitive to rotenone in the presence or absence of the drug in a glycerol medium.

	Generation time with rotenone (hr)	Without rotenone (hr)
Class I 194-5C/R12/21	9	3
194-5C/R12/3	8	5
194-5C/R21	10	8
iL126-1B/R91	11	8
iL126-1B/RIII/3	11	8
Class II 194-5C/RII/53	7 hr for 3 generations, stops afterwards	4.5

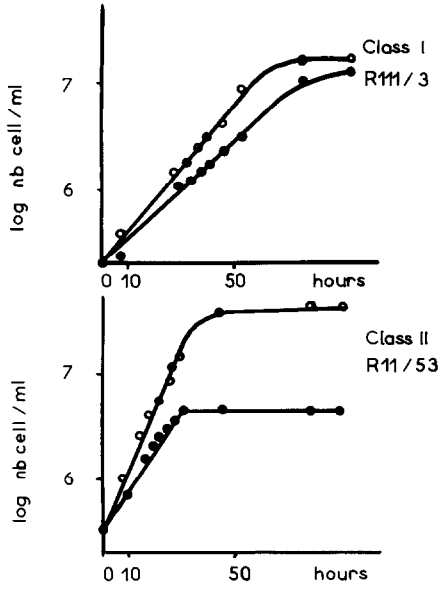

Fig. 1. Growth of two mutants sensitive to rotenone in presence (•–•–•) or in absence (o–o–o) of the drug (10^{-5} M). The carbon source is 3% glycerol, the medium also contains 1% yeast extract, 0.12% $(NH_4)_2 SO_4$, 5×10^{-2} M Na-phosphate buffer, pH = 6.8, adenine 40 mg/ℓ, histidine 40 mg/ℓ and uracil 40 mg/ℓ. Flasks are steadily shaken at 29°.

various carbon sources. Inhibition of growth is found only in the presence of non fermentable substrates, namely glycerol or ethanol; on the other hand rotenone has no effect on cells when they are grown in the presence of glucose or galactose (fig. 2). This result indicates that rotenone inhibits the normal

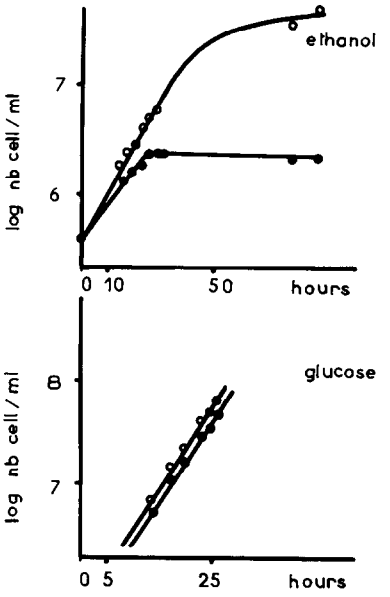

Fig. 2. Effect of 10^{-5} M rotenone (•–•–•) on growth of the sensitive mutant 194-5C/RII/53 according to the carbon source available, 2% ethanol or 3% glucose. Controls are run in the absence of rotenone (o–o–o). Conditions are the same as in fig. 1.

mitochondrial functions in these mutant cells, for its action can only be revealed when mitochondrial activities are compulsory for growth.

In vitro the $NADH_2$-oxidase activity of the mitochondrial fraction is partially sensitive to rotenone (10^{-6} M), the inhibition is not complete (27% in the conditions of the test), table 2. Attempts to examine accurately the pattern of inhibition have not been made. In the absence of such a piece of information the value of inhibition can only be considered as a crude qualitative characterization. Several phenomena can interfere in the measure of the activity; one is the multiplicity of $NADH_2$ dehydrogenase activities in the crude mitochondrial fraction used [7, 8], secondly sensitivity to rotenone in other yeast species is known to vary according to the physiological state of the cells [13–15] and this analysis has not been done in the case of this mutant especially in correlation with the lag of action observed *in vivo*.

However the effect of the drug appears to be specific of the $NADH_2$-oxidase activity. No similar inhibition in the same mitochondrial preparations has been observed on various mitochondrial activ-

Table 2
$NADH_2$-oxidase activity in the presence or absence of rotenone of mitochondrial fractions of different sensitive and resistant strains.

		$NADH_2$-oxidase (mM/min/mg protein)	Inhibition by 10^{-6}M rotenone (%)
Sensitive Class I	194-5C/R12/21	4.4×10^{-2}	5
	194-5C/R12/3	3.7×10^{-2}	1
	iL126-1B/RIII/3	5.1×10^{-2}	4
Sensitive Class II	194-5C/RII/53	6.9×10^{-2}	27*
Resistant	194-5C	2.8×10^{-2}	0
	iL126-1B	3.8×10^{-2}	3

* Mean of 6 determinations from 3 independent experiments.

ities: $NADH_2$-cytochrome *c* oxidoreductase [7], $NADH_2$-ferricyanide oxidoreductase, $NADH_2$-dichlorophenolindophenol dehydrogenase, succinate-cytochrome *c* oxidoreductase or succinate-ferricyanide oxidoreductase.

It seems clear that the nature of the sensitivity to rotenone is different in the mutants of class I and in 194-5C/R11/53. In this mutant sensitivity could be due to the interaction of rotenone with the $NADH_2$-dehydrogenase of the inner membrane of the mitochondria.

3.3. Genetic analysis

Owing to the tight association of the $NADH_2$-dehydrogenase with the mitochondrial membrane [6–8] the genetic analysis has been carried on especially for the mutant 194-5C/RII/53 to determine whether sensitivity and resistance are under mitochondrial or nuclear genetic control. Two criteria are used to distinguish between the two possibilities. One is the mitotic segregation of the mitochondrial genes and the absence of mitotic segregation of nuclear genes when diploid cells heterozygous for mitochondrial and nuclear markers multiply [9]. The second one bears on the Mendelian segregation of nuclear genes at the meiosis of these diploids and the absence of segregation of mitochondrial genes [9].

Crosses between haploid resistant and sensitive strains yield a diploid progeny of cells all resistant to rotenone. No mitotic segregation of this character is observed although mitochondrial markers segregate and recombine normally (table 3).

When meiosis is induced in pure diploid cells issued from a cross between the sensitive strain 194-5C/RII/53 and the resistant strain iL126-1B, 16 haploid spores out of 71 studied at random appear to inherit the sensitivity to rotenone. This result suggests a 3/1 segregation and is supported by the analysis of a few tetrads [6] where only one product out of four can be shown to be sensitive to the drug. Two mutated nuclear genes are probably responsible for sensitivity in the original parent. No further attempt has yet been made to elucidate the precise function of each one of these genes. One of them at least could control the mitochondrial $NADH_2$-dehydrogenase. Crosses between compatible sensitive strains of class I, or between 194-5C/RII/53 and a compatible mutant of class I give diploid cells resistant to rotenone in all cases. No mitotic segregation appears when these diploid cells divide. This complementation between different genotypes suggests that several cellular activities can be turned sensitive to rotenone by mutation, this is consistent with the classification of the mutants in two types.

A simple hypothesis for the control of sensitivity by two genes would be to assume that one of them is responsible for the absence of permeability of the cell to the drug and the other for the synthesis of an enzyme which inactivates this product. Mutation in the first gene would allow rotenone to enter the cell, mutation in the second one would leave the drug in an active form and $NADH_2$-oxidase would appear sensitive. If such an interpretation is true the enzyme able to inactivate rotenone should be a mitochondrial protein for the $NADH_2$-oxidase activity *in vitro* of the mitochondrial fraction of wild type cells is resistant to the highest concentrations available of rotenone.

Anyhow the mutants obtained can be useful in two ways: one is to reexamine the properties and the eventual sensitivity to rotenone of the mitochondrial $NADH_2$-dehydrogenase of yeast. The second deals with the integration of mitochondrial and nuclear genetic information for, although one of the proteins responsible for sensitivity is mitochondrial, it is under the control of the nuclear genome.

Table 3

Absence of mitotic segregation of resistance and sensitivity to rotenone in diploid cells issued from the cross of haploid resistant and sensitive parents, and comparison with the behaviour of the mitochondrial genes of resistance to erythromycin.

Cross	Rotenone		Erythromycin		No. of colonies examined
	Sensitive (%)	Resistant (%)	E^S (%)	E^R (%)	
194-5C/RII/53 × iL126-1B	0	100	96	4	391
194-5C/R12/21 × iL126-1B	0	100	75	25	347
194-5C/R12/3 × iL126-1B	0	100	64	36	343
194-5C/R21 × iL126-1B	0	100	90	10	187
194-5C × iL126-1B/RIII/3	0	100	66	34	337
Control with resistant strains 194-5C × iL126-1B	0	100	81	19	397

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