INDUCTION OF RESPIRATORY-DEFICIENT MUTANTS IN A "PETITE NEGATIVE" YEAST SPECIES KLUYVEROMYCES LACTIS WITH N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

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Summary: Thirty-nine nitrosoguanidine-induced respiratory-deficient mutants of Kluyveromyces lactis strain CBS 2359 were isolated. These mutants do not grow on glycerol and have a lower respiration rate associated with a reduced capacity to react with tetrazolium salts. Mitochondria isolated from these mutants are altered in the oxidation of several substrates of the respiratory chain. From the spectroscopic analysis of the cytochromes it is possible to distinguish four groups of mutants in which: first group) all cytochromes are missing; second group) only cytochrome a is missing; third group) cytochromes a, b, c_1 are missing; fourth group) no cytochrome is absent. Tetrade analyses show that the respiratory-deficient mutants are of chromosomal type. The existence of these mutants demonstrates that the failure in obtaining cytoplasmic respiratory-deficient mutants in Kluyveromyces lactis does not depend on the lethality of the alteration of the respiratory chain.

INTRODUCTION

In growing cultures of Saccharomyces cerevisiae and related species, there are approximately 2-4% of respiratory-deficient (RD) cells called vegetative petites known to be due to the loss of the cytoplasmic hereditary factor rho (1,2). These mutants exhibit a non-Mendelian pattern of inheritance and can be selectively induced by specific mutagenic agents such as acriflavine, 5-fluorouracile, ethidium-bromide with a frequency near to 100% (3,4).

The RD cells cannot utilize non-fermentable carbon sources and are unable to reduce tetrazolium salts (5). Spectroscopic examination of the intact cells of these mutants has revealed alterations in either the amount or kind of cytochromes, or both, which can account for the absence of a number of enzymatic activities involved in the respiratory chain.

In addition to the vegetative mutants, there exist phenotypically similar mutants resulting from a mutation which, on the basis of its Mendelian pattern of inheritance, have been mapped on the chromosomal loci (segregational petites) (1,2).

Bulder (6) classified several yeast species as "petite positive" and "petite negative" depending on whether or not viable RD mutants could be

obtained by acriflavine treatment. In addition, Del Giudice et al. (7) confirming Bulder's classification by means of 5-fluorouracil , ethidium bromide, U.V. rays, demonstrated that the lack of production of RD mutants in the "petite negative" species does not depend on the mutagens used but reflect a structural property of the mitochondrial information.

In this paper we report the isolation by means of nitrosoguanidine treatment of several RD mutants from a "petite negative" strain, Kluyveromyces lactis, showing a Mendelian segregation (chromosomal petites), the existence of which excludes that the lack of induction of cytoplasmic RD mutants in this species depends on the lethal effect of the alteration of the respiratory chain.

MATERIALS AND METHODS

Yeast strain. The prototrophic haploid yeast strain Kluyveromyces lactis CBS 2359 was used. This strain comes from the Centraalbureau voor Schimmelcultures, Yeast Division, Baarn, The Netherlands.

Media. YEPD broth 0.5% yeast extract (Difco), 1% bactopeptone (Difco), 2% glucose. YEPG broth with 2% glycerol substituted for glucose. For maintenance of yeast stocks and cell plating, 2% agar (Difco) was added.

Nitrosoguanidine treatment. Mutagenesis was performed at 30°C. Nitrosoguanidine stock solution (1 mg/ml) was prepared in sterile citric-phosphate buffer 0.15 M (pH 6.1).

Stationary-phase cells, grown at 30°C, were centrifuged for 10 min at 2,000 x g and washed twice with sterile distilled water. The pellet was resuspended in citric-phosphate buffer 0.15 M (pH 6.1) at a final concentration of about 1×10^9 cells per milliliter.

Nitrosoguanidine solution was added to give the desired final mutagen concentration and the mixture was incubated in a 30°C water bath without shaking. Samples were withdrawn at different intervals of time, washed twice and resuspended in sterile distilled water at appropriate dilutions for plating on YEPD agar for survivors.

2,3,5-Triphenyltetrazolium chloride (TTC) overlay. Colonies grown on YEPD agar plates were overlaid with TTC following the methods described by Ogur et al. (5); respiratory competent (RD^+) cells turned red within a few hours.

Genetical analysis. The conditions for mating, the isolation of spores and determination of mating type were carried out according to Herman and Halvorson's work (8).

Preparation of mitochondria. The A method of Kellems (10) was used with certain modifications. Cells were grown to early stationary-phase at 30°C in YEPD broth, harvested by centrifugation and washed once with sterile distilled water and once with a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and

0.25 M Mannitol (MTE buffer). Cells (about 20 g) were resuspended in 40 ml of MTE buffer and 80 g of glass beads (0.5 mm diameter) were added. The suspension was homogenized in a Virtis-45 homogenizer at maximum velocity for 20 min. The tube containing the cell suspension was cooled in an ice-bath. The suspension was decanted and the beads rinsed with 3 x 5 ml of MTE buffer. The pooled rinses and suspensions were centrifuged for 10 min at 2,000 x g to remove unbroken cells and cell debris. The supernatant suspensions were centrifuged at 12,000 x g for 10 min and the pellet resuspended in 50 ml of MTE buffer and centrifuged at 2,000 g for 10 min for further cleaning.

Mitochondria were pelleted from the supernatant suspensions by centrifugation at 12,000 x g for 10 min and resuspended in 2 ml MTE buffer.

Respiration measurement. The oxygen uptake of the whole cells was measured polarographically with a Gilson Oxygraph Mod. KM; the mixture contained 3.0 ml of 67 mM KH_2PO_4 (pH 4.5), 0.1 ml of 40% solution of glucose, 0.1 ml of cell suspension. Respiration rates (QO_2) were expressed as microliters of O_2 consumed per hour per 1 x 10^8 cells.

The mitochondrial oxygen uptake was measured in a mixture containing 3.0 ml of 0.1 M Tris-HCl (pH 7.4), 0.1 ml of the mitochondrial fraction with about 50 mg per ml of protein, and 0.1 ml of one of the following substrate solutions: a) 0.1 M sodium succinate, b) 0.5 mM NADH, c) 0.1 M sodium L-lactate, d) 0.1 M sodium D-lactate. Respiration rates were expressed as microliters of O_2 per hour per milligram of protein.

Cytochrome spectra. The cytochrome content was examined using the low-temperature spectroscopic technique described by Sherman (11). Cells were grown for 3 days at 30°C on YEPD agar plates and sodium dithionite grains were added to a paste of blotted yeast which was then cooled in liquid N_2. Cyto-

Table I. Spectroscopic analysis of the cytochrome of the wild strain and respiratory-deficient mutants of Kluyveromyces lactis

Mutant groups	Number of mutants analyzed	Cytochromes a	b	c_1	c
1	2	-	-	-	-
2	7	-	+	+	+
3	12	-	-	-	+
4	18	+	+	+	+
Parental strain Kluyv. lactis 2359		+	+	+	+

Table II. Respiration rates (QO_2) of intact cells of the wild strain and respiratory-deficient mutants of Kluyveromyces lactis*

	QO_2 ($\mu l\ O_2 x h x 1.10^8$ cells)	% QO_2 respect to the wild strain
Parental strain		
Kluyv.lactis 2359	40.8	100
Mutant strains		
First group: No. 1	5.3	13
2	11.0	27
Second group: No. 3	5.3	13
4	5.7	14
5	6.1	15
6	6.1	15
7	6.9	17
8	7.7	19
9	7.7	19
Third group: No.10	3.4	8.5
11	3.67	9
12	4.0	10
13	4.4	11
14	4.8	12
15	6.4	15.8
16	6.9	17
17	6.9	17
18	7.3	18
19	11.0	27
20	11.8	29
21	12.5	30.5
Fourth group: No.22	3.26	8
23	5.7	14
24	7.3	18
25	13.05	32
26	16.7	41
27	16.7	41
28	17.9	43.8
29	18.7	46
30	18.7	46
31	19.5	48
32	19.5	48
33	20.4	50
34	22.4	55
35	23.46	57.5
36	28.15	69
37	28.56	70
38	31	76
39	35.5	87

*Glucose was used as respiratory substrate

chrome spectra of cells were examined with a Pupillaire Zeiss spectroscope fitted on a microscope.

RESULTS

Strain CBS 2359 was treated with nitrosoguanidine at a final concentra-

tion of 25 µg/ml for 30 min, sufficient to give 10-20% of survivors. Out of 10,000 colonies, 39 RD mutants were selected for their inability to grow on YEPG media.

All the mutants, purified three times by single-colony isolation, showed a stable RD phenotype which was confirmed by their diminished ability to reduce TTC.

Table I shows the results of the low-temperature spectroscopic analysis of the cytochrome content of the mutants compared to that of the parental wild-type strain.

RD mutants were divided into four groups: in the first two mutants (1 and 2), all the cytochromes are missing; in the second, seven mutants (3 to 9) are deficient only in cytochrome a; in the third, twelve mutants (10 to 21) have cytochrome c as the only component; in the fourth, eighteen mutants (22 to 39) show a normal cytochrome spectra.

Table II shows the respiration rates of intact cells of all the RD mutants and of the parental wild-type strain, using glucose as substrate.

The first three groups of the RD mutants show low respiration rates, while in the fourth group there are RD mutants with low respiration rates and RD mutants with respiration rates comparable to that of the parental wild-type strain.

Respiration rates of mitochondrial fractions extracted from some mutants of each group compared to that of the parental strain has been determined (Table III). Succinate, NADH, L-lactate, D-lactate were used as substrates and the respiratory rates were sufficiently low with respect to that of the wild strain. The tetrade analysis on two independent RD mutants for each group of cytochrome spectra was made. At least seven asci for RD^+/RD segregation were analyzed for each cross and it was found that all gave a 2:2 ratio.

Table III. Oxygen uptake by mitochondrial fractions of wild strain and respiratory-deficient mutants of Kluyveromyces lactis*

Substrates	Parental strain	Respiratory-deficient mutants No.					
	Kluyv.lactis 2359	2	3	11	20	23	24
Succinate	108	46	39	17	58.4	20.1	33.2
NADH	108	46	39	17	58.4	20.1	33.2
L-lactate	51	26.2	16.4	11.3	41.3	14.6	24.5
D-lactate	51	26.2	16.4	11.3	41.3	14.6	24.5

*Results expressed as µl O_2 respired per hour per milligram of protein.

CONCLUSION

Despite the fact that classical mutagens of the cytoplasmic genetic complement are unable to induce RD mutants in petite negative species, viable RD mutants were isolated by nitrosoguanidine treatment of the "petite negative" yeast Kluyveromyces lactis CBS 2359.

Out of 39 independent RD mutants isolated by the replica-plating technique, four different groups have been identified by low temperature spectroscopic analysis of the cytochrome content. The first three groups of the mutants showed an altered cytochrome spectra, while the fourth had a normal cytochrome spectra.

Our results agree with those obtained on RD mutants induced by U.V. rays in a Kluyveromyces lactis yeast strain (12) and suggest that the alterations of the respiratory chain is not per se a lethal event. This fact indicates that in this yeast the mitochondrial particle is probably associated with a series of information or functions, the integrity of which is essential for the life of the cell and that does not coincide with the respiratory mechanisms of ATP synthesis. This hypothesis agrees with the results obtained on Kluyveromyces lactis strain CBS 2360 in which erythromycin and rifampicin inhibit in vivo the cytoplasmic system for the synthesis of RNA and proteins, in spite of the fact that in vitro the two biosynthetic systems are resistent to the above antibiotics which affect only the mitochondrial system for RNA and protein synthesis (13,14,15).

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