BBA 45 699

OXIDATIVE PHOSPHORYLATION IN YEAST

IV. COMBINATION OF A NUCLEAR MUTATION AFFECTING OXIDATIVE PHOSPHORYLATION WITH CYTOPLASMIC MUTATION TO RESPIRATORY DEFICIENCY

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(Received April 16th, 1968)

SUMMARY

1. An attempt was made to superimpose a cytoplasmic mutation for respiratory deficiency ρ^- over a nuclear mutation for oxidative phosphorylation deficiency by treatment of an oxidative phosphorylation-deficient yeast mutant op_1 with acriflavine. Unlike wild-type yeast, cells of the mutant op_1 grown in the presence of acriflavine did not form visible colonies after plating on solid medium with glucose as carbon source.

2. Growth of the op_1 mutant in acriflavine-supplemented medium was limited. After about 10 generations the cells of the mutant ceased to multiply. They were then unable to respire, lacked cytochrome *b* and aa_3 but seemed not to be dead since they fermented and assimilated glucose and did not stain with methylene blue.

3. In the culture of the op_1 cells growing in the presence of acriflavine a considerable fraction of cytoplasmic respiration-deficient mutants was detected by crossing the cells with a neutral cytoplasmic mutant of opposite mating type.

4. Revertants from the op_1 mutant which had grown in the presence of acriflavine gave rise to normal, viable respiration-deficient colonies after plating on solid medium.

5. It has been concluded that specific combination of the cytoplasmic ρ^- and nuclear op_1 mutations in a cell resulted in loss of multiplication ability. Implications of such a cumulative effect for the nature of op_1 and ρ^- mutations are discussed.

INTRODUCTION

The use of biochemical mutants in oxidative phosphorylation research has been suggested earlier[1]. Cytoplasmic mutation to respiratory deficiency in the yeast cell[2] (from ρ^+ to ρ^- genotype[3]) brings about loss of oligomycin sensitivity of the mitochondrial ATPase system[4,5] as a consequence of a structural modification or a lack of some components in this part of the oxidative phosphorylation machinery. A chromosomal mutation affecting the gene OP (from OP to op_1 genotype[1,6]) results in a mutant deficient in oxidative phosphorylation which possesses the complete respiratory chain

and intact coupling step but displays some lesions in subsequent reactions of mitochondrial ATP synthesis[7]. Since the mitochondrial ATPase activity of the deficient mutant carrying the mutated recessive gene op_1 has preserved oligomycin sensitivity[7], the superimposition of the cytoplasmic ρ^- mutation over the nuclear op_1 mutation should give rise to a new cell type with a still more profoundly affected oxidative phosphorylation system. Such cells may provide more insight both into the nature of the op_1 and ρ^- mutations and the mechanism of oxidative phosphorylation. An attempt to prepare such variants by acriflavine treatment[2] of the op_1 cells is described in this paper.

EXPERIMENTAL

The following strains of *Saccharomyces cerevisiae* were employed: DT XII (diploid, wild-type, originating from a baker's yeast factory in Trenčín), H 3 C (haploid, a op_1 ad_1 (see ref. 6)), DH 1 (diploid, homozygous for the gene op_1, adenine-requiring (see ref. 7)) and C 982-19 dA_1 (haploid, 'petite' neutral, α tr_1 $hi_1\rho^-$, from Dr. H. JAKOB, Gif-sur-Yvette). The yeasts were cultivated in glucose medium which contained 1 % peptone, 1.5 % yeast autolyzate, 0.004 % adenine and 2 % glucose. Glycerol medium had the same composition except that 2 % glycerol was used instead of glucose. Solid media contained 2 % agar in addition.

Acriflavine was purchased from British Drug Houses, chloramphenicol from Spofa and other chemicals from Lachema. Solutions of acriflavine and chloramphenicol were sterilized by filtration and added aseptically to the autoclaved media. Other methods are described in legends to figures and tables.

RESULTS

As expected[2], cells of the wild-type strain DT XII cultivated in liquid glucose medium in the presence of 5 to 25 μg/ml acriflavine were completely converted to respiratory-deficient variants as proved by differential plating on solid glucose and

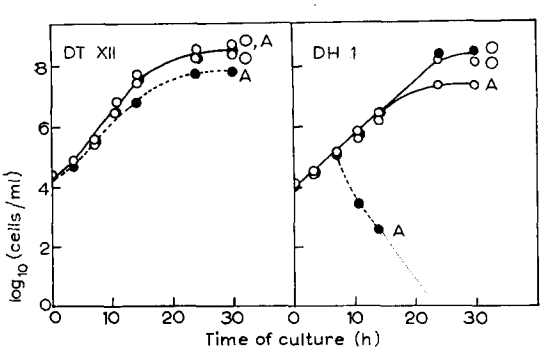

Fig. 1. Growth curves of cells in the presence of acriflavine. 24-h-old cells of the wild-type strain DT XII and of the oxidative phosphorylation deficient mutant DH 1 were inoculated into 20 ml of liquid glucose medium with or without acriflavine in 200-ml erlenmeyer flasks and cultivated on a shaker at 30°. Aliquots were taken periodically to estimate number of cells by direct counting in a haematocytometer chamber or by plating on solid glucose medium. O, control without acriflavine; A, in the presence of 5 μg/ml acriflavine; O—O, counting in haematocytometer; ● – – ●, counting by plating on solid medium.

glycerol media[8] and staining the colonies with triphenyltetrazolium chloride[9]. Surprisingly, when haploid or homozygous diploid strains of the op_1 mutants were grown in the same way in liquid glucose medium with acriflavine for 24 h and aliquots plated on solid glucose medium without acriflavine, no visible colonies appeared on agar plates even after several weeks of incubation. Control cells grown in the absence of acriflavine gave rise to normal colonies stainable with triphenyltetrazolium chloride.

On the other hand, the total count of the op_1 cells grown in liquid glucose medium with acriflavine (determined by counting the cells in a haematocytometer chamber) increased similarly as that of the wild-type cells and more than 99 % of the cells were found to be viable by the methylene blue staining procedure[10]. Only if the viable count was estimated by plating the cells from these liquid cultures, differences between the op_1 and wild-type cells became apparent (Fig. 1). Shortly after growth had started, the number of outgrown colonies was lower than the number of cells in the original inoculum indicating that not only daughter cells but even the cells used to inoculate the liquid culture were affected by the acriflavine.

The peculiar property of the op_1 mutant which enables it to grow in liquid glucose medium with acriflavine but not subsequently on solid acriflavine-free media became more clear after growing the wild-type and the op_1 cells in liquid glucose media with acriflavine starting from very diluted inocula (Fig. 2) and by transferring the cells grown in acriflavine-supplemented medium to a new batch of the same medium (Fig. 3). The cells of the wild-type strain could grow continuously in the presence of acriflavine although completely converted to respiratory-deficient variants. The growth of the op_1 cells in the presence of acriflavine was not unlimited; it could be calculated from Figs. 2 and 3 and from similar data that their growth stopped after about 10 generations in acriflavine-supplemented medium. Essentially in accord with

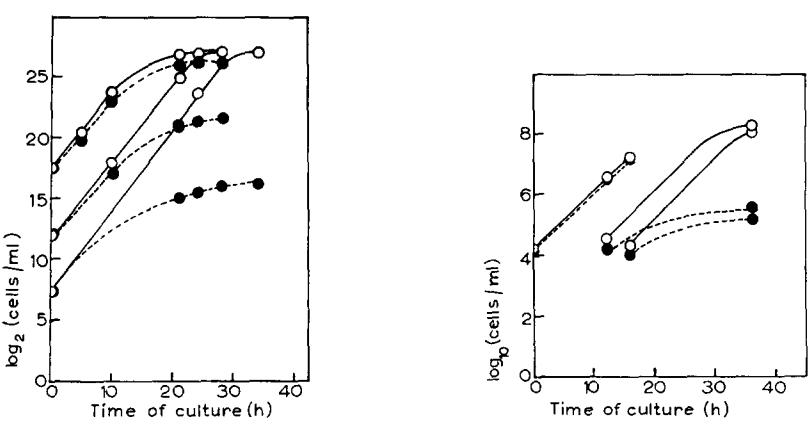

Fig. 2. Growth curves of mutant DH 1 growing from different inocula. Growth conditions similar as in Fig. 1 except that the amount of cells at the beginning of growth was 10^5, 10^3 and 10^2 cells/ml, respectively. Cell count was estimated by the haematocytometer method. O—O, control without acriflavine; ● – – ●, in the presence of 5 μg/ml acriflavine.

Fig. 3. Growth of mutant DH 1 after transfer into fresh medium. 24-h-old cells were inoculated into 20 ml liquid glucose medium with or without acriflavine in several 200-ml erlenmeyer flasks to a density of $1.8 \cdot 10^4$ cells/ml. After 12 and 16 h of cultivation at 30° the cells from some flasks were centrifuged, washed with water, reinoculated to new flasks with the same fresh medium to the original density and further cultured at 30°. Cell count was estimated by the haematocytometer method. O—O, control without acriflavine; ● – – ●, in the presence of 5 μg/ml acriflavine.

this observation, when the normally grown op_1 cells were plated directly on solid glucose medium containing 5 or 25 μg/ml acriflavine, barely visible microcolonies occurred on the plates. The microcolonies contained about 10^4 cells indicating that from each cell about 13 cell generations had been formed before growth stopped entirely.

The op_1 cells harvested after prolonged growth in glucose medium supplemented with acriflavine showed no O_2 uptake (Table I) and no cytochrome *b* or aa_3 in reduced spectra (Fig. 4). Thus, acriflavine had inhibited the synthesis of cytochrome *b* and aa_3 in the op_1 mutant similarily as in wild-type Saccharomyces and various other yeast species[11–14]. The harvested cells assimilated a part of added glucose (Table II) indicating that synthetic activity was not completely suppressed in these cells which were incapable of further multiplication.

The inability of the op_1 cells to multiply after prolonged growth in acriflavine

TABLE I

METABOLIC QUOTIENTS OF CELLS GROWN IN THE PRESENCE OF ACRIFLAVINE

Cells of wild-type *S. cerevisiae* strain DT XII and of the mutant DH 1 were grown from an original inoculum of 10^4 cells/ml for 48 h in liquid glucose medium with or without acriflavine on a shaker at 30° and washed four times with water. Despite extensive washing the cells cultivated with acriflavine retained a yellow colour. Metabolic quotients were determined manometrically at 30° in 80 mM citrate–phosphate buffer (pH 4.3) and 50 mM glucose.

Strain:	*DT XII*		*DH 1*	
Acriflavine in culture medium (μg/ml):	*0*	*5*	*0*	*5*
Q_{O_2}	133.0	5.6	42.0	0
$Q^{air}_{CO_2}$	—*	—*	325.0	173.0

* Not tested.

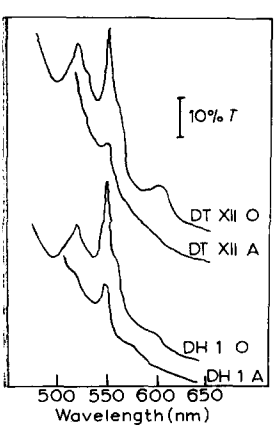

Fig. 4. Cytochrome spectra of cells grown in the presence of acriflavine. Cells of the wild-type strain DT XII and of the op_1 mutant DH 1 were cultured and harvested as described in Table I. They were then suspended in glycerol and their spectra measured in the SF 10 spectrophotometer against filter paper. O, control without acriflavine; A, cells cultivated in the presence of 5 μg/ml acriflavine.

medium could not be simply due to phenotypic absence of respiration since the op_1 mutant can grow anaerobically and since the op_1 cells plated on solid media with 4 mg/ml chloramphenicol, which is known to cause phenotypic suppression of cytochrome *b* and aa_3 synthesis in yeast[15], gave rise to similar colonies as the cells plated on medium without chloramphenicol. Accordingly, the effect of acriflavine on the op_1 cells does not seem to be associated with its ability to affect phenotypically the synthesis of respiratory pigments[11–14] but with its capacity to induce ρ^- mutation[2]. The op_1 cells might lose their multiplication ability after having assumed the ρ^- genotype.

In order to determine if ρ^- cells could be found in a culture of the op_1 mutant, haploid op_1 cells grown in liquid acriflavine-supplemented medium were taken periodically from the culture and crossed with a neutral ρ^- strain of the opposite mating type. The resulting zygotes were isolated and tested for respiration-deficient genotype by inoculating into glycerol medium. Crossing of the op_1 cells with the neutral ρ^- cells should result in respiration-competent ρ^+ zygotes capable of growth on glycerol, while respiration-deficient ρ^- zygotes unable to grow on glycerol should be formed from a cross between two ρ^- cells[3]. It is evident from Table III that a considerable part of

TABLE II

ASSIMILATION OF GLUCOSE BY CELLS GROWN IN THE PRESENCE OF ACRIFLAVINE

Cells of wild-type strain DT XII and op_1 mutant DH 1 were cultured and harvested as described in Table I. Fermentation of 5 μmoles glucose which, theoretically, should have furnished 224 μl CO_2 was measured manometrically in N_2. Differences between calculated and measured CO_2 are ascribed to glucose assimilated by the cells.

Strain	*Acriflavine in growth medium (μg/ml)*	*Addition to manometric flasks*	*CO_2 released (μl)*	*Glucose assimilated (%)*
DT XII	0	None	169	24
DT XII	0	50 μM 2,4-dinitrophenol	243	0
DT XII	5	None	201	14
DH 1	0	None	171	24
DH 1	5	None	182	19

TABLE III

OCCURRENCE OF ρ^- VARIANTS IN THE CULTURE OF op_1 CELLS GROWING IN THE PRESENCE OF ACRIFLAVINE

Samples of cells of haploid op_1 strain H 3 C growing in liquid glucose medium with 5 μg/ml acriflavine were taken at different time intervals and mixed with 24-h-old cells of ρ^- strain C 982-19 dA_1. After cultivation of the mixed culture for 24 h, zygotes were isolated and their respiration phenotype determined by differential plating on glucose and glycerol media.

Growth time (h)	*Number of zygotes examined*	*ρ^- zygotes (%)*
10.5	33	18
16	36	25
18	55	36
22	74	18
26	32	3

the op_1 cells could be converted to ρ^- cells in the presence of acriflavine. Even in the absence of the mutagen, cultures of the haploid op_1 strain H 3 C contained some ρ^- cells as proved by the crossing test. If the multiplication ability of op_1 ρ^- cells is suppressed they are still capable, at least in part, of crossing with cells of the opposite mating type.

Since acriflavine treatment of some yeast species also results in cells unable to multiply[12], it was necessary to demonstrate that, in our experiments, this inability was not a general property of the yeast strain from which the op_1 mutants were originally isolated, but that it results from a specific link between op_1 and ρ^- mutation. This was proved by the fact that revertants to wild phenotype, prepared from the op_1 cells by mutagenesis with ultraviolet light, plated on solid media after treatment with acriflavine produced viable respiration-deficient colonies just as the wild-type strain DT XII.

DISCUSSION

The cells carrying the mutated gene op_1, unlike the wild-type cells, lose the ability to multiply after growth for about 10 generations in glucose medium in the presence of acriflavine. They cannot, however, be considered as dead since they (a) did not stain with methylene blue, (b) actively fermented glucose and (c) assimilated a part of the fermentable substrate. In addition, they were able to cross with cells of the opposite mating type. The last argument may be ambiguous, however, since a mass mating technique was employed and only a minute fraction of the cell culture might be able to mate. The same ambiguity holds for the evaluation of the fraction of respiration-deficient ρ^- cells in the total population of the op_1 cells: this might be higher but most of the op_1 ρ^- cells might not cross.

It seems, however, to be established that the loss of the multiplication ability of the mutant after acriflavine treatment was due to the superposition of the cytoplasmic mutation over the nuclear op_1 mutation in a cell. The cell carrying both ρ^- and op_1 mutation, although equipped with fermenting and, at least in part, also with synthetic activity, is incapable of growth or multiplication. Since both mutations were supposed to affect mitochondrial reactions, their new cumulative effect would point out a function of mitochondria related to cell growth or multiplication. Such a function would not simply be connected with an energy supply by aerobic ATP synthesis.

Alternatively, ρ^- or op_1 mutation, although shown to affect mitochondria, may exert an additional effect on extramitochondrial structures or functions involved in cell multiplication, the extramitochondrial effect being hitherto evident only in the double op_1 ρ^- mutant. The pleiotropic effect on mitochondria of ρ^- mutation is well known[16] and has also been suggested for op_1 mutation[7]. WOODWARD AND MUNKRES[17] supposed that cytoplasmic 'mi' mutation in Neurospora, in many aspects similar to ρ^- mutation of yeast, affected both mitochondrial and extramitochondrial structural components.

In this respect, it is important to note that respiration-deficient cells prepared by acriflavine treatment from the so-called 'petite negative' species of yeasts resemble the respiration-deficient mutant of the op_1 strain of *S. cerevisiae* in their inability to multiply continuously[12]. In addition to its effect on oxidative phosphorylation, the op_1 mutation in *S. cerevisiae* might induce a state which is permanently present in wild-

type cells of the petite negative species. An unstable strain of *S. cerevisiae* should also be mentioned in which cytoplasmic respiratory deficient mutants were found to be non reproducing but gained the multiplication ability after a nuclear gene mutation[18].

Assuming that acriflavine induced ρ^- mutation in op_1 cells as effectively as in wild-type cells and that $op_1\ \rho^-$ cells could not multiply, the fact that about 10 generations had elapsed before growth of the op_1 cells in the acriflavine-supplemented medium stopped would be consistent with a dilution model of the cytoplasmic mutation to respiratory deficiency. The model, which considers dilution of cytoplasmic genetic determinants of cells proliferating in the presence of acriflavine, similar to that proposed by several investigators[19,21], will be discussed in another paper.

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