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INDUCTION OF RESPIRATORY DEFICIENCY BY REPRESSION OF THE RESPIRATORY SYSTEM IN A MUTANT OF *SACCHAROMYCES CEREVISIAE*

TERESA NEGROTTI* AND D. WILKIE
Department of Botany, University College, London (Great Britain)
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SUMMARY

Cells of a mutant strain of *Saccharomyces cerevisiae* produce daughter cells with respiratory deficiency of the cytoplasmic petite type when growing under conditions of respiratory repression, that is, in the presence of glucose or under anoxia, but otherwise produce respiratory normal cultures. Apart from inheriting the repression-petite induction character, the latter are normal in other respects such as response to specific mutagens and oxygen uptake under different physiological conditions.

Chloramphenicol, although specifically inhibiting the synthesis of mitochondria-bound cytochromes in this strain, does not induce the petite mutation in cultures growing on a non-repressing sugar.

The character of anoxia or glucose induction (*gi*) of petite appears to be under the control of a recessive nuclear gene and spontaneous reversion to normal takes place with a low frequency. Since petite cells show effective loss of mitochondrial DNA, these results are believed to provide evidence that the replication and/or transmission of the mitochondrial genetic information under respiratory repression is controlled by a system distinct from that operating under respiratory adaptation. In the latter, genetic continuity is probably achieved by auto-reproduction and passive inheritance of intact mitochondria—a mechanism not affected by chloramphenicol, while the former system is dependent on the induced activity of a nuclear gene or genes.

INTRODUCTION

In the facultative anaerobe *Saccharomyces cerevisiae*, repression of the respiratory system by anoxia or glucose, as well as the respiratory adaptation which follows the removal of these conditions, are accompanied by biochemical and morphological changes, the mechanisms of which are not clearly understood. Under glucose repression, aerobically growing cells have very few or no normal mitochondria, but aberrant membranous or vesicular structures are seen. In synchrony with these changes there is a decline in cellular oxygen uptake, low activity of respiratory enzymes and depletion of mitochondrial DNA per cell (JAYARAMAN *et al.*[1]; POLAKIS,

Abbreviation: YEP, 1% yeast extract — 2% Bactopeptone medium.

* Present address: Faculty of Science, University of Buenos Aires, Argentina.

BARTLEY AND MEEK[2]; MOUSTACCHI AND WILLIAMSON[3]). Cells grown anaerobically without a supply of fatty acids have no detectable mitochondria or cytochromes, but shortly after the resumption of aeration, the respiratory system is restored with the development of normal mitochondria and the appearance of characteristic bands for cytochromes *a*, *b* and *c* (SLONIMSKI[4]; LINDENMAYER AND ESTABROOK[5]; WALLACE AND LINNANE[6]). Although depleted, it is clear a system must operate to ensure the reproduction and transmission of the mitochondrial DNA under repression since this unit is generally believed to carry genetic information necessary for mitochondrial synthesis. The reproduction of this unit, assumed in this paper to be synonymous with the cytoplasmic ρ factor (SHERMAN[7]), is relatively inefficient under normal conditions in *S. cerevisiae* and is effectively lost with a frequency of between 10^{-2} and 10^{-3} in most strains (see MOUNOLOU, JAKOB AND SLONIMSKY[8]) to give the irreversible respiratory-deficient mutant known as "petite" (EPHRUSSI[9]). This mutant has aberrant mitochondria lacking both an organized inner membrane (YOTSUYANAGI[10]) and bound enzymes of the respiratory chain (SLONIMSKI[4]; ROODYN AND WILKIE[11]).

The case is described here of a mutant strain in which daughter cells formed under repression (but not otherwise), are all of the petite type. In other words, in this mutant there appears to be a complete breakdown of the replication–transmission mechanism of mitochondrial DNA under repression conditions. There is thus glucose induction (*gi*) and anoxia induction of petite in this strain.

A preliminary account of the *gi* mutant was given at the 2nd International Symposium on Yeast at Bratislava, 1966.

MATERIALS AND METHODS

Strains of S. cerevisiae

Strains from our laboratory stock were used, with their respective numbers and genotypes as follows:

4C1: me_2, le_1, tr_5, ur_3, α.
8C1: hi_6, me_2, tr_1, ly_1, ad_6, ur_1, a.
45: *hi*, *ur*, *a*.
57: ad_2, *a*.

me, *le*, *tr*, *hi*, *ly*, *ur* and *ad*, signify requirement for methionine, leucine, tryptophan, histidine, lysine, uracil, adenine, respectively; a/α = mating type.

Growth media

The normal growth medium consisted of: 2 % Bactopeptone, 1 % yeast extract (YEP) and either 2 % glucose or 4 % glycerol. When sugars other than glucose were used, they were added to the YEP medium to a concentration of 2 %. The pH was adjusted to 5.5 for the galactose medium and to 6 for the maltose one. The pyruvate medium was prepared with 4 % pyruvate. WICKERHAM's medium[12] with 4 % lactate as substrate was used instead of YEP for the oxygen uptake experiments.

Culture conditions

Liquid cultures were grown on a shaker at 30°. No additional aeration was given. Growth on solid medium was also at 30°. Respiratory-competent strains were always precultured on liquid YEP–glycerol until early stationary phase, before

plating on different media. For anaerobic growth, plates were placed in a Fildes cylinder under a nitrogen atmosphere, and kept for 7 days at 30°.

Crossing and sporulation

Auxotrophic parents with different biochemical requirements were crossed on minimal medium where only the resulting heterozygous diploids could grow. Respiratory-competent diploids, selected on YEP–glycerol medium, were sporulated by placing them in thick suspension on solid medium containing 0.3 % sodium acetate, 0.4 % raffinose and 2 % agar (pH adjusted to 6.5).

Tetrad and random spore analysis

Spore tetrads were separated with a De Fonbrunne micromanipulator. Random spore analysis was performed according to the technique of BEAVAN AND WOODS[13]. For further details of these procedures see HAWTHORNE AND MORTIMER[14].

Studies of cell lineages

Stationary-phase, glycerol-grown cells were allowed to bud on YEP–glucose or glycerol solid media at 30°. Daughter cells were removed as soon as they were budded off, using the De Fonbrunne micromanipulator. In some cases buds formed on glucose were transferred to glycerol. Proliferation of each cell to form a colony proceeded on the isolation medium and the respiratory capacity of those isolated on the glucose medium was tested. As in all cases, this was determined in the first instance by ability or inability to utilize the non-fermentable substrate glycerol.

Spectroscopic analysis of cytochromes

This was carried out in the Unicam SP800 spectrometer. Whole cells were analysed against a blank of cell walls (see THOMAS AND WILKIE[15]).

Enzymatic analysis

Activities of NADH, D(—)-lactate, L(+)-lactate and succinate dehydrogenases were determined using the Technicon Auto-analyzer equipment and the method of "Multiple enzyme analysis" (ROODYN AND WILKIE[11]). The tetrazolium test (OGUR, ST JOHN AND NAGAI[16]) for oxidase activity was used on whole colonies.

Irradiation with ultraviolet light

Monochromatic light at wavelength 265 mμ was produced in the Hilger Uvispek with a Siemens Type MB/D mercury lamp and monolayers of cells irradiated according to the method of WILKIE AND LEE[17]. Cell suspensions in distilled water were irradiated under the Philips 6-W T.U.V. discharge tube (95 % output at 254 mμ).

Acriflavin

The dye was added to the medium in appropriate amounts before autoclaving.

Chloramphenicol

Concentrated solutions were made by boiling the antibiotic (as the base) in water before adding to hot medium to give a final concentration of 4 mg/ml.

Oxygen uptake

Oxygen uptake in cultures was measured by means of the oxygen electrode. Cultures were kept in a bath at 30°, vigorously stirred and aerated throughout the experiment. Growth rate was followed by absorbance measurements and cell samples were taken at intervals to determine dry weights.

RESULTS

Isolation of the gi mutant and the glucose effect

The *gi* mutant was isolated from the respiratory normal strain 4C1 on a YEP–glucose medium containing 2 mM cobalt sulphate, which, at this concentration, inhibits the growth of respiratory-competent cells but not that of the cytoplasmic petites (HORN AND WILKIE[18]). Therefore, the proportion of cells growing into colonies on this medium, namely 2 %, corresponded as expected to the spontaneous petite frequency of the 4C1 strain. However, a few of these colonies were not entirely petite but a mixture of petite cells and respiratory-competent cells, the latter growing as a central papilla on a basal mass of petite cells (Fig. 1). That the base was in fact, comprised of petite cells of the cytoplasmic type was verified by (a) inability to utilize non-fermentable substrate, (b) irreversible nature, (c) response to the tetrazolium test, (d) genetic analysis (see below), (e) enzyme analysis (see below).

Fig. 1. Colonies from *gi* cells plated on yeast extract, peptone medium containing 2 % glucose after 4 days incubation. Stages in the emergence of *gi* papillae from the petite base can be seen.

When respiratory-competent cells of the papillae of the mixed colonies were transferred onto YEP–glucose medium, they developed into colonies of petite cells from which central papillae of respiratory-competent cells again began to emerge after the fourth day (Fig. 1). Cells from the papillae always repeated this pattern when plated on YEP–glucose. On YEP–glycerol on the other hand, the colonies that came up were apparently normal in all respects. The papillated condition will be referred to as the *gi* phenotype.

A more detailed analysis of the phenomenon was made in a study of cell lineages (see METHODS). It was found that when *gi* cells are budding on glycerol medium, daughter cells are all respiratory competent, but when they are put down on a glucose medium all cells budded off are petite, including the first bud. A minimum of 4 daughter cells were micromanipulated in each case and a total of 10 cell lineages were analysed. It is likely that resistance to cobalt results at least in part from early production of petite cells on glucose–cobalt plates.

By varying the amount of glucose in the medium, a correlation between the time of emergence of papillae and glucose concentration was established. With concentrations as low as 0.1 %, petite cells were still induced, but papillae emerged after the second day and overgrew the base at this concentration.

A more accurate account of the glucose effect was obtained by analysing *gi* cultures in liquid YEP–glucose medium on a shaker. Cells were sampled at intervals during growth of the culture and the ratio of petite/*gi* determined. This ratio increased exponentially to a maximum, this maximum being proportional to glucose concentration, after which it continued to fall until stationary phase was reached. The interpretation of these findings was that *gi* cells in the presence of glucose bud off mainly or entirely petite cells while retaining the ability to bud off respiratory-normal (but *gi*) cells when glucose repression is removed. This comes about after the proliferating petite cells have utilized all the sugar in the medium or reduced it to a low level.

Galactose-grown colonies of the *gi* mutant did not show the *gi* phenotype. On analysis, these colonies were found to be comprised of *gi* cells and very few petite cells were identified and no more than would be expected on a spontaneous mutation basis. It is known that galactose does not repress the respiratory system (TUSTANOFF AND BARTLEY[19]; POLAKIS, BARTLEY AND MEEK[20]).

Maltose, like galactose, did not induce petiteness in the *gi* strain. However, in one particular plating about 10 % of colonies on a maltose medium showed the *gi* phenotype, although papillae developed very early.

Effect of anoxia on the gi mutant

Respiratory repression by anoxia was studied in the *gi* mutant to see whether, like glucose repression, there was petite induction. A maltose medium was used since the *gi* mutant was found to be unable to grow anaerobically on galactose. The reason for this is not known. It was found that colonies fully grown anaerobically on YEP–maltose had no detectable papillae and that they were comprised almost entirely of petite cells. In the aerobic controls on the other hand, colonies were respiratory normal and comprised of *gi* cells.

It was clear that both glucose- and anoxia-induced petiteness in *gi* cultures, that is, repression of the respiratory system by either of these agencies in the *gi* cells, led to the failure to transmit the mitochondrial genetic information, or at least a functional replica of this unit, thereby inducing the petite condition in daughter cells.

Effect of chloramphenicol on the gi mutant

Chloramphenicol has been shown to block amino acid incorporation into the protein of isolated mitochondria (MAGER[21]; KROON[22]; WINTERSBERGER[23]) and, in intact cells of *S. cerevisiae*, to inhibit specifically the synthesis of particulate respiratory enzymes resulting in a petite phenotype (CLARK-WALKER AND LINNANE[24]). The possibility of this inhibitory action on mitochondrial synthesis having the same effect as repression in the *gi* mutant was investigated.

YEP–glycerol medium containing 4 mg chloramphenicol per ml was first shown to inhibit the growth of *gi* cells. A culture of *gi* cells was then grown to stationary phase on a YEP–galactose–chloramphenicol medium; cell samples were taken and tested (a) for respiratory capacity, and (b) for cytochromes. It was found in analysis

(b) that cells had no detectable cytochromes *a* and *b* when scanned in the recording spectrophotometer while a galactose-grown control culture had a normal spectrum of cytochromes *a*, *b*, and *c*. For the test (a), cells were placed in shaking culture on liquid YEP–galactose for 6 h to dilute out chloramphenicol (see CLARK-WALKER AND LINNANE[24]), washed and plated on YEP–glycerol. More or less all cells developed into colonies (shown subsequently to be *gi* type) showing there had been no petite induction by chloramphenicol. This means that although the antibiotic inhibited the synthesis of mitochondrial proteins in *gi* cells, this was not equivalent to repressive action, since the genetic information was still transmitted.

Relative stability of gi cells to the mutagenic agents ultraviolet light and acriflavin

Irradiation of cells with ultraviolet light increases the mutation rate to petite and at high doses the increase is usually many-fold (RAUT AND SIMPSON[25]; WILKIE[26]). The results of low doses of ultraviolet on *gi* cells and the parent strain 4C1 are given in Table I. At the lowest dose there was no difference in petite induction in the two strains but at the higher doses there were fewer petites among survivors in the *gi* strain and all colonies that came up showed the *gi* phenotype. At the same time it can be seen that *gi* cells are highly susceptible to the killing effect of ultraviolet light which introduces a complication to this aspect of the investigation.

TABLE I

PETITE INDUCTION BY ULTRAVIOLET IRRADIATION AT 265 mμ IN THE *gi* MUTANT AND STRAIN 4C1

Strain	*Ultra-violet dose (min)*	*No. of cells plated* $\times 10^{-2}$	*Total colonies*	% *Kill*	% *Petites*
4C1	0	6	590		1.2
	1	2.7	255	3	2.4
	2	4.5	420	7	3.3
	4	4.8	431	10	3.6
gi	0	4.5	448		1
	1	4.1	384	6	2
	2	3.5	172	50	0
	4	3.5	116	63	0

Acriflavin in low amount can differentially induce cytoplasmic petites in growing cultures of *S. cerevisiae* (see EPHRUSSI[9]). If there is 100% induction among daughter cells by the dye this will show up as inhibition of growth if the substrate is non-fermentable. Fully adapted cells of 4C1 and *gi* were tested on dye-containing YEP–glucose and YEP–glycerol plates. In both strains there was 100% petite induction at a concentration of 0.5 μg acriflavin per ml, that is, there was no growth on the glycerol plates and no *gi* phenotypes on glucose at this concentration of the dye.

It was concluded that there was no difference in the relative stability of the respiratory systems of the 4C1 and *gi* strains to the mutagenic action of ultraviolet light and acriflavin.

Effect of glucose on oxygen uptake in gi cells

The oxygen uptake of fully adapted cells growing on lactate medium was measured throughout the growth cycle of strains 4C1, 45 and *gi*. Glucose was added

to duplicate cultures at mid-log phase to a maximum concentration of 2 % and changes in respiration noted. It was found that equal amounts of glucose produced similar decreases in oxygen uptake in all three strains. In this respect, then, the *gi* mutant was no different to the two respiratory normal strains.

Enzymatic analysis of glucose-induced petite cells of the gi mutant

Cells of the petite base of colonies showing the *gi* phenotype were isolated, cultured and tested in the autoanalyser. They were found to have the same deficiencies in NADH-, L(+)-lactate-, and succinate-ferricyanide reductases and increased D(—)-lactate-ferricyanide reductase associated with known cytoplasmic petite mutants (RODYN AND WILKIE[11]).

Genetic analysis of the gi mutant

The *gi* mutant was crossed to the two respiratory normal strains 8Cl and 45 both on a galactose minimal medium and on a glucose minimal medium. Cells from the resulting diploids were sampled and tested. All diploid cells were respiratory competent and none gave rise to colonies showing the *gi* phenotype. The diploid from the cross *gi* × 45 was sporulated and spores analysed both randomly and in tetrads. Tetrad analysis was incomplete because of low viability of spores. Of twelve tetrads dissected in one particular series, only a total of six spores germinated to give rise to colonies: in a single tetrad two germinated and gave *gi* colonies but only one spore gave a colony in each of four other tetrads and these were all of normal type. From random spore platings it was estimated that the viability of spores was 20 %, and out of a total of 63 haploid colonies analysed on YEP–glucose medium, 11 developed the *gi* phenotype and the remainder were normal. Cells were sampled from the papilla of one of the *gi* colonies and, after plating, gave rise entirely to *gi* colonies demonstrating the stability of the *gi* mutation through the cross to the segregant. On the basis of a recessive nuclear gene controlling the *gi* character, segregation of *gi* to normal among random spores should be 1:1. The deviation obtained (11:52) could be explained by differential viability of normal spores, and support for this hypothesis is provided in experiments described later.

Genetic analysis of the respiratory-deficient cells of the base of the *gi* colony was carried out. The diploid resulting from the cross to strain 45 was respiratory competent and all ascospores tested were likewise respiratory competent. Crosses were also made to a known cytoplasmic petite of strain 8Cl of spontaneous origin and to an acriflavin-induced petite of strain 45. In both cases the resulting diploids were petite indicating that there was the same genetic lesion in the *gi* base cells as in the other petite mutants, namely in the cytoplasmic factor.

Spontaneous reversion to normal in the gi mutant

Although the *gi* mutation was apparently stable and *gi* cells generally gave rise to colonies having the *gi* phenotype on glucose medium, a search was made to find back mutation to normal. This was done by plating cells on YEP–glucose and after two days incubation, subjecting colonies to the tetrazolium chloride test. One colony was found out of a large number tested, to give a positive reaction to the test. This was a true revertant having the nuclear markers of the *gi* strain, and did not transmit the *gi* factor to ascospores in either the cross to strain 45 or to strain 57.

A diploid gi strain

Mitotic recombination in heterozygous diploid yeast results in the homozygous condition and this event is much increased by irradiation with ultraviolet light (see WILKIE AND LEWIS[27]). Diploid cells from the cross *gi* × 45 (presumed to be heterozygous at the *gi* locus) were irradiated in suspension with a ultraviolet dose giving about 50 % survivors and plated on YEP–glucose medium containing 2 mM cobalt sulphate. A total of 23 colonies came up of an estimated 2000 cells plated. 20 of the colonies were wholly petite but the remaining 3 were variously papillated (see HORN AND WILKIE[18]). When tested on YEP–glucose one of the three showed the *gi* phenotype, and was considered to be a mitotic recombinant homozygous at the *gi* locus. This diploid was sporulated but out of 10 tetrads dissected, not one ascospore was viable thus confirming the earlier hypothesis that the *gi* factor in some way adversely affects spore viability.

DISCUSSION

The genetic evidence suggests that a recessive nuclear gene controls the *gi* character. From the various comparative tests it appears that the *gi* strain is mutant only in respect to the genetic continuity of the mitochondrion under respiratory repression. This leads to the fundamental point that a nuclear factor or, more likely, nuclear factors of which the *gi* gene is one, function in a system for perpetuating mitochondrial DNA as soon as repression conditions are applied and only then. Under conditions inducing respiration this system does not operate and genetic continuity is probably achieved by autoreproduction of intact mitochondria with passive inheritance of the organelle. This system is unimpaired by chloramphenicol activity.

In electron microscope studies with cells of *S. cerevisiae*, YOTSUYANAGI[28] finds that glucose-repressed cells elaborate a membranous structure in the mitochondrion and he postulates this may be an attachment site for mitochondrial DNA in analogy with the bacterial mesosome. It is possible the *gi* gene is involved in specifying a protein which becomes attached to the DNA not only resulting in the repression of this unit, but also rendering it stable and reproducible as a DNA–protein entity under respiratory repression. Thus glucose and anoxia could act as inducing agents in the first instance with the repression of respiratory enzyme synthesis as a secondary effect. With mutation of the *gi* gene the system would not be elaborated but the deficiency would be detected only when repression was operating.

This mutant is of obvious importance in problems of mitochondrial continuity in the facultative anaerobe and at least one further field of investigation would be the electron microscopy of *gi* cells entering into a repression phase.

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