Properties of a Mitochondrial Suppressor Mutation Restoring Oxidative Phosphorylation in a Nuclear Mutant of the Yeast *Schizosaccharomyces pombe*

(Received for publication, November 11, 1976, and in revised form, May 2, 1977)

FRANÇOISE LABAILLE, ANNE-MARIE COLSON, LÉON PETIT, AND ANDRÉ GOFFEAU

From the Laboratoire d’Enzymologie, Université Catholique de Louvain, Place Croix du Sud, 1, 1348 Louvain-la-Neuve, Belgium

The growth in glucose of the nuclear pleiotropic respiratory deficient mutant pet1 of the "petite-negative" yeast *Schizosaccharomyces pombe* is limited to a few cell generations after the addition of either 0.2 μM bongkrekic acid or 0.1 μM antimycin to the growth medium. The mutation sup-2, which is of extra nuclear heredity, restores totally the resistance to both inhibitors for growth in glucose. Even though the cellular respiration and the content of cytochromes absorbing at 555.7, 560.5, and 605.8 nm at 77 K were almost totally restored in raffinose-grown pet1 sup-2, the growth on glycerol is not restored in this strain.

The oxidative phosphorylation and other mitochondrial activities such as oligomycin-sensitive ATPase, NADH-cytochrome c reductase, and cytochrome c oxidase activities, which are deficient in pet1, are 30 to 70% restored in pet1 sup-2 grown in 1% glucose or 10% raffinose.

A pronounced repression of respiration is observed when pet1 sup-2 is grown in 10% glucose. Under these conditions, pet1 sup-2 as well as pet1 exhibit a small but significant resistance to antimycin-sensitive respiration. Addition of antimycin to these cultures, while inhibiting the antimycin-sensitive respiration, allows continued expression of an antimycin-insensitive respiration, the rate of which is lower in pet1 (Q_o2 = 1.3 μl of O_2 x min^-1 x mg^-1, dry weight) than in pet1 sup-2 (Q_o2 = 2.8). This respiration is unusual in that it is insensitive to cyanide and to hydroxamates but sensitive to azide. l-Malate as well as glucose may be utilized as respiratory substrate with both cytochromes b_{558} and b_{560} (77 K) being reduced in the presence of antimycin.

Subsequent addition of azide oxidizes both cytochromes b even in the presence of cyanide plus antimycin, indicating oxidation-reduction equilibrium with a terminal antimycin and cytidine-insensitive oxidase.

It is concluded that continuous cellular multiplication of *S. pombe* requires a critical level of intramitochondrial ATP which can be supplied either by oxidative phosphorylation or by the influx of the extramitochondrial ATP. In addition, a low but critical level of oxidations (Q_o of about 2.0) is required which can be supplied by any combination of antimycin-sensitive and antimycin-insensitive respirations.

Regulation of the coordinated synthesis and the assembly of the components of the inner mitochondrial membrane is poorly understood. A possible approach to study this phenomenon is to investigate situations in which this regulation is upset. This might be the case in pleiotropic yeast mutants where one single-gene nuclear mutation produces several protein deficiencies in the inner mitochondrial membrane. Since their first observation by Sherman and Sionimski in 1964 (1), several nuclear pleiotropic respiratory-deficient mutants deficient in a set of proteins (such as cytochrome aa_3, cytochrome b, and oligomycin-sensitive ATPase) containing mitochondrially synthesized peptides have been described in *Saccharomyces cerevisiae* (2-9) and *Schizosaccharomyces pombe* (10-15). In *S. cerevisiae*, these nuclear pleiotropic respiratory-deficient mutants do often exhibit a high frequency of spontaneous induction of mitochondrial ρ- mutations which complicates their genetic and biochemical study (1, 4, 6, 9). In contrast, no viable ρ- can be induced in the "petite-negative" yeast *S. pombe* (10, 16, 17). This property has greatly facilitated the study of the nuclear pleiotropic mutant *S. pombe* pet1 which was previously reported to be deficient in cytochrome aa_3, cytochrome b, and the oligomycin-sensitive ATPase (13). The present paper describes two additional characteristics of pet1. Its growth on glucose is inhibited by addition of either antimycin, a respiratory chain inhibitor (18) or of bongkrekic acid, an inhibitor of mitochondrial translocation of adenine nucleotides (19). Both traits are suppressed by the introduction into pet1 of the sup-2 mutation. The latter mutation was recently reported to be of mitochondrial heredity (20). We describe here that the suppressor mutation largely restores the deficient oxidative phosphorylation of pet1. With the possible exception of a rather similar situation in *Paramecium* recently reported (21), such mitochondrial-nuclear interaction has not been recognized so far. Furthermore, investigation of the effects of the suppressor mutation on the sensitivity of respiration to antimycin leads to the description of a new type of "alternative" respiration which is characterized by sensitivity to azide and by insensitivity not only to cyanide and antimycin but also to hydroxamates.

* This work is Publication 1401 of the EURATOM Biology Division.
† EURATOM Grantee.
§ Chercheur Fonds Recherche Fondamentale Collective.
MATERIALS AND METHODS

Yeast Strains—Schizosaccharomyces pombe 972h- is the wild strain from which the chromosomal pleotropic respiratory-deficient mutant previously referred to as M196 has been isolated (19, 13). This mutant has been reconstituted pet-l26. The strain pet-l26 sup-126 contains a spontaneous deletion of the pet1 gene and is auxotrophic for methionine, adenine, and uracil. To construct the temperature-sensitive mutants, 10 cells from a preculture were inoculated into 7 ml of medium with and without 10 mM imidazole/HCl, pH 4.5. The cultures were grown on a rotary shaker at 30 °C. At the end of exponential growth, 0.5 g of glucose/100 ml was added to the medium and the growth was continued for 15 h with aeration at 30 °C. The cultures were centrifuged at 10,000 x g for 15 min. All cultures were inoculated into 10 cells/ml from an actively growing preculture, and grown aerobically at 30 °C.

Growth Conditions—Unless otherwise indicated, the culture medium contained 3 mg of glucose (or raffinose), 2 g of yeast extract/100 ml and HCl to bring to pH 4.5. All cultures were inoculated with 10 cells/ml from an actively growing preculture, and grown aerobically at 30 °C on a rotary shaker at 50 rounds/min. The cell density was estimated with a Thomas haemocytometer.

Preparation of Mitochondrial Fractions and of Isolated Mitochondria—About 10 g wet weight of cells were suspended at 4 °C in 2 volumes of 0.6 M sorbitol, 1 mM ethylenediaminetetraacetic acid, 50 mM Tris/acetate, pH 7.5. The suspension was homogenized for 45 s with 2 volumes of glass beads, 0.45 to 0.55 mm in diameter, in a refrigerated Braun cell homogenizer. The homogenate was centrifuged at 1000 x g for 5 min. The supernatant was centrifuged at 25,000 x g for 20 min and the final pellet was suspended in 1 volume of 0.6 M sorbitol, 50 mM Tris/acetate, pH 7.5, and centrifuged at 25,000 x g for 20 min and the final pellet was suspended in 1 volume of 0.6 M sorbitol, 50 mM Tris/acetate, pH 7.5.

When the ratios of ATP/oxgen were determined, mitochondria were isolated from proplastoplasts. In this case, the cells were grown on 1 g of glucose, 1 g of yeast extract/100 ml, and HCl to bring to pH 4.5. The following procedure modified from that of Foury and Goffeau (22) was used. Two liters or 1.5 liters of culture medium were inoculated with 10 cells/ml and incubated at 30 °C in 6-liter Erlenmeyer flasks on a rotary shaker at 120 rounds/min. After 19 h of growth, 0.5 g of glucose/100 ml was added to the medium and the culture was further incubated for 30 min. A final concentration of 1.5 mM 2-deoxyglucose was then added to the culture and the cells were collected 10 min later by centrifugation at 3500 x g for 5 min and washed twice with 25 mM potassium phosphate buffer, pH 6.5, and centrifuged at 25,000 x g for 20 min and the final pellet was suspended in 1 volume of 0.6 M sorbitol, 50 mM Tris/acetate, pH 7.5.

The enzymatic digestion was stopped by centrifugation at 1250 x g for 3 min when approximately 95% of the cells of an aliquot were disrupted in hypo-osmotic 0.3 M sorbitol. The spheroplasts were suspended at 4 °C and washed twice in 1.3 M sorbitol, 50 mM Tris/acetate, pH 7.5. The pellet of washed spheroplasts was suspended in 5 μl of 0.3 M sorbitol, 50 mM Tris/acetate, pH 7.5, 1% bovine serum albumin, pH 6.4, and submitted to vigorous magnetic stirring for 5 min. One volume of 1 M sorbitol, 10 mM imidazole/acetate, 1 mM ethylenediaminetetraacetic acid, 0.1 g of bovine serum albumin/100 ml, 25 mM potassium phosphate buffer, pH 6.4, was added and the suspension was centrifuged at 1,250 x g for 5 min. The supernatant was centrifuged at 15,000 x g for 15 min, giving a pellet which was suspended in a few ml of 0.6 M mannitol, 10 mM imidazole/HCl, 0.1% bovine serum albumin, pH 6.4, and centrifuged again at 1250 x g for 5 min. The latter supernatant was centrifuged at 15,000 x g for 15 min and used for the study of isolated mitochondria.

Production of NADPH, stoichiometric to the ATP content, was followed by NADP reduction using 0.1 ml of 10 mM NADPH. The reaction mixture contained 0.1 ml of the mitochondrial preparation, 10 mM Hepes buffer of pH 7.5, and 0.1 ml of 10 mM NADP. The reaction was followed by an absorption index of 19.2 mm -1 cm -1 for cytochrome c.

Cytochrome c oxidase (ferrocytochrome c oxidoreductase (EC 1.9.3.1)) was measured at room temperature by decrease in the absorbance at 550 nm due to the reduction of cytochrome c. One-milliliter cuvettes contained 0.1 ml of potassium phosphate buffer, pH 7.2, 0.5 x 10 -3 M NADH, 1 mg/ml of cytochrome c, and mitochondrial proteins (0.016 mg for the wild type, 0.058 mg of pet-l26, and 0.16 mg for pet-l). The reaction was carried out at 30 °C and started by addition in the sample cuvette of 10 mM succinate, pH 7.2, or 0.1 M NADH. The specific activity is expressed in nanomoles of cytochrome c oxidized per minute of protein using an absorbance index of 19.2 mm -1 cm -1 for cytochrome c.

Cytochrome c oxidase (EC 1.9.3.1) was measured at room temperature by decrease in the absorbance at 550 nm due to the reduction of cytochrome c. The reaction mixture contained 0.1 ml of potassium phosphate buffer, pH 7.2, 5 x 10 -3 M cytochrome c reduced at 96% by ascorbate. The reaction was started by addition of mitochondrial proteins to the sample cuvette. The activity of cytochrome c oxidase is defined by the first order velocity constant in mm -1 x mg -1 of proteins.

ATPase activity (ATP phosphohydrolase EC 3.6.1.3) was measured at 30 °C for 8 min in a total volume of 1.0 ml containing 3 mM ATP, 50 mM Tris/HCl, 6 mM MgCl 2, 4 mM phosphoenolpyruvate, 5 μl of an (NH 4 ) 2 SO 4 suspension of pyruvate kinase (EC 2.7.1.40) containing 15 IU. The final pH of the reaction mixture was 9.0. Five microliters of 0.8 mg/ml of oligomycin were added to one sample while 5 μl of methanol were added to the reference. The difference between the two activities is the oligomycin-sensitive ATPase. The reaction was started by addition of 50 to 100 μg of proteins to the reaction mixture, and stopped by 3 ml of 10% sodium dodecyl sulfate. Inorganic phosphate was measured as described previously (13). The ATPase activity is expressed in micromoles of phosphate liberated per min per mg of proteins.

Protein Determination—the methods of Lowry et al. (23) or of Waddel (24) were used with bovine serum albumin as standard.

Chemicals—The yeast extract from Difco, Antimycin A was from Boehringer and used as a methanolic solution. Bongkrekic acid was a gift from Professor Berends, Technological University of Delft. The stock solution was kept in 2 N NaOH and diluted with methanol. Oligomycin was from Sigma and was dissolved in methanol. Zymolyase-5000 was from Kaken Chemical Co., Ltd, Tokyo, Japan. β-Gluconoylase/aryl sulfatase as well as hexokinase, glucose-6-phosphate dehydrogenase and pyruvate kinase were from Boehringer Mannheim.

RESULTS

Effects of Bongkrekic Acid and Antimycin on Growth in Glucose—Fig. 1A shows that concentrations as low as 0.4 μM bongkrekic acid added to the culture medium containing 10% glucose limit the growth of the nuclear respiratory-deficient mutant pet-l to four generations compared to nine generations in the absence of inhibitor. Table I shows that the growth yield in cells/ml from an actively growing preculture, and grown aerobically at 30 °C.

Mitochondrial Suppressor of a Yeast Nuclear “Petite” 5717
addition of 0.18 μM antimycin to the culture medium, the cellular growth yield is decreased by further addition of bongkrekic acid, even though antimycin alone has no effect on cell multiplication. Similar situations have been described for Saccharomyces cerevisiae and have been interpreted as indicating the existence of a requirement for cell division of an unknown metabolite or process which is diluted out when the intramitochondrial ATP supply is limited (25). The sensitivity to bongkrekic acid of the growth on glucose of pet1 provides thus a convenient screening test to select suppressor mutations such as pet1 sup-2 able to grow on glucose in the presence of bongkrekic acid (Table I).

In addition to the inhibition by bongkrekic acid, the cellular division in glucose of the respiratory-deficient mutant pet1 is sensitive to the presence of antimycin in the culture medium. Fig. 1B shows that the addition of 0.1 μM antimycin to the culture medium limits the growth of pet1 in glucose to six generations compared to nine for the wild type. In the same conditions, the strain pet1 sup-2 behaves like the wild type.

Table I also demonstrates marked synergistic effects of the combination of bongkrekic acid and antimycin in all strains. We therefore conclude that both inhibitors reach their intracellular target and that the suppressions of sensitivity to bongkrekic acid or antimycin in pet1 sup-2 is not due to a modification of the cellular permeability or to a modification of the (intracellular) inhibitors binding sites.

The antimycin resistance of the growth in glucose of pet1 sup-2 was used to demonstrate that the sup-2 gene is of extranuclear (and probably mitochondrial) heredity (20). The resistance to antimycin given by sup-2 must not be confused with the ANT<sup>+</sup> trait recently described in S. pombe (26). The latter gene is also of mitochondrial heredity but is expressed by resistance to antimycin of the growth in glycerol while the suppressor mutations here described confer resistance of the growth in glucose but not that in glycerol (20).

Oxidative Phosphorylation in Wild Type—Suppression of bongkrekic sensitivity in pet1 sup-2 suggests that in this strain, the mitochondria are independent of the cytosol for the supply of their intramitochondrial ATP and therefore that they are able to carry out oxidative phosphorylation. Until now it was not possible to measure mitochondrial ATP/oxygen in S. pombe because of the lack of availability of a method for isolation of intact mitochondria. We have derived, therefore, a new method to prepare S. pombe proplasmoplasts, rendering the cell wall fragile by addition of 2 deoxy-d-glucose to glucose-growing cells. These proplasmoplasts were further treated with a mixture of small gut and Arthrobacter enzymes as described under "Materials and Methods." After osmotic lysis, fairly intact mitochondria with a respiratory control of 2.6 for NADH (see Fig. 2) and 1.3 for α-glycerophosphate were isolated from the wild type. Respiratory controls of 2.6, 2.4, 2.3, and 1.8 were obtained with α-ketoglutarate, citrate, malate, and succinate, respectively, provided that 1.6 mm pyruvate (which alone is poorly oxidized) was added to each of the above substrates (data shown for succinate in Fig. 2). Table II demonstrates that ATP/oxygen ratios between 1.70 to 1.95 were observed for the wild type mitochondria oxidizing NADH, succinate, malate, or citrate. These values are similar to those reported for S. cerevisiae where the phosphorylation Site I is absent (27). The high antimycin-sensitive oxidation rate of externally added NADH and its ATP/oxygen value of 1.75 suggest that, like in S. cerevisiae (28), an external NADH dehydrogenase is directly hooked to the second phosphorylation site in the inner mitochondrial membrane of S. pombe. On the other hand, the rather low ATP/oxygen ratios obtained with α-ketoglutarate are unexplained. Substrate level phosphorylation, however, is likely to be present because the oligomycin-insensitive ATP formed during the oxidation of α-ketoglutarate is significantly higher than that with external NADH (Table II).

### Table I

| Addition                          | pet1 sup-2 | pet1 + sup-2 |
|----------------------------------|------------|--------------|
| Cell density after 48 h          | 972 cells/ml | 1000 cells/ml |
| 0.5% Methanol                    | 382        | 325          | 394        |
| 0.85 μM Bongkrekic acid          | 340        | 10           | 374        |
| 0.18 μM Antimycin                | 442        | 75           | 276        |
| 0.18 μM Antimycin + 0.80 μM      | 125        | 3            | 10         |
| μM bongkrekic acid               |            |              |            |

### Oxidative Phosphorylation in Mutants—Table II shows that no appreciable oxidations or phosphorylations are detected in pet1 mitochondria and that both oxidations and phosphorylations are very significantly restored in pet1 sup-2. The phosphorylation associated with the oxidation of α-ketoglutarate is largely oligomycin-insensitive in pet1 sup-2 suggesting that the substrate level phosphorylation is also functioning. It must be noted that no endogenous mitochondrial ATP is detected in any of the strains when no substrates are added.

Presence of Antimycin-insensitive and Antimycin-sensitive Respirations—Table III shows that a small antimycin-sensitive respiration (Q<sub>0.2</sub> = 0.8) is observed in pet1 grown in highly repressive conditions, such as exponential growth in 10% glucose. Under the same conditions, the rate of antimycin-sensitive respiration of pet1 sup-2 is significantly higher (Q<sub>0.2</sub> = 1.6).

In addition, Table III shows that S. pombe develops an antimycin-insensitive respiration under conditions where the antimycin sensitive respiration is reduced. The rates of the antimycin-insensitive respiration of pet1 sup-2 (Q<sub>0.2</sub> = 2.8) is significantly higher than that of pet1 (Q<sub>0.2</sub> = 1.3). Table IV shows that the antimycin-insensitive respiration observed in the mutants is not inhibited by 3 mm salicyldihymanic acid, a typical inhibitor of the so-called "alternative-respiration" observed in many organisms and plant mitochondria (29-34).
Mitochondrial Suppressor of a Yeast Nuclear "Petite"

Fig. 2. Oxidation of NADH and succinate in the presence or absence of pyruvate in isolated mitochondria from Schizosaccharomyces pombe 972h. The oxidation rates were measured as described under "Materials and Methods" using 3.6 mg of protein. When indicated, 3 mM NADH, 16 mM succinate, and 1.6 mM pyruvate were added. Mitoplast.

Table II
Oxidation rates and phosphorylations by mitochondria isolated from Schizosaccharomyces pombe strains

The mitochondria were isolated and the ATP/O oxygen ratios were measured as described under "Materials and Methods." For each respiratory substrate, except NADH, 1.6 mM pyruvate was added. The respiratory rates were measured in the presence of ADP and phosphate. No endogenous ATP was detected in the absence of substrates. In each assay, 1 to 3.0 mg of protein were used.

| Addition                  | 972h   | pet1   | pet1 sup-2 |
|---------------------------|--------|--------|------------|
|                           | nmol O2 ATP/O x min⁻¹ | nmol O2 ATP/O x min⁻¹ | nmol O2 ATP/O x mg⁻¹ |
| 16 mM NADH                | 90.0   | <0.5   | <0.05      | 16.100 |
| 16 mM NADH + 100 µg of NADH | 50.0   | n.d.   | n.d.       | n.d. |
| 23 µM α-Ketoglutarate     | 25.0   | <0.5   | <0.05      | 6.45  |
| 35 µM α-Ketoglutarate + 100 µg of oligomycin | 25.0 | <0.5 | <0.05 | 6.15 |
| 35 mM Malate              | 45.0   | <0.5   | <0.05      | 16.90 |
| 16 mM Succinate           | 45.0   | <0.5   | <0.05      | 16.100 |
| 16 mM Citrate             | 30.0   | <0.5   | <0.05      | 10.80 |

* n.d.: no measurements carried out.

Furthermore, while the "alternative respirations" described so far are insensitive to NaN₃ (33), it appears that the antimycin-insensitive oxygen uptake of S. pombe is inhibited by NaN₃. As illustrated in Fig. 3, the levels of sensitivity to azide of the antimycin-sensitive and antimycin-insensitive respirations are markedly different. Half-inhibition of the first one requires 7.5 µM azide compared to 175 µM for the latter. We conclude that cytochrome oxidase, which is sensitive to low concentrations of azide, is not involved in the antimycin-insensitive respiration. This is supported by the observation that 1 mM KCN which totally inhibits the respiration of the wild type is not more inhibitory than antimycin in the mutants.

The development of an antimycin-insensitive respiration is not specifically dependent on the pet1 mutations since Table III shows that an antimycin-insensitive respiration also develops in the wild type grown in 10% glucose when the antimycin-sensitive respiration has been blocked by the presence of antimycin during the growth as well as during the harvest and the respiration measurements. Under these conditions also, the

Table III
Antimycin-sensitive and antimycin-insensitive respirations in Schizosaccharomyces pombe mutants

Cellular Qₒ values were measured in the presence of 320 mM glucose and in the presence and absence of 0.18 µM antimycin as described under "Materials and Methods." The cells grown in 10% glucose were harvested after 14 h and those in 10% raffinose were grown for 24 h. The cells were harvested, washed twice with cold distilled water and used immediately for respiratory measurements. When grown in the presence of 0.18 µM antimycin, the harvest and the respiratory measurements were also carried out in the presence of 0.18 µM of antimycin. The limit of sensitivity of the oxygen uptake measurements is estimated to be about 0.1 µl of O₂/h·mg dry weight. The antimycin-insensitive respiration is the oxygen uptake measured in the presence of 0.18 µM antimycin. The antimycin-sensitive respiration is the difference of oxygen uptakes obtained in the presence and absence of 0.18 µM antimycin.

| Growth conditions | 972h | pet1 | pet1 sup-2 |
|-------------------|------|------|------------|
|                   | µl O₂ x h⁻¹ x mg⁻¹ | dry weight |
| 10% Glucose       | 19.1 | <0.1 | 0.8        | 1.3 | 1.6 | 2.8 |
| 10% Glucose + 0.18 µM antimycin | 0.8 | 1.3 | 1.6 | 2.8 |
| 10% Raffinose     | 37.0 | <0.1 | 3.9 | 1.3 | 23.0 | <0.1 |

Table IV
Effects of respiratory inhibitors on oxygen intake of Schizosaccharomyces pombe wild type and mutants

The strains were grown for 14 h on 10% glucose, 2% yeast extract, pH 4.5. The Qₒ values of washed cells were measured immediately after harvest as described under "Materials and Methods" except cells were preincubated for 6 min at 30° with the inhibitors in the respiratory medium.

| Inhibitors                  | 972h | pet1 | pet1 sup-2 |
|-----------------------------|------|------|------------|
| None                        | 18.3 | 2.1  | 4.4        |
| +0.18 µM Antimycin          | <0.1 | <0.1 | <0.1       |
| +0.5 mM NaN₃                | <0.1 | <0.1 | <0.1       |
| +1 mM KCN                   | n.d. | 1.2  | 2.2        |
| +0.18 µM Antimycin + 3 mM salicylhydroxamate | <0.1 | 1.7 | 2.8 |
| +0.18 µM Antimycin + 1 mM KCN | <0.1 | 1.6 | 2.5 |
| +0.18 µM Antimycin + 0.5 mM NaN₃ | <0.1 | <0.1 | <0.1 |

Qₒ of pet1 is lower than that of the wild type and pet1 sup-2. 1.3 compared to 2.3 and 2.4, respectively.

Glucose Repression of Expression of Suppressive Mutation – Up to now, we have considered experimental conditions in which the strains were submitted to glucose repression. The expression of sup-2, however, is much more pronounced in nonrepressive conditions. Table III shows that, when the wild type is grown in 10% raffinose for 24 h, the antimycin-sensitive cellular Qₒ reaches 37.0, compared to 19.1 for exponential cells in 10% glucose. The mutant pet1 does derepress significantly when grown on raffinose, but the rate of its antimycin-sensitive respiration remains low (Qₒ = 3.9). When grown in 1% glucose, the antimycin-sensitive Qₒ of pet1 is even lower (Qₒ = 1.2) which explains why no appreciable oxidative phosphorylation was observed in mitochondria isolated from pet1
Mitochondrial Suppressor of a Yeast Nuclear "Petite"

cells grown under these conditions. On the other hand, pet1 sup-2 derepresses markedly when grown in raffinose since its antimycin-sensitive $Q_0$ reaches 23.0 compared to 1.6 in 10% glucose. It also becomes clear that when the antimycin-sensitive respiration reaches a sufficient level, as in wild type in glucose or raffinose or pet1 sup-2 in raffinose, the antimycin-insensitive respiration disappears.

Restoration of respiration in pet1 sup-2 under nonrepressive conditions is confirmed by mitochondrial enzyme-activity measurements. Table V shows that when isolated from raffinose-grown cells, the mitochondrial succinate:cytochrome c reductase and oligomycin-sensitive ATPase of pet1 sup-2 reach about one-third of the wild type activities, while the NADH:cytochrome c reductase and cytochrome c oxidase activities of the suppressed strain reach about 75% that of the wild type. Under the same growth conditions, these activities are barely detectable in pet1.

The pronounced restoration of mitochondrial activities in pet1 sup-2 is further illustrated by Fig. 4, showing absorption spectra of mitochondria isolated from raffinose-grown stationary phase cells. At liquid nitrogen temperature, the wild type exhibits the cytochrome $c$ absorption bands ($a_2 = 548.7$ nm and $a_1 = 543.8$ nm), two cytochrome $b$ bands absorbing at 553.3 and 560.5 nm and a cytochrome $aa_3$ band at 605.8 nm. The cytochrome $e_1$ absorbing at 550.5 nm is masked by cytochrome $c$ (see Ref. 35 for discussion of mitochondrial pigments in S. pombe). The cytochromes $b$ and $c$ are very low in pet1. On the other hand, the pet1 sup-2 absorption spectrum is similar to that of the wild type; the 553.3, 560.5, and 605.8 nm peaks being even more prominent in the suppressed strain than in the wild type.

Involvement of Cytochromes $b$ in Antimycin-insensitive Respiration - Antimycin-insensitive respiration rates of about 2.0 $\mu$L of $O_2$ h$^{-1}$ mg$^{-1}$ dry weight, were elicited not only by glucose but also by L-malate as respiratory substrates for wild type cells grown in glucose plus antimycin. Under the same conditions, oxalacetate, succinate, fumarate, citrate, pyruvate, L- and D-lactate, $\alpha$-ketoglutarate, glyceral, $\alpha$-glycerophosphate, ethanol, formate, acetate, glutamate, aspartate, $\beta$-hydroxybutyrate either were oxidized poorly or not at all. Fig. 5 (b and d) shows that the onset of antimycin-insensitive respiration by L-malate or glucose in wild type cells grown in glucose plus antimycin produces a marked increase of absorbance at about 554.5 and 560 nm (77 K). These absorption peaks

---

**Table V**

Mitochondrial enzymatic activities in Schizosaccharomyces pombe wild type and respiratory deficient mutants grown in nonrepressive conditions

The strains were grown for 48 h in 100- or 250-ml Erlenmeyer flasks containing 10 or 25 ml of 10% raffinose and 2% yeast extract, pH 4.5. The mitochondrial fractions were obtained after mechanical grinding and their enzymatic specific activities were measured as described under "Materials and Methods."

| Enzymatic activities                      | 972h | pet1 | pet1 sup-2 |
|-------------------------------------------|------|------|------------|
| Succinate:cytochrome c reductase (nmol x min$^{-1}$ x mg$^{-1}$) | 340  | 24   | 111        |
| NADH:cytochrome c reductase (nmol)        | 900  | 31   | 682        |
| Cytochrome c oxidase (nmol x min$^{-1}$ x mg$^{-1}$) | 5.6  | 1.0  | 4.4        |
| Oligomycin-sensitive ATPase (mU)          | 15/0 | 5    | 1/4        |
|                                           |      |      |            |

---

**Fig. 3.** Inhibitions by sodium azide of oxygen uptake of Schizosaccharomyces pombe 972h- grown in glucose with and without antimycin. S. pombe 972h- was grown for 14 h in 1-liter Erlenmeyer flasks containing 100 ml of 10% glucose, 2% yeast extract, pH 4.5, supplemented (□) or not (○) with 0.18 $\mu$L antimycin. The oxygen uptake was measured in the presence of 320 mM glucose and 0.1 M phthalate, pH 4.5, as indicated under "Materials and Methods." $I_{az}$ is the concentration of azide giving 50% inhibition.

**Fig. 4.** Differential absorption spectra of mitochondrial fractions isolated from Schizosaccharomyces pombe wild type and mutants grown in nonrepressive conditions. Dithionite-reduced minus oxidized spectra of mitochondrial fractions prepared by mechanical grinding were carried out at liquid nitrogen temperature in 2-mm cuvettes with the Aminco DW2 spectrophotometer. The strains 972h- and pet1 sup-2 were oxidized with molecular oxygen. To oxidize pet1, 2 mm potassium ferricyanide was used. The slit width was 1.5 nm for 972h- and pet1 and 0.5 nm for pet1 sup-2. The scan speed was 1.0 nm x s$^{-1}$ and the chart speed was 25 nm x inch$^{-1}$. The protein concentrations were 6.3 mg x ml$^{-1}$ for 972h-, 5.8 mg x ml$^{-1}$ for pet1 sup-2, and 8.2 mg x ml$^{-1}$ for pet1.
Mitochondrial Suppressor of a Yeast Nuclear “Petite”

Fig. 5. Absorption spectra of Schizosaccharomyces pombe cells grown in glucose in the presence of antimycin. Schizosaccharomyces pombe 972b was grown for 14 h in 1% glucose supplemented with 0.18 μM antimycin. The cells were harvested and washed in the presence of 0.18 μM antimycin and their respiration was immediately measured for 5 min with a Clark electrode in a 3-ml vessel containing 30 mg dry weight of yeast cells, 0.1 M phthalate, pH 4.5, 0.18 μM antimycin, and the following components when indicated: a, no addition (endogenous);  Êm = 0.4; b, 320 mM glucose;  Êm = 2.0; c, a few grains of dithionite; d, 320 mM l-malate,  Êm = 2.1; e, 320 mM l-malate for 5 min, followed by 0.5 mM KCN for 3 min; f, 320 mM l-malate for 5 min, followed by 0.5 mM KCN for 3 min, followed by 0.5 mM NaNO₃ during 3 min.  Êm < 0.1. The total contents of the Clark electrode vessel were centrifuged for 15 s in an Eppendorf microfuge. The pellets were quickly resuspended in 0.4 ml of their respective respiratory medium supplemented with 50% sucrose, transferred in a 2-mm cuvette, and frozen in liquid nitrogen. The spectra were taken with Aminco DW2 spectrophotometer in dual wavelength the reference being set at 575 nm. The slit width was 1.0 nm and the scan speed was 0.5 nm/s.

are not at the exact same wavelengths as the cytochrome b3331 and b3333 observed in mitochondria isolated from raffinose-grown cells. However, it seems reasonable to assume that these two cytochromes b which were clearly induced by the sup-2 mutation are at least partly responsible of the spectral changes observed during the antimycin-insensitive respiration. Furthermore, oxidation of the two cytochromes b was induced by azide, even in the presence of both cyanide and antimycin (Fig. 5 Trace f). It is also obvious from Trace c and e that the bulk of cytochrome c absorbing at 547 nm becomes oxidized when dithionite or KCN are present and therefore is largely oxidized when the antimycin-insensitive respiration is operating (as in Traces b or d).

These observations can be explained as follows. In the presence of antimycin, the two cytochromes b (but not cytochrome c) are in oxidation-reduction equilibrium with the "alternative-oxidase." The latter is operating slowly, so the two cytochromes b are reduced during the antimycin-insensitive respiration (Traces b and d). One also has to postulate that azide blocks the pathway on the substrate side of the cytochromes b in order to explain their oxidation when azide is added (Trace f).

DISCUSSION

Pet1 is a single-gene nuclear mutant of Schizosaccharomyces pombe which does not grow on glycerol (12, 13). When grown on glucose, pet1 exhibits marked deficiencies in several enzymes containing products of the mitochondrial protein synthesis. No significant oligomycin-sensitivity of the ATPase can be detected, and cytochrome c oxidase and cytochrome b activities are markedly decreased when compared to the wild type under similar conditions. The mitochondrial protein synthesis, however, is functioning in pet1 since the cycloheximide-resistant incorporation of L-[3H]leucine is inhibited by chloramphenicol and since the growth of pet1 in glucose is markedly reduced by the addition of chloramphenicol and ethidium bromide to the growth medium (13, 35). However, the mitochondrial protein synthesis of pet1 is leaky, especially for the high molecular weight products (36) rather similarly to what has been reported for the nuclear pleiotropic respiratory-deficient mutants pet936 and N9-168 of Saccharomyces cerevisiae (4, 9). A working hypothesis concerning the primary gene-deficient products of pet1 and of the suppressor mutation has been presented recently (36). It was postulated that pet1 is modified in one of the numerous nuclear-coded proteins of the mitochondrial protein synthesis machinery. The resulting leaking mitochondrial protein synthesis would be particularly abnormal for the high molecular weight components and would be partly corrected by mitochondrial mutations affecting either ribosomal or transfer mitochondrial RNA.

This paper describes two new properties of pet1. Its growth on glucose is restricted by the presence of bongkrekic acid or antimycin in the culture medium. These phenotypic traits provide very convenient screening test to select for nuclear respiratory-deficient mutants in S. pombe. Furthermore, restoration of the antimycin or bongkrekic resistance of growth on glucose, for which there is a strong selective pressure in a population incubated in the presence of inhibitors provides a very efficient procedure for large scale screening of revertants or suppressors of respiratory-deficient mutants. A case of a functional suppression by the mitochondrial mutation sup-2 is reported here. Several types of suppression of nuclear respiratory-deficient mutations have been previously described (37-46) but the only other cases of mitochondrial suppression of nuclear-coded respiratory traits reported so far are the suppression of nuclear-venturicidin resistance in S. cerevisiae (47) and suppression of slow growth in Paramecium (34). The latter suppression might well be similar to those reported in this paper for S. pombe, but the much higher rate of antimycin-insensitive respiration in Paramecium (34) makes comparisons between the two species difficult.

Suppression of Bongkrekic Acid Sensitivity of Growth on Glucose—The bongkrekic acid sensitivity of glucose-grown S. pombe pet1 and its suppression in sup-2 are easily explained in the light of the hypothesis of Subik et al. (25) and Kovac (48) proposing that, in S. cerevisiae, a continuous supply of intramitochondrial ATP is required for the synthesis of an unknown component which dilutes out in a few cell generations and is required for cellular multiplication in glucose. Pet1 is obviously unable to carry out oxidative phosphorylation. Its cellular multiplication in glucose, therefore, is expected to be dependent on the translocation of glycolytic ATP into mitochondria and consequently be inhibited by bongkrekic acid. This is indeed the case, not only in pet1 but also in the S. pombe wild type when the respiration is decreased by the presence of antimycin in the culture medium. In pet1 sup-2, the mitochondrial oxidations and phosphorylations are markedly restored and the resistance to bongkrekic acid, therefore, is conferred. It must be pointed out however that the suppression is partial; pet1 sup-2 does not grow on glycerol probably because, even in nonrepressive conditions, only one-third of the succinate:cytochrome c reductase and oligomycin-sensitive ATPase activities are restored. In exponential glucose-growing pet1 sup-2 cells, less than 10% of the antimycin-sensitive
oxidative phosphorylation rate of the wild type is obtained. This is however sufficient to restore resistance of growth to bongkrekic acid. The latter trait is therefore a powerful test for screening strains with very low or no oxidative phosphorylation.

Suppression of Antimycin Sensitivity of Growth on Glucose – Glucose-repressed pet1 and pet1 sup-2 exhibit total (antimycin-sensitive plus antimycin-insensitive) respiration rates of 2.1 and 4.4 µl of O2 x h⁻¹ x mg⁻¹ dry weight, respectively. They, respectively, carry out six and nine generations in 10% glucose supplemented with 0.18 µM antimycin. Such differences are clearly detected not only by the cell density of liquid cultures but also by the size of colonies on agar plates containing 3% glucose and 0.5 µg/ml of antimycin (20). The test of antimycin sensitivity of the growth on glucose is thus remarkably precise since it allows discrimination of strains with such small rates of respiration.

The presence in glucose-grown pet1 of a low residual antimycin-sensitive cellular respiration (Qₒₒ = 0.8) strongly suggests that this process is the inhibitory target of antimycin when added in the culture medium. If so, this low antimycin-sensitive respiration must be essential for the cellular multiplication in glucose of the mutant pet1 since addition of antimycin restricts its growth. A similar suggestion has previoulsy been made for the pleiotropic S. cerevisiae pet 936 respiratory-deficient strain (4). This implies that in the wild type as well as in the suppressed strains pet1 sup-2 which grows well in the presence of antimycin, the inhibition of the antimycin-sensitive oxygen uptake is compensated by a distinct process which cannot fully develop in pet1. This process might well be the antimycin-insensitive respiration which is functioning in glucose-grown pet1 at a lower rate (Qₒₒ = 1.3) than that of glucose-grown pet1 sup-2 (Qₒₒ = 2.6) or of the wild type grown in the presence of antimycin (Qₒₒ = 2.3).

The antimycin-insensitive respiration rates observed in respiratory-deficient conditions are rather low in S. pombe. The highest antimycin-insensitive Qₒₒ obtained in this work, is 2.8 µl of O2 x h⁻¹ x mg⁻¹, dry weight, which is 10 to 50 times lower than the so-called alternative respiration observed in other species such as Neutrosperma crassa (28), Candida lipolytica (30), Monilia laevis (31), or Paramecium tetraurelia (32). Furthermore, in contrast to the above species, the S. pombe antimycin and cyanide-insensitive respiratory is not sensitive to salicylhydroxamic acid and is sensitive to sodium azide. To our knowledge, the presence of such antimycin- and hydroxamate-insensitive, azide-sensitive oxidation has not been reported so far. It must be mentioned that this new type of alternative respiration is not restricted to the strains described in this paper; we have also observed its presence in 14 distinct mitochondrial mit-strains of S. pombe kindly provided by Dr. K. Wolf and G. Seitz from Munich University.

The inhibition by antimycin of the growth of respiratory-deficient strains could be explained by a requirement for continuous growth in glucose of a minimum level of either a "normal" antimycin-sensitive respiration or of an antimycin-insensitive, azide-sensitive oxygen uptake or of a combination of both. This is in agreement with inhibition of growth in glucose of S. pombe by anaerobiosis (17) or by azide (data not shown). In the growth conditions used in this work, the minimum level of the total cellular respiration yielding continuous cellular multiplication in glucose is estimated to be about 2.0 µl of O2 x h⁻¹ x mg⁻¹, dry weight, e.g., slightly lower or equal to that of glucose-grown pet1 (Qₒₒ = 2.1) but higher than that observed in the same strain put in presence of antimycin (Qₒₒ = 1.4).

If this hypothesis is correct, the limited growth of pet1 in glucose in the presence of antimycin is due to its insufficient level of the antimycin-insensitive, azide-sensitive respiration. Conversely, the suppression by sup-2 of the antimycin sensitivity of the growth on glucose of pet1 must then result from the potentiality of the suppressed strain to induce a sufficient antimycin-insensitive modified respiration when grown on glucose in the presence of antimycin. It is not excluded that this might result from a higher content in cytochrome(s) b.

Cytochromes b are generally considered not to participate in the cyanide-insensitive pathway in plants (see Ref. 33 for discussion). However, it has recently been suggested that, in Paramecium, the branching of the antimycin-insensitive pathway is posterior to both cytochrome b₃₅ and cytochrome b₈₇ (77 K), the latter possibly being an autooxidizable pigment (49). The situation might be similar in S. pombe. Indeed, the fact that azide oxidizes cytochromes b₃₅₁,₅ and b₈₅ (77 K) which were both previously reduced by l-malate in the presence of antimycin plus cyanide, indicates that azide blocks the flow of reducing equivalents prior to the two cytochromes b and also that the latter are in equilibrium with a cyanide-insensitive oxidase. Therefore, we cannot exclude the possibility that at least one of the two cytochromes b is directly involved in the antimycin-sensitive respiration of S. pombe and is directly or indirectly controlled by the mitochondrial sup-2 mutation.

REFERENCES
1. Sherman, F., and Slonimaki, P. P. (1964) Biochim. Biophys. Acta 90, 1-15
2. Lachowicz, T. M. (1968) Arch. Immunol. Thér. Exp. 16, 603-701
3. Lachowicz, T. M., Kotyla, Z., Kolodynski, J., and Sniegocka, Z. (1969) Arch. Immunol. Ther. Exp. 17, 72-85
4. Ebner, E., and Schatz, G. (1973) J. Biol. Chem. 248, 5379-5384
5. Ebner, E., Mennucci, L., and Schatz, G. (1973) J. Biol. Chem. 248, 5385-5388
6. Subik, J., Kovač, L., Kolarov, J., Kováč, L., and Lachowicz, T. M. (1970) Biochim. Biophys. Acta 205, 513-519
7. Subik, J., Kovač, L., and Kolarov, J. (1972) Biochim. Biophys. Acta 289, 146-154
8. Thomas, D. Y., and Scragg, A. H. (1973) Eur. J. Biochem. 37, 585-588
9. Teagoloff, A., Akai, A., and Needleman, R. D. (1975) J. Biol. Chem. 250, 8228-8235
10. Heslot, H., Louis, C., and Goffeau, A. (1970) J. Bacteriol. 104, 462-463
11. Wolf, K., Sebald-Althaus, M., Schweyen, R. J., and Kaudewitz, F. (1971) Mol. & Gen. Genet. 110, 101-109
12. Goffeau, A., Colson, A.-M., Landry, Y., and Fourié, F. (1972) Biochem. Biophys. Res. Commun. 48, 1448-1454
13. Goffeau, A., Landry, Y., Fourié, F., Briquelet, M., and Colson, A.-M. (1973) J. Biol. Chem. 248, 7097-7100
14. Bandlow, W., Wolf, K., Kaudewitz, F., and Slater, E. C. (1974) Biochim. Biophys. Acta 333, 445-459
15. Bandlow, W. (1974) In The Biochemistry of Mitochondria (Kroon, A. M., and Sacccone, C., eds) pp. 225-230, Academic Press, New York
16. Schwab, K., Sebald, M., and Kaudewitz, F. (1971) Mol. & Gen. Genet. 110, 361-366
17. Heslot, H., Goffeau, A., and Louis, C. (1970) J. Bacteriol. 104, 473-481
18. Altmann, K., Schneider, H. G., and Strong, F. M. (1960) Arch. Biochem. Biophys. 8, 281-294
19. Henderson, P. J. F., and Lardy, H. A. (1970) J. Biol. Chem. 245, 1319-1326
20. Colson, A.-M., Labaille, F., and Goffeau, A. (1976) Mol. & Gen. Genet. 149, 101-109
21. Sainsard, A. (1975) Nature 257, 312-314
22. Fourié, F., and Goffeau, A. (1973) J. Gen. Microbiol. 74, 227-229
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
24. Waddel, W. J. (1957) J. Lab. Clin. Med. 48, 311–314
25. Subik, J., Kolarov, J., and Kovac, L. (1972) Biochem. Biophys. Res. Commun. 49, 192–198
26. Wolf, K., Burger, G., Lang, B., and Kaudewitz, F. (1976) Mol. Gen. Genet. 118, 1203
27. Ohrnishi, T., Sottocass, G., and Ernst, L. (1966) Bull. Soc. Chim. Biol. 48, 311–1203
28. von Jagow, G., and Klingenberg, M. (1970) Eur. J. Biochem. 12, 583–592
29. Lambowitz, A. M., Smith, E. W., and Slayman, C. W. (1972) J. Biol. Chem. 247, 4580–4588
30. Henry, M. F., Hamaide-Deplus, M. C., and Nyns, E. J. (1974) Antonie van Leeuwenhoek 50, 79–91
31. Hanssens, L., D’Hondt, E., and Verachtert, H. (1974) Arch. Microbiol. 98, 339–349
32. Sainsard, A., Claisee, M., and Balméfrézol, M. (1974) Mol. & Gen. Genet. 130, 113–125
33. Henry, M. F., and Nyns, E. J. (1975) Sub-Cell Biochem., 1–65
34. Lloyd, D. (1974) in The Mitochondria of Microorganisms, pp. 137–158, Academic Press, London
35. Goffeau, A., Briquet, M., Colson, A.-M., Delhez, J., Fourny, F., Labaille, F., Landry, Y., Mohar, O. and Mrena, E. (1975) in Membrane Biogenesis (Tzagoloff, A., ed) pp. 63-97, Plenum Press, New York
36. Goffeau, A., Labaille, F., Mohar, O., and Colson, A.-M. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th., Neupert, W., Sebald, W., and Werner, S., eds) pp. 851–856, North Holland Publishing Co., Amsterdam
37. Mitchell, M. B., and Mitchell, K. H. (1956) J. Gen. Microbiol. 14, 84–89
38. Sherman, F. (1963) Genetics 48, 375–385
39. Kotylak, Z. (1971) Acta Microbiol. Pol. 3–4, 109–112
40. Kotylak, Z., Maszewski, S., Msiiewicz, M., and Czerwinska, K. (1973) Acta Microbiol. Pol. 5, 31–36
41. Kotylak, Z. (1973) Mol. & Gen. Genet. 125, 265–273
42. Maszewski, S., and Lachowicz, T. M. (1974) Mol. & Gen. Genet. 131, 69–77
43. Ono, B.-I., Fink, G., and Schatz, G. (1975) J. Biol. Chem. 250, 775–782
44. Maszewski, S., and Lachowicz, T. M. (1975) Acta Microbiol. Pol. 7, 77–85
45. Storm, E. M., and Marmur, J. (1975) Biochem. Biophys. Res. Commun. 64, 752–759
46. Trembath, M. K., Monk, B. C., Kellerman, G. H., and Linnane, A. W. (1975) Mol. & Gen. Genet. 140, 333–337
47. Colson, A.-M., Goffeau, A., Briquet, M., Weigel, P., and Mattone, J. R. (1974) Mol. & Gen. Genet. 135, 309–326
48. Kovac, L. (1974) Biochim. Biophys. Acta 346, 101–135
49. Doussié, J., Adoutte, A., Sainsard, A., Ruiz, F., Beisson, J. and de Vignais, P. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th., Neupert, W., Sebald, W., and Werner, S., eds) pp. 873–880, North Holland Publishing Co., Amsterdam
Properties of a mitochondrial suppressor mutation restoring oxidative phosphorylation in a nuclear mutant of the yeast Schizosaccharomyces pombe.

F Labaille, A M Colson, L Petit and A Goffeau

J. Biol. Chem. 1977, 252:5716-5723.

Access the most updated version of this article at http://www.jbc.org/content/252/16/5716.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/16/5716.citation.full.html#ref-list-1