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Germination of Yeast Spores Lacking Mitochondrial Deoxyribonucleic Acid

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A population of petite ascospores (mitochondrial deoxyribonucleic acid [mtDNA]-less), produced by brief ethidium bromide (EthBr) mutagenesis prior to transfer to sporulation medium, was used to examine the role of the mitochondrial genetic system on germination and outgrowth in *Saccharomyces cerevisiae*. Petite ascospores, which are morphologically indistinguishable by phase-contrast microscopy from wild-type spores, germinate and proceed through outgrowth at a rate and extent only slightly less than that of wild-type spores. Both developmental processes occurred in the absence of mtDNA synthesis and measurable cytochrome oxidase activity. These results indicate that neither respiration nor a functional mitochondrial genome are required for germination and outgrowth. The properties of the petite clones were typical of petites formed during vegetative growth. Individual sporal clones differed markedly from each other in suppressiveness. Petite sporal clones which exhibited a high degree of supressiveness also contained a reduced but detectable amount of mtDNA of altered buoyant density. One clone contained a unique mtDNA with a buoyant density higher than that of wild-type mtDNA.

Although sporulation of *Saccharomyces cerevisiae* has been used as a model system to study development (see reviews 3, 13), relatively little information is available on the transition of the semidormant spore to the vegetative state. Optimal conditions have now been established to obtain a synchronously germinating yeast spore population (12), and the sequence of macromolecular events during germination and outgrowth of ascospore formation have been defined (10).

We have reported on the role of the mitochondrial genetic system during meiosis and ascospore formation (4). The results indicate that, although full respiratory activity is required to initiate sporulation, no mitochondrial genetic information needs to be expressed during sporulation. In this paper we extend our studies to the subsequent stages in the life cycle, germination, and outgrowth.

Earlier reports on mitochondrial activity during germination are conflicting. Although oxygen uptake increases during germination (11), reports that germination can occur anaerobically have been made (9). Ethidium bromide (EthBr), a potent inhibitor of mitochondrial deoxyribonucleic acid (mtDNA) replication, delays outgrowth of spores (P. Rousseau, Ph.D. thesis, Univ. of Wisconsin, 1972).

The work presented here shows that petite ascospores, which are devoid of mtDNA and cytochrome oxidase activity, germinate and proceed through outgrowth at a rate and extent similar to wild-type spores. Furthermore, the primary petite clones arising after meiosis and germination exhibit properties typical of petites formed during vegetative growth.

MATERIALS AND METHODS

Organism. The heterothallic diploid strain of *S. cerevisiae* Y185 used in this study has the following genotype:

$$\frac{\alpha \; his_2}{a} \; \frac{+}{+} \; \frac{+}{his_8} \; \frac{+}{lys_2} \; \frac{+}{ade_2}$$

Production of spores. The conditions for growth, sporulation, and formation of wild-type and petite ascospores have been described (4). The petite spores were produced by a culture which was mutagenized with EthBr one generation prior to transfer to sporulation medium. No mtDNA was detectable in the EthBr-treated culture or in the spores. The spores were harvested by centrifugation, washed, and stored as aqueous suspension at 4 C.

Preparation and germination of a single spore population. The asci and unsporulated cells were suspended in 0.067 M potassium phosphate buffer (pH 6.8) containing 0.1% of a saturated solution of mercaptoethylamine, 1% Tween 80 and 1 mg of

"protoplasting enzyme" per ml (generously supplied by the Kirin Brewery Co. of Takasaki, Japan). After incubation for 1 h at 30 C, all the unsporulated cells had lysed and asci walls were digested. The spores were washed five times with 0.067 M potassium phosphate buffer (pH 6.8) containing 1% Tween 80. The spores were suspended in ice-cold yeast extract (1%), peptone (2%), and Tween 80 (1%) medium and passed through a French pressure cell at 7,000 psi. The resulting single-spore suspension was adjusted to a concentration of 3×10^7 spores per ml and placed in a 5-liter fermentor (Chemapec, Inc., Hoboken, N.J.). After equilibration of the temperature to 30 C, glucose (2%) was added as a germination agent and the culture was aerated at 600 rpm. Samples were taken at various intervals and analyzed for total DNA, the percentage of mtDNA, enzyme activities, and protein as described previously (4).

Terminology. Germination is defined as the transition period from a refractile to a nonrefractile spore. Outgrowth is defined as the development of a budded cell from a phase-dark spore.

Determination of suppressiveness. Cytoplasmic petites (*rho*$^-$) can be classified as either neutral (*rho*$^-$[n]) or suppressive (*rho*$^-$[s]), depending on whether or not they transfer their phenotype when crossed to respiratory competent (*rho*$^+$) strains. Neutral petites do not impose their phenotype on their mates, and only (*rho*$^+$) zygote colonies are formed. Suppressive petites, when crossed to (*rho*$^+$) strains, produce (*rho*$^-$) zygote colonies, the frequency of which determines their degree of suppressiveness. The auxotrophic requirements and mating types of clones arising from single petite spores were identified. The petite strains were mated with a (*rho*$^+$) strain D 243-16C (α, ade_1, trp_1, lys_1), and the percentage of suppressiveness was determined after plating and prototrophic selection of diploid clones as described previously (8). The values for the degree of suppressiveness are corrected for the number of petite cells in strain D 243-16C (7).

RESULTS

Properties of wild-type and petite spores. Suspensions of wild-type and petite spores were prepared and examined by phase-contrast microscopy. No difference in size, shape, or refractility were observed between the two types of ascospores. Extracts were prepared from the spore suspensions and the total DNA was isolated as described previously (4). The percentage of mtDNA to total DNA was 11% in the wild-type spores. No mtDNA was detectable in the petite spores after CsCl analytical ultracentrifugation.

Germination and outgrowth in the absence of mtDNA. The kinetics of germination and outgrowth of wild-type and petite spores were followed spectrophotometrically and microscopically. As shown in Fig. 1 both types of spores germinated rapidly and completely. In both cultures, there was a decrease of about 15% in light absorbance during the first 2 h. This change in optical density was paralleled by phase darkening of the spores. After 2 h the optical density of the wild-type culture increased and the percentage of cells containing buds started to rise. Increases in absorbance and bud formation were slightly delayed in the petite culture. The extent of outgrowth of the petite spores was only slightly less than the wild-type spores.

mtDNA synthesis during germination and outgrowth. As shown in Fig. 2, in wild-type cells nuclear DNA synthesis (measured colorimetrically) begins late in outgrowth whereas mtDNA synthesis is observed early in germination. The percentage of mtDNA rises until chromosomal DNA synthesis occurs, and then it returns to the value originally present in the spore. Nuclear DNA synthesis was observed during outgrowth of the petite culture but no mtDNA was detectable in samples taken up to 5.0 h.

Respiratory activity during germination and outgrowth. Both the wild-type and EthBr-treated cultures were highly respiratory active when transferred to sporulation medium (4). In both cultures the cytochrome oxidase activity was maximal at T_0 and declined gradually

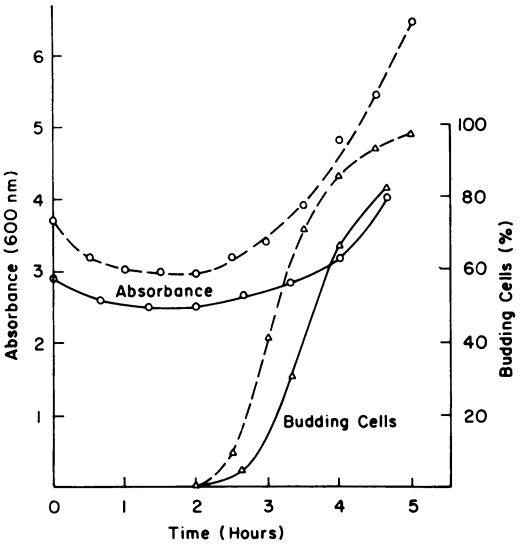

FIG. 1. *Kinetics of germination and outgrowth of wild-type and petite spores. Decrease in absorbance at 600 nm was used as an index of germination. The extent of outgrowth was followed by counting the percentage of budded cells. Control culture, ----; petite culture, ——.*

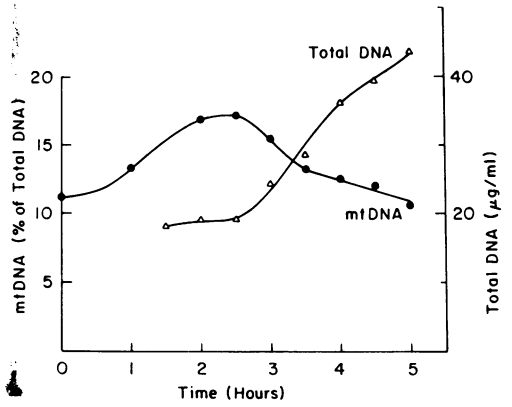

FIG. 2. *Percentage of mtDNA to total DNA during germination and outgrowth of the control culture. Total DNA synthesis was determined by the diphenylamine method. The percentage of mtDNA to total DNA was measured after CsCl analytical centrifugation as described in Materials and Methods. Samples were taken from the culture described in Fig. 1.*

during sporulation. Since it was previously reported that the rate of oxygen uptake increases again during germination and outgrowth in strain Y-55 (11), we have determined the activity of cytochrome oxidase during these stages in both wild-type and petite spores. The results are shown in Fig. 3. Cytochrome oxidase activity increases continuously during germination and outgrowth of the wild-type spores. The low activity originally present in the petite spores drops early in germination and remains at a low value during outgrowth. This decrease in cytochrome oxidase activity is in agreement with unpublished oxygen uptake experiments which showed a complete loss of the small amount of respiratory activity originally present in the petite spore. Loss of cytochrome oxidase activity could also be explained if synthesis of this enzyme was dependent on the functioning of both the nuclear and mitochondrial genetic systems as has been suggested (6).

The rapid synthesis of mtDNA early in germination followed by the rise in cytochrome activity is unusual for two reasons. First, germination and outgrowth can occur in their absence, and second because these processes occur under conditions of high glucose repression. The amount of glucose present in the medium is sufficient to cause severe repression of isocitrate lyase, a soluble enzyme involved in acetate metabolism. The small activities present in both wild-type and petite spores drop to minimal values early in germination and do not increase during outgrowth (see Fig. 3).

Characteristics of primary clones arising from petite spores. Since the petite ascospores germinate normally, we were able to compare for the first time the petite mutants formed after meiosis and spore germination with the typical petites produced and maintained in the vegetative state.

Over 1,000 clones arising from single petite spores were examined. None of the clones were able to grow when replica-plated to media containing nonfermentable carbon sources, glycerol or acetate. All of the clones gave a negative reaction when tested with the redox dye 2-3-5 triphenyltetrazolium chloride. Thus, all of the spores tested gave rise to petite clones. As we have shown before, after EthBr treatment of vegetative cells only 98% of the colonies were

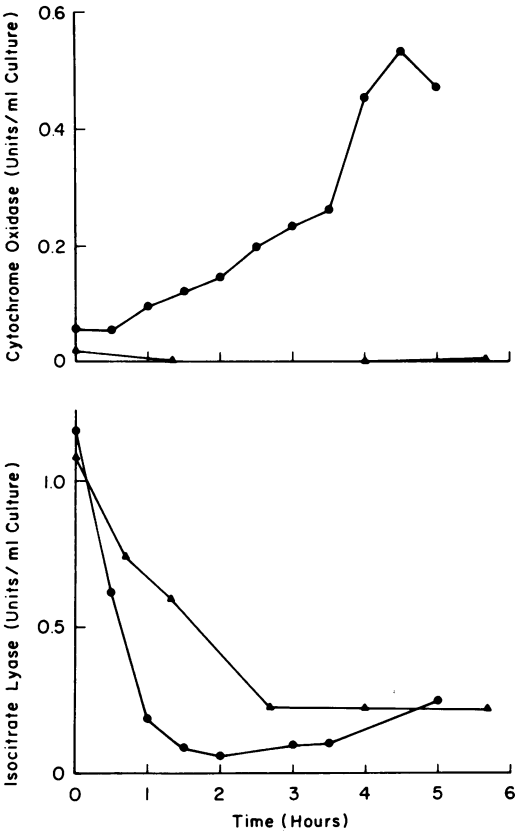

FIG. 3. *Cytochrome oxidase and isocitrate lyase activities during germination and outgrowth of wild-type (●) and petite (▲) spores. Enzyme activities were measured as described in reference 4. Units are expressed as nanomoles of substrate utilized per minute. Samples were taken from cultures described in Fig. 1.*

petite (4). Consequently, during sporulation and outgrowth, the number of petite cells increased.

From studies on petites found after EthBr mutagenesis during vegetative growth, it has been suggested that an association exists between the degree of suppressiveness of a petite and the presence of mtDNA in that clone (7). The degree of suppressiveness, percent of mtDNA, and buoyant density of mtDNA for several random sporal clones are shown in Table 1. The clones displayed a wide range of suppressiveness. Total cell DNA was extracted from these clones and subjected to CsCl ultracentrifugation. Two clones, numbers 3 and 41, which were devoid of detectable mtDNA, appeared to be neutral petites. The three remaining clones, 34, 44, and 134, contained significant but reduced amounts of mtDNA and also showed a measurable degree of suppressiveness. In two of the clones, 34 and 44, the buoyant density of the petite mtDNA was less than that found for the original diploid strain Y-185. However, the mtDNA of clone 134 had a buoyant density greater than that of the wild-type strain.

DISCUSSION

The ability to produce and to germinate a pure population of petite yeast spores has offered the opportunity (i) to evaluate the role of the mitochondria and mtDNA on germination and outgrowth and (ii) to compare the characteristics of the petite mutants after meiosis and germination with those obtained and maintained in a vegetative state.

The first result to emerge from these studies is that germination and outgrowth are not dependent on a functional mitochondrial genome. This conclusion is based on the fact that only small differences were observed in either the rate or extent of germination and outgrowth between wild-type and petite spores. Since we have previously shown that meiosis and ascospore formation can occur in the absence of mtDNA (4), it appears that the mitochondrial genome does not contain information which must be expressed during the sexual stages of the yeast life cycle.

TABLE 1. *Characteristics of mtDNA from parent and petite clones of S. cerevisiae*

Clone no.	Buoyant density (g/cm^3)	% of total DNA	Degree of suppressiveness (%)
Petite			
3		<1	<1
34	1.681	6	8.5
41		<1	<1
44	1.680	9	29.0
134	1.688	4	19.5
Wild type			
Y-185	1.683	10	

Respiration, which is a prerequisite for initiation of sporulation (4), is not required for germination and outgrowth of yeast spores. Although a rise in oxygen uptake has previously been observed during spore germination (11) and an increase in cytochrome oxidase activity occurs during germination of wild-type spores, neither activity is essential for germination since the petite spores contained only negligible amounts of cytochrome oxidase throughout the entire process.

Despite the fact that mtDNA is not a functional necessity for sporulation and germination, mtDNA synthesis occurs during these developmental stages. During sporulation, the percentage of mtDNA decreases from 11% to a minimal value of 6% after meiotic DNA replication (4). In germinating spores, a maximal value of 17% was observed early in germination. After nuclear DNA synthesis, this value quickly returned to 11%, approximately the same percentage found in diploid cells prior to sporulation. These results suggest that some regulatory mechanism(s) is operative which assures that the original cellular content of mtDNA is maintained during the alternation of haploid and diploid phases.

Another interesting result is that petites induced by EthBr and immediately subjected to meiosis, spore formation, and germination do not differ drastically from petites which have not undergone these developmental processes. In both cases, individual petite clones vary quite markedly from one another not only in the amount of mtDNA with altered physical properties but also in the degree of suppressiveness. In our studies, suppressiveness was eliminated in neutral petite clones, whereas suppressive clones contained mtDNA of altered buoyant density. Our results are consistent with the notion that suppressiveness is determined by mtDNA. The primary effects of EthBr mutagenesis are basically not altered by the process of sporulation and germination.

As a general rule, petite mutants contain mtDNA with a buoyant density equal to or lower than the wild-type parent (1). The isolation of a suppressive clone with a mtDNA of a higher density than the parental mtDNA may be fortuitous or it could have resulted from the

different intercellular conditions during sporulation and germination. In any event, characterization of the physical properties of this exceptional mtDNA and determination of the genetic functions which may have been lost should prove interesting and are presently underway.

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