Respiratory-deficient Mutants in
Saccharomyces lactis

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Six independent ultraviolet-induced respiratory-deficient mutants (petites) of Saccharomyces lactis were isolated and characterized. Two possessed a normal cytochrome spectrum, another displayed an increased level of all the cytochromes, and three suffered from a partial or complete loss of one or more of the cytochromes \(a, b, c,\) and \(c_1\). All of the mutants were segregational petites; none was vegetative. Determination of linkage relationships between mutants was restricted because matings between mutants, homozygous or heterozygous, for loci affecting cytochrome content were blocked at various stages in the mating-sporeulating sequence. At least three of the petites were genetically nonidentical. Three of the mutations appeared to occur loci within the same linkage group; two of the three mutations that mapped within this region were cytochrome-deficient. Growth at high or low temperatures, under increased osmotic pressure or in media supplemented with various fatty acids or sterols, did not relieve the physiological defects in these mutants. Reasons for the differences in survival of segregational and vegetative petites within this species are examined.

In Saccharomyces cerevisiae and related species, respiratory-incompetent cells (petites) may arise either from mutation of chromosomal genes (segregational petites) or from the loss of an essential cytoplasmic determinant (vegetative petites). Both types of cells have modifications in their ability to oxidize nonfermentable carbon sources, and usually show alterations in either the amount or kind of cytochromes produced, or both. In addition, the mitochondrial organelles are generally nonfunctional and may have structural abnormalities. Mutations that cause segregational petites map as discrete loci scattered throughout the genome. On the other hand, vegetative petites do not follow a Mendelian pattern of inheritance but possess characteristics of a cytoplasmically determined mechanism of heredity. The spontaneous rate of formation of vegetative petites \((\rho^-)\) can be quite high, and in the presence of acriflavine or 5-fluorouracil the rate of conversion from wild type (grandes) to \(\rho^-\) may approach 100% (3).

Bulder (1) investigated the effect of acriflavine on the formation of \(\rho^-\) cells in yeasts that are taxonomically unrelated to S. cerevisiae, and found several genera in which no \(\rho^-\) types were recovered. Further studies (2) indicated that in these acriflavine-treated stocks, \(\rho^-\) petites were generated but were nonviable. Could a yeast, in which loss of the cytoplasmic factor leads to lethality, mutate and produce viable segregational petites? What would be the characteristics of these mutants? Answers to these questions were sought through studies of the heterothallic yeast S. lactis (Kluyveromyces lactis) (11), since this species is one in which vegetative petites are nonviable (1). The results showed that (i) viable segregational petites are formed following ultraviolet mutagenesis; (ii) these mutations map at several independent loci; (iii) the cytochrome spectra of some of the petites exhibit modifications from the wild type; and (iv) several of the mutations that cause respiratory incompetence are pleiotropic and affect zygote formation and sporulation.

MATERIALS AND METHODS

Organism and cultivation. Parental stocks NRRL Y-1140 and NRRL Y-1118 of S. lactis, or mutants derived from them, were used. In the genetic analyses, segregations of mutations to histidine or adenine dependence were followed as supplementary genetic markers. Maintenance of the stocks was as previously described (5). Cells were grown in yeast-malt extract (YM) broth (12) or in a glycerol medium (Gly) containing 20 g of glycerol, 5 g of peptone (Difco), and

\(\text{fluorouracil}\) and in the presence of high, taxonomically unrelated of either from the cytochromes characteristics of the cells. Mutations that affect cytochrome content were blocked at various stages in the mating-sporeulating sequence. At least three of the petites were genetically nonidentical. Three of the mutations appeared to occur loci within the same linkage group; two of the three mutations that mapped within this region were cytochrome-deficient. Growth at high or low temperatures, under increased osmotic pressure or in media supplemented with various fatty acids or sterols, did not relieve the physiological defects in these mutants. Reasons for the differences in survival of segregational and vegetative petites within this species are examined.

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water to 1,000 ml. All media were solidified as needed with 20 g of agar (Difco) per liter. Malt extract-agar (ME) was used as a combination mating and sporulation medium (12). Diploids were isolated according to the method described by Tingle et al. (10).

Selection of petites. Washed, stationary-phase NRRL Y-1140 cells were exposed to irradiation from a Sylvania germicidal lamp for an interval sufficient to effect a 90% kill. Irradiated cells were diluted to a suitable concentration, plated on YM agar, and incubated at 25°C for 2 to 3 days. Colonies formed by survivors were replica-plated to media containing glycerol as the sole carbon source. Clones unable to grow on the Gly replicas were restreaked from the master plates onto fresh YM agar and incubated at 25°C. Inocula from the ensuing isolated colonies were transferred to YM agar-stock tubes and stored as potential petites at 10°C.

Triphenyl tetrazolium chloride (TTC) overlay. The potential petites were examined for their ability to reduce TTC. For this assay, each culture was transferred as an isolated streak to a YM plate, incubated overnight at 25°C, and overlaid with TTC following the method described by Ogur et al. (6). Clones that showed diminished ability, or inability, to reduce TTC were retained as petite stocks.

Reagents. 2,3,5-Triphenyl tetrazolium chloride was purchased from General Biochemicals Corp., Chagrin Falls, Ohio. Tweens 20, 40, and 80 were obtained from Emulsion Engineering, Inc., Melrose Park, III.

RESULTS

Characteristics of ultraviolet-induced respiratory mutants. Mutants were identified as presumptive petites if they (i) formed small colonies on YM agar, (ii) were unable to utilize glycerol as a sole carbon source, and (iii) sustained a partial or complete loss in ability to reduce TTC. Nine independent isolates with these characteristics were recovered. The frequency with which these presumptive petites appeared was equivalent to 4.5 per 10⁷ irradiated cells, and approached the value (3.2 per 10⁶ cells) with which amino acid auxotrophs were formed in the same population. Three of the nine mutants were lost during storage; consequently, only six stocks were examined.

Warburg determinations. The aerobic respiratory capacity, determined by using standard Warburg procedures, was established for each of the six petites. No detectable rate of glucose oxidation was noted in any of the isolates; apparently, each mutant is respiratory deficient.

Cytochrome spectra. Low-temperature spectroscopic studies of the cytochrome spectra of the six petites were compared with that of the normal stock (Y-1104) by Fred Sherman. Dr. Sherman's studies (personal communication) indicate that S. lactis Y-1104 possesses the same cytochromes as those found in bakers' yeast, namely, a, b, c, and c₁. Three of the presumptive petites, Pet 1, 7, and 12, have normal cytochrome spectra. In Pet 12, however, the concentration of all the cytochromes is slightly elevated. The remaining three mutants, Pet 5, 10, and 16, contain cytochrome deficiencies: Pet 10 is deficient in cytochromes a and b, and partially deficient in cytochrome c₁; Pet 5 is deficient in cytochrome a, and partially deficient in cytochromes b and c₁; and Pet 16 is partially deficient in cytochromes a and b, and slightly higher in cytochrome c. For comparison, the cytochrome deficiency pattern present in Pet 5 most closely resembles the spectrum of Type V petites of S. cerevisiae; Pet 16, that of Type IV; and Pet 10, that of Type II (9).

Segregational analyses (petite × grande). Each of the six petites was mass-mated with grande cells of complementary mating type (NRRL Y-1118) and sporulated; the tetrads were dissected as in procedures described earlier (5). Each segregant was examined for the presence or absence of the respiratory-deficient characteristics: small colony morphology and inability to utilize glycerol (Table I). With the exception of the Pet 5 × Grande and Pet 12 × Grande crosses, each of the heterozygotes produced four viable spores per ascus. From the matings of Pet 5 × Grande and Pet 12 × Grande, asci with three viable spores were formed. A characteristic 2:2 ratio of petite to grande in the zygotcs with four spores per ascus, and the 2:1 and 1:2 ratios in the zygotes with three spores, indicated that the respiratory deficiency in each of these mutants was of genic origin and represented the expression of an alternate allele at an individual locus.

Dominance. Respiratory competence, measured by ability to grow on Gly medium, was determined for heterozygous diploids formed from crosses between each of the petite stocks with grande cells of complementary mating type. Two of the diploids, Pet 10 × Grande and Pet 16 × Grande, sporulated when they were transferred from YM to Gly, and an accurate estimate of

<table>
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<th>Table 1. Petite × grande segregations</th>
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<td>Cross, petite × grande (+):a</td>
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<td>-------------------------------------</td>
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<tr>
<td>Pet 1 × +</td>
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<td>Pet 5 × +</td>
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<td>Pet 7 × +</td>
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<td>Pet 10 × +</td>
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<td>Pet 12 × +</td>
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<td>Pet 16 × +</td>
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*a Plus sign represents respiratory sufficiency (grande). Procedures used in dissection and determination of petite vs. grande characteristics are described in the text.
their ability to utilize glycerol was impossible. Each of the four diploids remaining, i.e., Pet 1 × Grande, Pet 5 × Grande, Pet 7 × Grande, and Pet 12 × Grande, grew well on Gly. One assumes, therefore, that each of these four mutations is recessive to its grande allele.

Identification of independent mutant sites. Identity or nonidentity between mutants was determined by crossing pairs of petites in all possible combinations. Mutants were considered to be nonidentical if grande recombinants were recovered among the segregants from a mating. The success of this undertaking was limited because viable zygotes were produced only from matings between certain mutant combinations. Heterozygous matings which were fertile are listed in Table 2; infertile combinations appear in a footnote to the table.

Grande recombinants occurred among the segregants from each of the fertile matings. An examination of the genotypes of the heterozygotes that generated grande recombinants leads to the conclusions that Pet 1 occupies a site independent from that of each of the other five mutants, and that the Pet 16 locus is not identical to that of Pet 5, 7, or 12. Relationships of the remaining mutants to one another could not be established because of infertility.

Heterozygous diploids from the fertile mutant combinations were not stable and their respiratory phenotype could not be established.

Linkage relationships. Since all meiotic products of a cell are recovered in the four spores of a yeast ascus, one can detect linkage between two factors undergoing segregation by analyzing the distribution ratio of parental ditype (PD) to nonparental ditype (NPD) to tetratype (TT) among tetrads. If there is no linkage between two factors, the ratio of PD to NPD to TT approaches 1:1:4. On the other hand, linked markers segregate more frequently as a unit, fewer NPD tetrads are formed, and a marked deviation from the anticipated 1:1 ratio of PD to NPD occurs in the tetrads recovered (7).

Linkage relationships were determined between those mutants that generated fertile zygotes. The tetrad distribution ratios obtained from these matings are summarized in Table 2. Usually, only those matings in which Pet 1 participated as one of the parents produced numerous zygotes capable of forming four viable spores per ascus. A predominance of PD compared to NPD tetrads from the Pet 1 × Pet 5, Pet 1 × Pet 10, and Pet 1 × Pet 16 zygotes (Table 2) suggests that Pet 5, Pet 10, and Pet 16 are linked to Pet 1.

In the mating Pet 16 × Pet 5, zygote formation and spore viability were extremely poor; the viable tetrads probably represent a selective rather than a representative sample of the population. Zygotes produced from the Pet 10 × Pet 5 and Pet 10 × Pet 16 crosses were not viable. For these reasons, the relationships of Pet 5, Pet 10, and Pet 16 to one another are unresolved.

In Table 2, the ratio of PD to NPD to TT tetrads from Pet 1 × Pet 7 and Pet 1 × Pet 12 zygotes (1:3:9 and 4:3:10, respectively) approaches the 1:1:4 ratio expected for unlinked markers, and implies that Pet 1 probably occupies a linkage group independent from that of Pet 7 or Pet 12. Whether Pet 7 and Pet 12 are identical is not known. Limited zygote formation and poor spore viability in the Pet 16 × Pet 7 combination prohibits an analysis for linkage between these mutations.

For the last cross in Table 2 (Pet 12 × Pet 16) only the PD tetrads contained four viable spores. In the NPD and TT tetrads, recombinants presumably possessing the double mutant genotype were nonviable. As in the two preceding crosses, zygote formation was confined to only a small percentage of the population.

Effect of homozygosity on conjugation and sporulation. Matings between complementary stocks carrying identical respiratory-deficient markers generally were nonfertile. Only two of the six homozygous matings of pairs (Pet 1 × Pet 1 and Pet 7 × Pet 7) produced zygotes capable of forming viable spores. In both of these crosses, all of the segregants were petite. It will be recalled that the cytochrome pattern in these two petites is normal. In two other homozygous crosses (Pet 5 × Pet 5 and Pet 12 × Pet 12), zygotes were not formed, whereas in the two remaining combinations (Pet 10 × Pet 10 and Pet 16 × Pet 16),

<table>
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<th>Zygote genotype</th>
<th>No. of tetrads^a</th>
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<td>PD</td>
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<td>Pet 16 × Pet 12</td>
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^a Heterozygotes Pet 5 × Pet 7, 10, or 12; Pet 10 × Pet 7 or Pet 12; and Pet 7 × Pet 12 were not viable.

^b PD = parental ditype; NPD = nonparental ditype; and TT = tetratype. Ratio of petite: + in PD tetrads is 4:0; in NPD tetrads 2:2; and in TT tetrads 3:1.
aborted zygotes appeared. Briefly, matings between stocks homozygous for mutations that cause alterations in the cytochrome spectra (Pet 5, 10, 12, and 16) are infertile, although petites with unaltered cytochromes (Pet 1 and Pet 7) sporulate normally in homozygous crosses.

Response of the petites to environmental modifications. Each mutant was examined for its response to several modifications in cultural conditions. For these studies, effects on vegetative growth were determined with a modified glycerol medium. Mating-sporulation responses were measured on a modified ME medium.

Resnick and Mortimer (8) isolated ultraviolet-induced mutants of S. cerevisiae that were petite, and required either oleic acid or ergosterol for growth, or both. We wished to determine whether the S. lactis petites suffered from similar defects. Following their procedure (8), several sterols and fatty acids were added as individual supplements and the effects on each of the six mutants were noted. Neither palmitic, oleic, nor linoleic acid, when added alone or in combination with Tween 20, 40, or 80, had any effect either on growth or on mating-sporulation defects. Myoinositol and ergosterol stimulated the vegetative growth of Pet 10 slightly but did not relieve the inhibition associated with sporulation.

The addition of yeast extract (2% final concentration) to the two test media increased the vegetative growth response of Pet 12 but did not remedy blocks in sporulation.

Studies were made to determine whether the mutants were osmotically remedial (4). For this purpose, YM and Gly media were supplemented with 0.1 M KCl, and the response of each mutant was measured. In every one, the increase in osmotic pressure inhibited the growth of the mutants. Apparently all of the petites were non-remedial.

None of the mutants appeared to be temperature-sensitive since incubation at 15, 20, and 30°C did not relieve the defects associated with the growth of each of the mutants at 25°C.

In summary, each of the environmental modifications tested proved ineffective in relieving the physiological defects associated with respiratory-deficient mutations.

**DISCUSSION**

Each of the six ultraviolet-induced respiratory-deficient mutants (petites) of S. lactis that was isolated and characterized was of genic origin. At least three independent loci were represented. Four of the six mutants possessed altered cytochrome spectra. As a whole, the spectra displayed by the respiratory-deficient S. lactis mutants resembled those seen in segregational petites of S. cerevisiae. Unfortunately, S. lactis and S. cerevisiae are noninterbreeding, and tests for homology between these two species are impossible.

Our results are consistent with Bulder's (2) earlier observations that viable vegetative petites are not isolated from this species. What distinguishing features enable the segregational petites to survive while vegetative petites cannot? Perhaps segregational petites possess unique patterns of cytochrome alterations that differ from those encountered in vegetative petites. These differences may determine the lethality or non-lethality of the mutation.

On the other hand, ρ− cells of S. lactis may be nonviable because of modifications other than cytochrome composition that occur in the mitochondria of vegetative petites. Compared to S. cerevisiae, the fermentative capacity of S. lactis is weak and, undoubtedly, most of the physiological requirements of this organism are met by an oxidative type of metabolism. Mitochondrial damage (which leads, for example, to deficiencies in fatty acid biosynthesis or the generation of tricarboxylic acid cycle intermediates), may have much more serious, even lethal, effects in a more obligately aerobic yeast such as S. lactis.

It is interesting to compare the pleiotrophic effects that mutations to respiratory deficiency have on the various stages of the life cycles of the two yeasts S. cerevisiae and S. lactis. In S. cerevisiae, complementary haploids homozygous for ρ− or the p genes, mate normally but the diploids are unable to sporulate.

In the S. lactis petites studied, all of the homozygous and several of the heterozygous combinations of mutations leading to cytochrome modifications block the life cycle; diploids homozygous for respiratory-deficient mutations in which the cytochrome composition is normal (Pet 1 and Pet 7) suffer no such interference. Some homozygous combinations of cytochrome-deficient mutants (Pet 5 and Pet 12) are unable to form zygotes, whereas others (Pet 10 and Pet 16) are blocked later in the sporulation portion of the life cycle. In the nonviable heterozygotes, the block occurs during conjugation.

Sherman and Slonimski (9) noted that the presence or absence of ρ, the cytoplasmic factor determining respiratory competence in S. cerevisiae, alters the phenotypic expression of some segregational petites. Since ρ− cells arise spontaneously in this species, any sizable population usually consists of a mixture of ρ+ and ρ− types. If biochemical studies of the segregational petites are attempted with mixed populations such as these, additional laboratory manipulations and controls are required. We suggest that S. lactis
mutants would be the stocks of choice for biochemical investigations of segregational petites, since viable $p^-$ cells are not formed in this species and since the complications associated with using a mixed $p^+$ and $p^-$ population would not occur.

ACKNOWLEDGMENTS

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LITERATURE CITED