Induction of petite mutation and inhibition of synthesis of respiratory enzymes in various yeasts

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A systematic investigation covering a wide diversity of yeast species was made on the appearance of respiratory deficient (*petite*) mutants after treatment with acriflavine. Petite mutants were obtained from certain species only, but in these species all strains were found to have in common the property of giving rise to petite mutants; such species were designated as "petite positive". Species failing to give rise to petite mutants were accordingly called "petite negative". The primary action of acriflavine, namely the inhibition of the synthesis of the respiratory system, was shown to occur not only in petite positive yeasts, but also in petite negative ones. Some implications of the results are discussed.

INTRODUCTION

Acriflavine is known to produce in *Saccharomyces cerevisiae* and in several related species a mutation to respiratory deficiency, the so-called petite mutation (Ephrussi et al., 1949; see also Ephrussi, 1953 and Nagai, Yanagishima and Nagai, 1961). Besides its mutagenic action acriflavine inhibits, at least in *S. cerevisiae*, the synthesis of some cytochrome components of the respiratory system in cells which are adapting to oxygen; the inhibition of this synthesis precedes mutagenesis, and in all probability some stage or reaction is common to both (Slonimski, 1953). Systematic investigations on the mutagenic action of acriflavine on yeast species in general are lacking. The following is a report on the screening of a large variety of yeast species with respect to susceptibility to the mutagenic action of acriflavine. When it was found that from several yeast species respiratory deficient mutants could not be obtained by acriflavine treatment, if at all, it was subsequently investigated whether acriflavine inhibits the synthesis of cytochromes in these yeasts in the same way as it does in *S. cerevisiae*.

In the present work, the inhibition of the synthesis of the respiratory system

was not studied in anaerobically grown resting cells, as done by Slonimski, but instead, in a population of cells growing aerobically. In such a growing population no distinction can be made between the mutagenic effect of acriflavine and the inhibitory effect with respect to the synthesis of the respiratory system, as the mutagenic effect by its very nature comprises an inhibition of the synthesis of respiratory enzymes in a growing population. Such a distinction, however, need not be made if one is interested only in the possible inhibitory effect of acriflavine. Besides, in many species of yeasts, it is more difficult to evaluate the adaptation of resting cells to oxygen than it is in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains. The yeast strains used were obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft. Except in a few cases, mentioned in the text, only the type strains were used.

Mutagenic treatment with acriflavine. The strain under investigation was transferred with a platinum wire from a malt agar slant to 100 ml flasks containing 30 ml of malt extract to which had been added 1 ppm, 10 ppm, and 100 ppm, respectively, of acriflavine (Trypaflavin, registered trade name of Bayer–Meister Lucius). The cultures were incubated at 30 C, but strains that did not grow at 30 C were incubated at 25 C.

As soon as growth became visible, generally after 1 or 2 days, streaks from each culture were made on a malt agar plate. The plates were incubated at the same temperature as the flasks and examined from time to time. Mutant colonies of the respiratory deficient, or petite, type, if present, can be easily detected by their characteristic appearance: they are smaller, less opaque, and more glistening than the normal colonies. Pure cultures of mutants were obtained by suspending some material of a mutant colony in sterile water, plating out on malt agar and repeating this procedure once, or as many times as was considered desirable. The pure cultures thus obtained were kept on malt agar slants.

Checks for respiratory deficiency. The yeast strain concerned was cultivated on a malt agar plate at a suitable temperature. After 2 days the cells were scraped from the surface, suspended in tap water and centrifuged in a 50 ml tube. The water was discarded and the tube with the sediment was placed into the tubus of a microscope from which all lenses, except those of the condensor, had been removed. A low-voltage microscope lamp served as a light source. The absorption spectrum was observed with the Hartridge reversion spectroscope of Beck, London. Absence of the absorption bands of cytochromes

a and b, with the simultaneous presence of the bands of cytochrome c, was considered to indicate respiratory deficiency. If the absorption spectrum was ambiguous as to respiratory deficiency, a final decision was based on the results of a manometric determination of oxygen uptake and carbon dioxide production (Umbreit, Burris and Stauffer, 1957).

Inhibition of the synthesis of respiratory enzymes. The effect of acriflavine on the synthesis of respiratory enzymes was investigated by measuring manometrically the uptake of oxygen in a growing population. The production of carbon dioxide was measured as well, in order to observe the effect acriflavine might have on the synthesis of the fermentative system. Care was taken to use only populations that were actively growing from the beginning of the measurements. As a rule, cells from an agar slant were inoculated in 30 ml of medium. After incubation for ca. 16 hr, 3 ml of this culture was used as an inoculum for 30 ml of fresh medium; 0.9 ml of this suspension was brought into the Warburg vessels; 0.1 ml of acriflavine solution, or of water in the controls, was tipped in from the side bulb immediately before the first readings.

RESULTS

Occurrence of respiratory deficient mutants after acriflavine treatment

Respiratory deficient mutants could be obtained from the species listed below. After treatment with acriflavine the populations contained generally 50% to 100% deficient mutants. In the following, these species will be designated as "petite positive":

Saccharomyces cerevisiae; S. pastorianus; S. exiguus; S. logos; S. bayanus; S. willianus; S. uvarum; S. carlsbergensis; S. chevalieri; S. heterogenicus; S. oviformis; S. italicus; S. steineri; S. fructuum;
Torulopsis holmii; T. glabrata;
Candida robusta;
Kloeckera africana;
Brettanomyces claussenii; B. anomalus.

Respiratory deficient mutants were also obtained from *Schizosaccharomyces pombe*, *Saccharomyces florentinus*, and *Brettanomyces lambicus;* mutants from these species, however, occurred rather infrequently and were harder to isolate. From *Saccharomyces fragilis*, incidentally an aberrant respiratory deficient mutant was isolated, presumably not due to the acriflavine treatment; besides a strong band of cytochrome c, the band of cytochrome b was also visible in the absorption spectrum.

In these experiments respiratory deficient mutants could not be obtained from the species mentioned below, designated in the following as "petite negative":

Schizosaccharomyces octosporus;
Saccharomyces rouxii; S. marxianus; S. bailii; S. lactis; S. rosei; S. bisporus; S. fermentati; S. microellipsodes; S. mellis; S. acidifaciens; S. elegans; S. veronae;
Hansenula anomala; H. saturnus; H. suaveolens; H. schneggii; H. subpelliculosa; H. mrakii; H. silvicola;
Pichia fermentans;
Schwanniomyces occidentalis;
Saccharomycodes ludwigii;
Hanseniaspora valbyensis;
Nadsonia fulvescens; N. elongata;
Torulopsis colliculosa; T. molischiana; T. dattila; T. sphaerica; T. sake; T. globosa; T. versatilis; T. etchellsii; T. anomala; T. magnoliae; T. stellata; T. stellata var. *cambresieri; T. bacillaris;*
Candida utilis; C. pulcherrima; C. pelliculosa; C. intermedia; C. stellatoidea; C. pseudotropicalis; C. catenulata; C. parapsilosis; C. tenuis; C. claussenii; C. solani; C. albicans; C. tropicalis; C. reukaufii; C. macedoniensis; C. guilliermondii;
Kloeckera apiculata; K. antillarum; K. corticis; K. javanica; K. jensenii; K. lafarii; K. magna;
Brettanomyces bruxellensis.

Of the species *Schizosaccharomyces versatilis*, *Torulopsis lactis-condensi*, *Torulopsis pintolopesii* and *Candida slooffii* the type strain itself was found to be respiratory deficient, and in several of these species strains possessing a complete respiratory system may not occur at all. Of only a few species, some strains besides the type strain were subjected to the mutagenic treatment, and it appeared that in general, strains which belong to one species, irrespective of ploidy, respond in the same manner: they are either all petite positive or all petite negative. Only in one case, that of *Saccharomyces delbrueckii*, a group of petite positive strains and a group of petite negative strains were encountered. It appeared that these two groups could also be distinguished by other characteristics, the strains of the petite negative group resembling the strain of *S. delbrueckii* originally described as *Torulaspora delbrueckii* by Lindner, and the strains of the petite positive group resembling the strain originally described as *Saccharomyces unisporus* by Jörgensen (Kreger-van Rij, personal communication; see also Lodder and Kreger-van Rij, 1952). The different responses towards acriflavine give additional evidence that in *S. delbrueckii* two species

have been lumped together that might better be regarded as separate species. If the *Torulaspora*-like strains are separated from the others, there will be no exception to the rule that all strains belonging to a species are either petite positive or petite negative.

Inhibition of the synthesis of the respiratory system

The results of manometric experiments on the specific influence of acriflavine on the synthesis of the respiratory system are reported in Figs. 1 and 2. It appears from these graphs that acriflavine inhibited the increase in the rate of oxygen uptake not only in growing cultures of the petite positive yeast *S. cerevisiae* but also in those of the petite negative yeast *S. rosei*. The prevailing conditions were the same as those under which petite positive yeasts produced respiratory deficient mutants and petite negative strains did not.

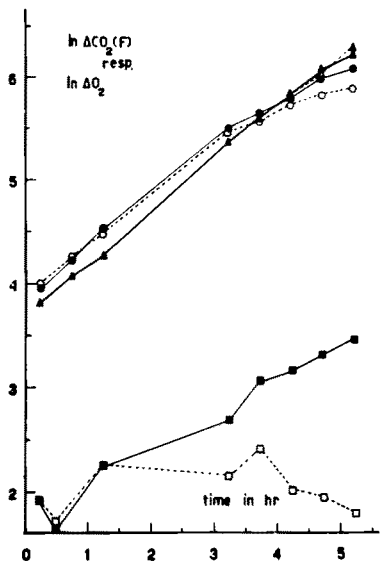

Fig. 1. *Saccharomyces cerevisiae.*

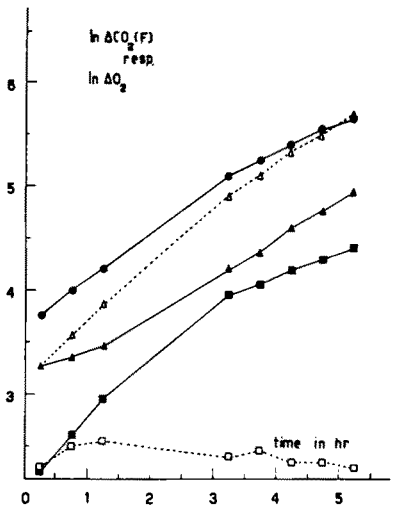

Fig. 2. *Saccharomyces rosei.*

Figs. 1 and 2. Increase in rates of fermentation and respiration in populations of *S. cerevisiae* and of *S. rosei* growing in malt extract with acriflavine, 10 ppm. Fermentation and respiration rates, $\Delta CO_2(F)$ and ΔO_2, respectively, are expressed in μl per 30 min and plotted as their natural logarithms against time.

■———■ respiration without acriflavine.
□- - - - - -□ respiration in presence of acriflavine.
▲———▲ aerobic fermentation without acriflavine.
△- - - - - -△ aerobic fermentation in presence of acriflavine.
●———● anaerobic fermentation without acriflavine.
○- - - - - -○ anaerobic fermentation in presence of acriflavine.

} coincide in Fig. 2.

The inhibition can be regarded as specific for the synthesis of the respiratory system, since the increase of the fermentative activity was not adversily affected by acriflavine, either in *S. rosei* or in *S. cerevisiae*. Acriflavine even seems to stimulate in *S. rosei* the increase of the fermentative activity. As this was observed under aerobic, but not under anaerobic, conditions, the apparent stimulation probably is not due to direct action of acriflavine but may be a consequence of the impaired synthesis of the respiratory system. Results similar to those reported above were obtained with *S. elegans* and *S. lactis*, two other petite negative yeasts.

DISCUSSION

The results have shown that some yeasts, when grown in the presence of acriflavine, give easily rise to petite mutants, whereas other yeasts fail to do so. Except perhaps for a few species (*Schizosaccharomyces pombe*, *S. florentinus*, and *Brettanomyces lambicus*), the ability or the failure to produce petite mutants on acriflavine treatment were consistently reproducible for a given yeast; thus the properties petite positive and petite negative can be regarded as being inherent to the particular strain. Furthermore, all available evidence suggests that these properties are specific, since the only example of a species comprising both petite positive and petite negative strains concerned a case of apparently less opportune "lumping together" of two species (*Saccharomyces delbrueckii*). Additional support for the view that the strains belonging to a species are either all petite positive or all petite negative, is afforded by comparing the results obtained with sporogenous species and the corresponding asporogenous (imperfect) forms. The imperfect forms are found to be petite positive when the corresponding sporogenous species is petite positive, and petite negative when the sporogenous form is petite negative (see Table 1).

TABLE 1

Comparison of the ability of sporogenous yeast species to form petite colonie mutants, with that of the corresponding asporogenous (imperfect) forms

Sporogenous species	petite	Corresponding imperfect species	petite
Saccharomyces cerevisiae	+	*Candida robusta*	+
S. exiguus	+	*Torulopsis holmii*	+
S. marxianus	—	*Candida macedoniensis*	—
S. lactis	—	*Torulopsis sphaerica*	—
S. rosei	—	*T. stellata* var. *cambresieri*	—
S. fermentati	—	*T. colliculosa*	—
Hansenula anomala	—	*Candida pelliculosa*	—
Hanseniaspora valbyensis	—	*Kloeckera apiculata*	—

In the genus *Saccharomyces* sensu latiore various characteristics of the haploid-diploid alternation in the life cycles have sometimes been used to distinguish the genera or subgenera *Zygosaccharomyces* and *Torulaspora* from *Saccharomyces* sensu stricto. When these characteristics are compared with the properties petite positive and petite negative, a correlation is found. As *Torulaspora* species some yeasts have been described which according to Lodder and Kreger-van Rij belong to *S. delbrueckii*, *S. rosei*, and *S. fermentati*. Krumbholz (1933) rejected the genus *Torulaspora*, and brought these yeasts into *Zygosaccharomyces*. As *Zygosaccharomyces* have further been described yeasts belonging to *S. rouxii*, *S. marxianus*, *S. bailii*, *S. lactis*, *S. bisporus*, *S. pastori*, *S. mellis*, *S. florentinus*, and *S. acidifaciens*. In the species that had been brought to the genus *Torulaspora*, as a rule conjugation tubes are formed previous to ascus formation, but actual conjugation is seldom observed. The other *Zygosaccharomyces* species usually show a manifest conjugation of two cells immediately before ascus formation. These phenomena indicate that in *Zygosaccharomyces* (including *Torulaspora*) the vegetative cells are predominantly haploid. A comprehensive discussion of this matter is given by Lodder and Kreger-van Rij (1952). The species that might be classified as *Zygosaccharomyces* or *Torulaspora* are those found to be petite negative. The only exception is *S. florentinus* from which petite mutants were obtained, although with some difficulty.

Among the yeasts that cannot be classified as *Zygosaccharomyces* or *Torulaspora*, only *S. fragilis* is petite negative, all others are petite positive. *S. fragilis* displays some properties which render its place in the genus *Saccharomyces* rather questionable; this has led Kudrjawzew (1954) to separate *S. fragilis* from the genus *Saccharomyces* and to include it in his new genus *Fabospora*, while Wickerham (1955) has proposed a genus *Dekkeromyces* which should include *S. fragilis*, *S. marxianus*, and *S. lactis*.

Although the tendency of a *Saccharomyces* species to occur predominantly in either the diploid or the haploid phase was shown to be correlated with the property petite positive or petite negative, no correlation can be found between the actual ploidy of a strain and the said property. Ephrussi, Hottinguer and Chimenes (1949) have obtained petite mutants from haploid strains of *S. cerevisiae*. Though the present writer did not extensively investigate this matter, as has already been mentioned, he too, encountered haploid strains of species belonging to *Saccharomyces* sensu stricto which were petite positive, whereas all incidental diploid strains of species belonging to *Zygosaccharomyces* which were investigated, were petite negative.

Hence, within the genus *Saccharomyces* sensu latiore, the property petite positive possesses the peculiar feature of being essentially restricted to those

species that can be regarded as *Saccharomyces* species sensu stricto. In addition, petite positive yeasts were also found outside the genus *Saccharomyces* and several of these can hardly be regarded as closely related to *Saccharomyces* sensu stricto. Since the property petite positive is common to *Saccharomyces* species sensu stricto, it may serve as an additional help in solving taxonomic problems. As, however, it is not exclusively bound to that group of yeasts, this property probably depends on some other physiological properties that determine, in the genus *Saccharomyces* sensu latiore, the characteristics of the life cycle. The property petite positive is, therefore, not comparable with rather simple biochemical properties like vitamin and amino acid deficiencies, or the abilities to ferment particular sugars, which are found rather arbitrarily distributed over different species and genera. The situation depicted above renders a genetical analysis of the property petite positive or petite negative rather unpromising, and in the genus *Saccharomyces* it may even be impossible to cross a petite positive with a petite negative yeast. In the case of *S. florentinus* a cross with a petite negative yeast of the *Zygosaccharomyces* group may be feasible, but it must be kept in mind that *S. florentinus* is not definitely petite positive.

Clearly distinct as the petite positive yeasts may be from the petite negative yeasts in their ability to form petite mutants when exposed to acriflavine, the synthesis of respiratory enzymes is inhibited by the dye in *both* groups to the same extent. This observation excludes low permeability of yeast cell walls or cell membranes for acriflavine as a possible general cause of the failure to obtain petite mutants from petite negative yeasts.

A higher resistance of cell wall material to penetration by acriflavine has been assumed by Millbank (1962) to cause the lower proportion of petite mutants obtainable from *Saccharomyces carlsbergensis* on acriflavine treatment, as compared to *S. cerevisiae*. However, it is hard to see how such a higher resistance to penetration can be reconciled with a higher lethality as earlier observed in the strains that are more resistant to mutation (Millbank and Hough, 1961). Furthermore, the assumption of Millbank (1962) is based on a comparison of the inhibitory effect exerted by acriflavine on the respiration of protoplasts on the one hand, with the mutagenic effect of this agent on intact cells on the other hand. This comparison of different effects in different systems does not seem to allow the conclusion drawn. Whatever the situation may be, the differences in susceptibility towards acriflavine as observed by Millbank and Hough (1961) are of a quantitative nature and offer less sharp a distinction than that between petite positive and petite negative yeasts in terms of the present study.

The occurrence of a specific inhibition of the synthesis of respiratory enzymes

in both petite positive and petite negative yeasts demonstrates that acriflavine can penetrate the cells of petite negative yeasts as well. At the same time it suggests that the primary action of acriflavine is the same in all yeasts, irrespective of whether they are petite positive or petite negative (see also Introduction). It remains undecided, however, whether the stage of the mutational event is reached in the petite negative yeasts, and if so, at what stage the mutation fails to express itself. This matter will be dealt with in a following publication.

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REFERENCES

EPHRUSSI, B. 1953. Nucleo-cytoplasmic relations in micro-organisms. Clarendon Press, Oxford.

EPHRUSSI, B., HOTTINGUER, H. et CHIMENES, A.-M. 1949. Action de l'acriflavine sur les levures. I. La mutation "petite colonie". Ann. Inst. Pasteur **76**: 351-367.

KRUMBHOLZ, G. 1933. Ist die Beibehaltung einer Gattung *Torulaspora* berechtigt? Arch. Mikrobiol. **4**: 167-169.

KUDRJAWZEW, W. I. 1954. [German translation]. Die Systematik der Hefen. Akademie-Verlag, Berlin, 1960.

LODDER, J., and KREGER-VAN RIJ, N. J. W. 1952. The Yeasts. A taxonomic study. North-Holland Publishing Company, Amsterdam.

MILLBANK, J. W. 1962. The action of acriflavine on yeast protoplasts. Antonie van Leeuwenhoek **28**: 215-220.

MILLBANK, J. W., and HOUGH, J. S. 1961. The action of acriflavine on brewer's yeast. J. Gen. Microbiol. **25**: 191-199.

NAGAI, S., YANAGISHIMA, N. and NAGAI, H. 1961. Advances in the study of respiration-deficient (RD) mutation in yeast and other microorganisms. Bacteriol. Rev. **25**: 404-426.

SLONIMSKI, P. P. 1953. A specific relation between enzymic adaptation and cytoplasmic mutation, p. 76-79. *In* R. Davies and E. F. Gale, [ed.], Adaptation in micro-organisms. Symp. Soc. Gen. Microbiol., 3rd, London, 1953. University Press, Cambridge.

UMBREIT, W. W., BURRIS, R. H. and STAUFFER, J. F. 1957. Manometric techniques. Burgess Publishing Co., Minneapolis, Minn.

WICKERHAM, L. J. 1955. New materials and procedures for genetic studies of yeasts. Nature **176**: 22.